

Review

Plant biostimulants: physiological responses induced by protein hydrolyzed-based products and humic substances in plant metabolism

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ABSTRACT: In recent years, the use of biostimulants in sustainable agriculture has been growing. Biostimulants can be obtained from different organic materials and include humic substances (HS), complex organic materials, beneficial chemical elements, peptides and amino acids, inorganic salts, seaweed extracts, chitin and chitosan derivatives, antitranspirants, amino acids and other N-containing substances. The application of biostimulants to plants leads to higher content of nutrients in their tissue and positive metabolic changes. For these reasons, the development of new biostimulants has become a focus of scientific interest. Among their different functions, biostimulants influence plant growth and nitrogen metabolism, especially because of their content in hormones and other signalling molecules. A significant increase in root hair length and density is often observed in plants treated with biostimulants, suggesting that these substances induce a “nutrient acquisition response” that favors nutrient uptake in plants via an increase in the absorptive surface area. Furthermore, biostimulants positively influence the activity and gene expression of enzymes functioning in the primary and secondary plant metabolism. This article reviews the current literature on two main classes of biostimulants: humic substances and protein-based biostimulants. The characteristic of these biostimulants and their effects on plants are thoroughly described.

Keywords: hormones, nitrogen metabolism, carbon metabolism, phenylpropanoid pathway, stress

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Introduction

Pesticides, phosphorus and nitrate represent the main agricultural pollutants that pose concern for the environment. Agricultural practices, such as the application of nitrogen rich-fertilizers to crops, may increase the nitrate concentration in groundwater to the point that it can exceed the safe threshold limits proposed by the EPA (Goulding et al., 2000). In response to this, crop production over the last few decades has addressed cost-effective, sustainable and environmentally friendly systems to provide high yields and quality of plant derived-food (Vernieri et al., 2006). Despite the main approaches including genetic selection and creation of varieties displaying elevated capacity of nutrient uptake and/or resistance to biotic and abiotic stresses (Kauffman III et al., 2007), the use of biostimulants to promote plant growth has recently gained increasing attention worldwide (Nardi et al., 2002a; Ertani et al., 2009; Ertani et al., 2013). Biostimulants are defined as materials that contain one or more substances and/or microorganisms able to stimulate nutrient uptake and use efficiency by plants, increase plant tolerance to abiotic/biotic stress and improve crop quality when applied in small amounts (De Vasconcelos et al., 2009; Calvo et al., 2014). Furthermore, biostimulants can enhance the activity of rhizosphere microbes and soil enzymes, the production of hormones and/or growth regulators in soil and plants, and the photosynthetic process (Nardi et al., 2009; Gi-

annattasio et al., 2013). The mode of action of biostimulants is often unknown and hard to identify, because they derive mainly from complex sources containing multiple bioactive components that, together, may contribute to specific effects in plants (Ertani et al., 2011a, b). For instance, a number of biostimulants contain hormones, such as auxins (Muscolo et al., 1998; Nardi et al., 2000; Pizzeghello et al., 2001; Jindo et al., 2012; Ertani et al., 2012), gibberellins (Hussain and Boney, 1969), cytokines (Bentler-Mowat et al., 1968; Jennings, 1969; Augier and Harada, 1972; Pizzeghello et al., 2013), and triacontanol (Ries and Violet, 1977; Kumaravelu et al., 2000; Chen et al., 2002; Çavuşoğlu et al., 2008; Ertani et al., 2012), which are recognized as the main active components responsible for the beneficial effects on plant growth. In this review, the characteristics and mode of action of two classes of plant biostimulants, HS and protein hydrolysates, are thoroughly described.

Humic substances

Humic substances consist of organic material resulting from concerted reactions of various biotic and abiotic processes. This complex assemblage of molecules deriving from plant and animal debris, represents one of the most abundant organic materials on earth. Humic substances are present in both aquatic environments and the atmosphere (Graber and Rudich, 2006; Salma et al., 2010), and represent the major components of the mixture of materials that comprise soil organic matter.

Soil HS are known to perform several ecological functions, in both natural and anthropogenic ecosystems. They are responsible for soil fertility by influencing structure and porosity through an effect on particle aggregation (Bronick and Lal, 2005). In addition, HS supply nutrients, through both chelating minerals and their own degradation (Stevenson, 1994), and shape soil biota communities, representing the main source of available organic carbon (Kalbitz et al., 2000).

Biological activity of humic substances

Several authors have found evidence of HS improving plant growth and physiology (Bottomley, 1914a, 1914b, 1917; O'Donnell, 1973; Cacco and Dell'Agnola, 1984; Dell'Agnola and Nardi, 1987; Nardi et al., 1988; Schiavon et al., 2008; Pizzeghello et al., 2013). These positive effects on plants could be ascribed, in the main, to hormone-like activity, as a number of hormones enclosed in the humus structure have been identified (Muscolo et al., 1998; Nardi et al., 2000b; Pizzeghello et al., 2001). In particular, a combination of genetic and molecular biology techniques (Dobbs et al., 2010; Trevisan et al., 2009, 2011) and gas-chromatography mass spectrometry (Jindo et al., 2012) has confirmed the presence of physiologically active indoleacetic acid (IAA) concentrations in HS.

The capacity of HS auxin-like activity to induce root development in plants was first hypothesized by Concheri et al., (1996). This mechanism was further confirmed by Trevisan et al., (2009) using a mutant genotype (*aux1*) of *Arabidopsis thaliana* and specific inhibitors of auxin transport or action. The findings obtained showed that HS induced lateral root formation via auxin-like activity, as confirmed by activation of the auxin synthetic reporter DR5::GUS and enhanced transcription of the early auxin responsive gene *IAA19*.

How IAA is bound to, or in association with, HS is still not well understood (Schulten and Schnitzer, 1998). The carboxylic groups of HS seem to be key receptors for the IAA and regulate its availability (Canellas et al., 2010, 2002; Jindo et al., 2012; Muscolo et al., 2007a, b). IAA is produced by soil bacteria, fungi, and plant root exudates (Rademacher, 1992; Lebuhn and Hartmann, 1993) and might be in association with HS through hydrophobic interactions (van der Waals, π - π , ion-dipole) and hydrogen bonds. According to previous investigations (Piccolo, 2001, 2002), HS are supramolecular aggregates and their stability and reactivity depend on the solution's ionic strength and pH of the surrounding environment. Low molecular weight organic acids, as well as root exudates, break the macro aggregate structure and generate subunits of biological active molecules (Nardi et al., 1988; Nardi et al., 2002b, 2005; Canellas and Olivares, 2014).

Although the recognized importance of IAA to HS to promote plant growth, there is clear evidence that the simple presence of auxin in the bulk HS is not sufficient to justify all plant physiological responses to these aggregates. Indeed, plants treated with HS often display

different behavior in terms of growth and metabolism in comparison to plants treated with the equivalent concentration of IAA (Muscolo et al., 2007a, b). Furthermore, a transcriptomic study by Trevisan et al., (2011) showed that HS exert their effects on plant physiology by means of complex transcriptional networks and indicated that HS exert their function in plants through a multifaceted mechanism of action, partially connected to their recognized auxin activity, but involving also IAA-independent signaling pathways.

The presence of other signaling molecules in the HS structure, or the involvement of different metabolic messengers mediating HS effects was endorsed by other authors (Aguirre et al., 2009; Kovács et al., 2013). Zandonadi et al., (2010), in particular, showed that root development stimulation and the H⁺-ATPase activation elicited by HS depend on mechanisms that use NO as a messenger that is induced in the early stages of lateral root development.

Humic substances can also display gibberellin (Nardi et al., 2000; Pizzeghello et al., 2002) and cytokinin-like activities (Nardi et al., 1988; Piccolo et al., 1992; Muscolo et al., 1996). Pizzeghello et al., (2013), in particular, demonstrated that HS contain a cytokinin (Isopentenyladenosine, IPA) in physiologically active concentrations and that its content in HS can stimulate plant metabolism. On the contrary, physiologically active amounts of gibberellins have not yet been detected in HS.

Mora et al., (2010) hypothesized that the effects of HS in plants may involve a primary effect on the root H⁺-ATPase activity and nitrate root-shoot distribution that, in turn, would cause changes in the root-shoot distribution of certain cytokinins, polyamines and abscisic acid, thus affecting shoot growth. These effects were associated with higher shoot concentration of several cytokinins and polyamines, and a concomitant decrease in their content in roots.

Protein-based biostimulants

Protein-based products can be divided into two major categories: protein hydrolysates consisting of a mixture of peptides and amino acids of animal or plant origin, and individual amino acids such as glutamate, glutamine, proline and glycine betaine.

Protein hydrolysates are produced through enzymatic, chemical or thermal hydrolysis of a variety of animal and plant residues, including animal epithelial or connective tissues (Cavani et al., 2006; Ertani et al., 2009, 2013a), animal collagen and elastine (Cavani et al., 2006), carobgerm protein (Parrado et al., 2008) and alfalfa plants (Schiavon et al., 2008; Ertani et al., 2009, 2013b). Individual amino acids include the twenty structural amino acids involved in the synthesis of proteins and non-protein amino acids, which are abundant in some plant species (Vranova et al., 2011). These amino acids are adsorbed by both roots and leaves and then translocated into the plant (Watson and Fowden, 1975; Soldal and Nissen, 1978; Michonneau et al., 2004; Zhang et al., 2015).

Several studies testing the action of protein hydrolysates in plants, have demonstrated that their first effect was the stimulation of root and leaf biomass (Zhang et al., 2003; Schiavon et al., 2008; Ertani et al., 2009). For instance, Ertani et al., (2009) reported that short-time application of protein hydrolysates incremented the root dry weight of maize plants compared to the untreated plants. These effects were consistent with those induced by HS in plants, which notoriously increase root growth in the short term and promote shoot biomass over longer periods (Nardi et al., 2009). The increase in root dry weight may result in a more successful transplanting of plants, in higher overall plant-biomass productivity and higher yields (Zhang et al., 2003).

The addition of biostimulants to plants also modifies the morphology of plant roots in a similar way to IAA, suggesting that they induce a "nutrient acquisition response" that favors the uptake of nutrients via an increase in the absorptive surface area (Ertani et al., 2012). These effects on growth appear to be distinct from the nutritional effect of an additional nitrogen source (Ertani et al., 2009, 2014).

Mechanism of action

Despite the mode of action of protein-based biostimulants being mostly unknown, recent studies have identified their target metabolic pathways and some of the mechanisms through which they exert their effects on plants (Schiavon et al., 2008; Ertani et al., 2009; Ertani et al., 2011a; Ertani et al., 2013). In particular, data available so far suggest that protein hydrolysates may promote nitrogen assimilation in plants via a coordinated regulation of C and N metabolism. For instance, a protein hydrolysate derived from alfalfa plants, enhanced shoot biomass production, soluble sugar accumulation and nitrogen assimilation of hydroponically-grown maize plants (Schiavon et al., 2008). Specifically, this biostimulant increased the activity of three enzymes (malate dehydrogenase, isocitrate dehydrogenase and citrate synthase) functioning in the tricarboxylic acid cycle (TCA) and five enzymes (nitrate reductase, nitrite reductase, glutamine synthetase, glutamate synthase and aspartate aminotransferase) involved in N reduction and assimilation. The biostimulant-induced up-regulation of the genes coding for these enzymes was confirmed by RT-PCR experiments. In a further investigation, the same biostimulant was found to improve the growth of maize plants cultivated under salinity stress, by increasing the ratio Na^+ and K^+ in leaves, and the synthesis of flavonoids (Ertani et al., 2013b).

Similarly to the alfalfa protein hydrolysate, a meat hydrolysate derived from tanning residues increased short-term growth and the macro-element content of maize seedlings, and, concomitantly, decreased nitrate, phosphate and sulfate content (Ertani et al., 2013a). In addition, Vernieri et al., (2006) demonstrated that the application of a protein hydrolysate influenced nitrogen metabolism in plants, speeding up the incorporation of

nitrate into proteins, through the activation of N assimilation-related enzymes. The increased use efficiency of nitrogen was justified by the higher leaf chlorophyll content in treated plants.

Kramer (1980) reported that the perennial ryegrass plants treated with a product based protein and exposed to prolonged high air temperature stress exhibited both an improved photochemical efficiency and membrane thermostability than untreated plants. These results provided consistent and interesting results and showed that foliar applications of protein hydrolysates can positively affect plant tolerance to heat stress (Kauffman III et al., 2007).

In a recent work, Ertani et al., (2014) reported that two biostimulants, one derived from alfalfa plants (AH) provide by ILSA S.p.A., and the other obtained from red grapes (RG), were chemically characterized using enzyme linked immuno-sorbent assays, Fourier transform infrared (FT-IR) and Raman spectroscopies. Two doses (50 and 100 mL L⁻¹ for RG, and 25 and 50 mL L⁻¹ for AH) of biostimulants were applied to *Capsicum chinensis* L. plants cultivated in pots inside a tunnel. Both biostimulants contained different amounts of indoleacetic acid and isopentenyladenosine; the AH spectra exhibited amino acid functional groups in the peptidic structure, while the RG spectra showed the presence of polyphenols, such as resveratrol. These results revealed that at flowering, RG and AH increased the fresh weight of leaves and fruits and the number of green fruits, whereas at maturity the biostimulants affected mainly the fresh weight and number of red fruits. At flowering, the leaves of the biostimulant-treated plants contained high amounts of epicatechin, ascorbic acid, quercetin, and dihydrocapsaicin, while at maturity, they exhibited elevated quantities of fructose, glucose, chlorogenic, and ferulic acids. Furthermore, green fruits exhibited high contents of chlorogenic acid, p-hydroxybenzoic acid, p-coumaric acid and antioxidant activity, while both AH- and RG-treated red fruits were highly endowed in capsaicin. The ¹H high-resolution magic-angle spinning (HRMAS)-nuclear magnetic resonance (NMR) spectra of red fruits revealed that both products induced high amount of NADP⁺, whereas RG also increased glucose, fumarate, ascorbate, thymidine and high molecular weight species. These results suggested that AH and RG promoted plant growth and the production of secondary metabolites, such as phenols.

The cases of no-response

Besides the plant positive effects of biostimulants, there are also several studies (Ruiz et al., 2000; Cerdán et al., 2009; Lisiecka et al., 2011) reporting that foliar applications of commercial protein hydrolysate products from animal origin can cause phytotoxicity and plant growth depression. In some situations, wrong product concentrations or environmental aspects like field conditions may contribute to no-response to biostimulants. For instance, application of biostimulants in excess might induce no-response or negative responses in plants. Asli

and Neumann (2010) reported that multiple applications of humic acid inhibited the shoot growth of maize grown hydroponically. No-positive effects were also reported by Kirn et al., (2010) in a trial with okra (*Abelmoschus esculentus*) grown in field soil experiments, as no significant increases in fruits per plant were observed when the recommended dose was not applied. Other conditions which induced no-response to biostimulant application are reported by Calvo et al., (2014).

Final Remarks

Recent studies support the potential of different types of biostimulants to improve plant biomass, crop yield and resistance to multiple types of stress. In particular, primary and secondary metabolic pathways of leaves and root tissues are recognized as targets of biostimulants. Further research combining functional genomic and proteomic approaches may help to obtain more insights in how biostimulants elicit plant growth, nutrient uptake and stress-tolerance responses in different plant species. In addition, these studies could allow for the identification of markers for beneficial plant responses, which may be useful for the development of new biostimulants.

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