



REVIEW

Biostimulants in Modern Agriculture: A Comprehensive Review with Emphasis on Protein Hydrolysates

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ABSTRACT: Biostimulants, categorized as microbial or non-microbial, including humic substances, seaweed extracts, chitosan, or protein hydrolysates (PHs), have gained significant attention in modern agriculture for their ability to enhance crop productivity, improve nutrient use efficiency, and increase resilience to abiotic and biotic stresses, while reducing dependence on conventional agrochemicals. This review synthesizes the historical development, classification, mechanisms of action, and agronomic benefits of biostimulants, with a particular emphasis on PHs, which are mixtures of amino acids, peptides, and polypeptides derived from plant or animal proteins through enzymatic, chemical, or thermal hydrolysis. The concept of biostimulants has evolved considerably over the past century, transitioning from vague notions of biogenic stimulants to clear definitions guided by international councils and regulatory bodies. Among these, PHs demonstrated the ability to promote plant growth, improve soil microbial activity, and mitigate environmental stresses. Evidence across the literature shows that PHs can deliver measurable agronomic benefits: a meta-analysis of 47 field trials reported an average yield increase of 16.5%, while greenhouse studies documented 21%–35% increases in root and shoot biomass, together with improved chlorophyll content and enhanced uptake of nutrients such as N, Fe, and Zn. These responses are associated with stimulation of root development, nutrient assimilation, osmotic adjustment, antioxidant activity, and beneficial soil microbial processes. The effectiveness of PHs, however, varies with source material, hydrolysis method, crop species, environmental conditions, and application strategy. Plant-derived enzymatic PHs often contain more bioactive peptides, whereas animal-derived PHs may provide higher concentrations of free amino acids and nitrogen. These properties have been reported to reduce reliance on synthetic inputs and contribute to more resilient cropping systems. Despite their promise, inconsistent product composition, incomplete mechanistic understanding, and fragmented regulatory frameworks continue to limit reproducibility and wider adoption. Future research should focus on standardized product characterization, multi-omics approaches, and long-term multi-environment field studies to improve the reliability and practical integration of PHs into sustainable cropping systems.

KEYWORDS: Abiotic stress; biostimulants; crop productivity; plant growth; protein hydrolysates; regulatory frameworks; sustainable agriculture; and soil microbiome

1 Introduction

The objective of this systematic review was to critically evaluate the current state of knowledge on plant biostimulants, with a particular focus on protein hydrolysates (PHs). Specifically, this review aimed to trace the historical development and evolving definitions of plant biostimulants, with the goal of summarizing

biostimulant classification, with emphasis on microbial and non-microbial categories. In addition, the aim was to evaluate the mechanisms of action and agronomic benefits of PHs in improving plant productivity, nutrient use efficiency, soil health, and stress tolerance, and to identify limitations in current knowledge, including variability in product performance, regulatory inconsistencies, and mechanistic uncertainties. The overall goal was to highlight future research directions, refine our understanding of PHs, and enhance their application in sustainable agriculture. As farmers face climate change, soil degradation, nutrient inefficiency, and stricter limits on chemical inputs, the global biostimulant market was projected to exceed USD 4 billion by 2025 [1], and biostimulants had already been applied to approximately 6 million hectares in Europe before 2021 [2]. Plant biostimulants have been found to increase nutrient uptake and use efficiencies for macro and micronutrients [3] in both agriculture and horticulture crops [4] helping to increase yield and crop quality [5], while improving photosynthesis [6], elicit phytohormones responses [7], improve abiotic and biotic stress [8], and positively affecting the microbiome of the rhizosphere and phyllosphere [5]. Biostimulant products are usually applied to plants and crops as a foliar spray (the most common method), via fertigation, or as a seed treatment [9].

Among non-microbial biostimulants, PHs have received particular attention. Protein hydrolysates are mixtures of amino acids, peptides, and polypeptides obtained from plant or animal proteins through enzymatic, chemical, or thermal hydrolysis [4]. Their agronomic potential is supported by both controlled environment and field studies. For example, greenhouse experiments have reported 21%–35% increases in root and shoot biomass, along with improved uptake of nutrients such as N, Fe, and Zn [3], while a meta-analysis of 47 field trials found an average yield increase of 16.5% following biostimulant application [10] and have also been associated with improved chlorophyll content, nutrient assimilation, root development, stress tolerance, and beneficial interactions with rhizosphere and phyllosphere microbial communities [4–6,11].

Despite these promising results, PH performance remains variable because responses depend on source material, hydrolysis method, crop species, environmental conditions, and application strategy [4,5,11]. In addition, the mechanisms underlying PH effects within the soil–plant–microbiome system are not yet fully resolved, and inconsistencies in product composition and regulation continue to limit reproducibility and broader adoption [11–13]. This review therefore synthesizes the development, definitions, and classification of plant biostimulants, with particular emphasis on PHs, and critically examines their mechanisms of action, agronomic benefits, current limitations, and future research needs in sustainable agriculture.

1.1 Concept of Plant Biostimulants

Agricultural production relies heavily on inputs such as fertilizers, pesticides, herbicides, and growth regulators to optimize yield and crop performance, making them a staple of modern agriculture worldwide [14]. While these products have long been staples of farming systems, the growing demand for sustainable and innovative solutions has created new opportunities for biostimulants. The idea of using biologically derived compounds to improve plant health is not new; discussions of “biogenic stimulants” date back to the 1930s. Russian scientist V.P. Filatov was among the first to suggest that biological materials exposed to stressors could enhance metabolic processes in plants, animals, and humans [15]. His work was expanded upon by Blagoveshchensky in the 1950s, who defined biogenic stimulants as organic acids that enhance enzymatic activity [16]. However, little progress was made in defining or applying biostimulants until the late 20th century, when Hervé introduced the idea of “bio-rational products,” emphasizing their systemic role in plant physiology, agriculture, and ecology [17]. Over time, the concept evolved into a focus on low-dose applications that enhance crop productivity and deliver ecological benefits, such as

heavy metal remediation and improved soil fertility [18]. By the early 2000s, researchers began to describe biostimulants as prestress conditioners [19], linking them explicitly to stress mitigation. Later, formal definitions emerged. Kauffman et al. defined biostimulants as materials, other than fertilizers, that promote plant growth at low concentrations [20], while Basak took a systematic approach to classification [21]. Du Jardin offered what would become the most widely cited definition, describing biostimulants as any substance or microorganism that enhances nutrient efficiency, abiotic stress tolerance, or crop quality, regardless of nutrient content [12] as well as Calvo et al. [22]. Building on these frameworks, industry groups such as the European Biostimulants Industry Council (EBIC) and the Biostimulant Coalition in the United States further refined the terminology and pushed for regulatory recognition [23,24]. Their efforts contributed to official definitions in both the European Union [23] and the 2018 U.S. Farm Bill [24]. While no single definition has been universally adopted, these regulatory milestones highlight the complexity of classifying biostimulants and underscore their growing importance in agriculture.

1.2 Categorization of Plant Biostimulants

Just as the definitions of biostimulants have evolved, so too has their categorization. Early attempts grouped them loosely based on broad characteristics [15], but by the mid-2000s, more systematic frameworks began to emerge. Kauffman et al. [20] divided biostimulants into three groups: humic substances, seaweed extracts, and amino acid-based products. du Jardin [12] later expanded this categorization to seven groups, which included humic and fulvic acids, protein hydrolysates and other nitrogen-containing compounds, seaweed extracts and botanicals, chitosan and other biopolymers, inorganic compounds, beneficial fungi, and beneficial bacteria. This classification gained traction among researchers and practitioners because it captured the diversity of both microbial and non-microbial biostimulants. Further refinements were introduced by Colla et al. [4], who separated six non-microbial categories: seaweed extracts, protein hydrolysates, humic acids, chitosan, phosphates, and silicon from three microbial categories, such as arbuscular mycorrhizal fungi, plant growth-promoting rhizobacteria, and *Trichoderma* species. Bulgari et al. [13] also proposed classification systems based on modes of action, but this approach has been limited by the lack of mechanistic understanding. While imperfect, the source- and ingredient-based classification remains the most widely accepted and practical framework, serving as a foundation for research, regulation, and industry development. Table 1 summarizes the definitions and regulatory frameworks for biostimulants over the years.

Table 1: Definitions and Regulatory Frameworks for Biostimulants.

Author/Organization	Year	Definition/Contribution	Notes/Context
Filatov [15]	1933	Introduced the concept of “biogenic stimulants,” biological materials derived from stressed organisms could influence metabolic/energetic processes in humans, animals, and plants.	USSR; early conceptual stage, not agriculture-specific.
Blagoveshchensky [16]	1955	Defined biogenic stimulants as “organic acids with stimulating effects due to their dibasic properties, enhancing enzymatic activity in plants.”	First agricultural link to plant stimulation.
Hervé [17]	1994	Proposed “bio-rational products”—systemic approach grounded in chemistry, biochemistry, biotechnology; should be applied in small amounts.	Precursor to modern biostimulant definitions.

Table 1: *Cont.*

Author/Organization	Year	Definition/Contribution	Notes/Context
Kauffman et al. [20]	2007	Defined biostimulants as “materials, other than fertilizers, that promote plant growth when applied in low quantities.”	One of first formal agricultural definitions.
Basak [21]	2008	Systematic definition/classification of biostimulants, considering functions, categories, and regulation.	Early attempt at harmonization.
du Jardin [12]	2015	“Any substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance, and/or crop quality traits, regardless of its nutrient content.”	Widely accepted academic definition.
European Biostimulants Industry Council (EBIC) [23]	2012 (updated-2023)	“Substances and/or microorganisms stimulating natural processes to enhance nutrient uptake/efficiency, abiotic stress tolerance, and crop quality.” Expanded to include plant metabolism, water efficiency, and soil properties.	Influential in shaping EU legislation.
U.S. Biostimulant Coalition/Biostimulant Council [24]	2012–2018	Defined biostimulants as “substances, including microorganisms, applied to plant, seed, soil, or other media to enhance nutrient assimilation or provide plant benefits.” Codified in Agriculture Improvement Act of 2018 (Farm Bill).	U.S. regulatory framework; still less harmonized than EU.
EU Regulation 2019/1009 [23]	2019	Legal definition: “A plant biostimulant is 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: (i) nutrient use efficiency, (ii) tolerance to abiotic stress, (iii) quality traits, or (iv) availability of confined nutrients in soil/rhizosphere.”	Established biostimulants as distinct from fertilizers, pesticides, and PGRs in EU law.
U.S. Congress (Farm Bill) [24]	2018	“Substance or microorganism applied to seeds, plants, or rhizosphere stimulating natural processes to enhance nutrient uptake/efficiency, tolerance to abiotic stress, or crop quality and yield.”	First federal-level recognition of biostimulants in the U.S.

1.3 Protein Hydrolysates

Protein hydrolysates are a major subcategory of biostimulants and are increasingly studied for their roles in crop productivity and resilience. In agricultural systems, PHs are derived primarily from plant and animal feedstocks [4,11]. Common plant sources include soybean (*Glycine max*), alfalfa (*Medicago sativa*), wheat (*Triticum aestivum*), corn/maize (*Zea mays*), and rice (*Oryza sativa*). Animal-derived PHs are typically produced from fish-processing residues (various species), collagen-rich bovine (*Bos taurus*) or porcine (*Sus scrofa domestica*) tissues, poultry feathers, and offal (e.g., chicken, *Gallus gallus domesticus*) [5], and dairy proteins such as casein or whey (commonly from *Bos taurus*). In practice, plant-derived PHs are often more readily accepted under certain regulatory or market frameworks, whereas animal-derived PHs can provide highly concentrated amino acids and distinctive peptide profiles, although the magnitude and nature of “bioactivity” depend strongly on how the material is processed [3,12].

Production method is a key driver of PH composition and performance: enzymatic hydrolysis, typically conducted under milder conditions, tends to preserve short peptides and biologically active amino acids

more effectively than chemical hydrolysis, which can induce racemization and alter amino acid integrity [18]. Enzymatic processing may also require less energy, strengthening the sustainability case for environmentally conscious growers [9]. Across products, peptide molecular weight is a critical determinant of biological activity, with smaller peptides often more readily taken up by plants [25]; many plant-derived PHs produced enzymatically are enriched in these low-molecular-weight fractions [5]. From an applied perspective, PHs are typically used at low rates as foliar sprays, soil/root-zone treatments, or seed coatings, supporting cost-effective delivery and multifunctional benefits that may reduce reliance on synthetic fertilizers [18]. However, important trade-offs remain plant-derived PHs can show substantial batch-to-batch variability without robust compositional reporting and quality control, while animal-derived PHs may face additional traceability and compliance considerations, and, if produced via harsh chemical routes, may show reduced peptide integrity and even in cell cultures [4,26–28]. From a sustainability perspective, both categories can support circularity when they valorize by-products, but a case-study life cycle assessment (LCA) indicates that the processing route can dominate environmental outcomes, with higher energy use and carbon dioxide emissions reported for a chemically produced animal by-product hydrolysate compared with an enzymatically produced plant-derived hydrolysate [29]. Table 2 summarizes recent studies reporting the efficacy of PHs in agricultural production.

Table 2: Summary of Included Studies on Protein Hydrolysates (PHs).

Author (Year) & Country/ Chronological Order	Crop Species	Setting	PH Source (Plant/Animal)	Hydrolysis & Application Method	Outcomes Measured/Results
El-Gabierly & Mesbah (2011), Egypt [30]	Cotton (<i>Gossypium hirsutum</i>)	Field	Plant-based	Enzymatic, Foliar	↑ seed oil and protein content
Ertani et al. (2009), Italy [31]	Maize (<i>Zea mays</i>)	Greenhouse	Plant-based (alfalfa)	Enzymatic, Roots	Improved growth under salinity stress; ↑ proline accumulation
Colla et al. (2014), Italy [3]	Tomato (<i>Solanum lycopersicum</i>)	Greenhouse	Plant-based	Enzymatic, Foliar spray	↑ root & shoot biomass (21–35%); ↑ nutrient uptake (N, Fe, Zn); ↑ chlorophyll
Hammad & Ali (2014), Egypt [32]	Wheat (<i>Triticum aestivum</i>)	Greenhouse	Plant-based	Enzymatic, Foliar spray	↑ chlorophyll, photosynthesis; stress mitigation
Colla et al. (2015), Italy [4]	Maize (<i>Zea</i> spp.) Tomato (<i>Solanum</i> spp.) Lettuce (<i>Lactuca</i> spp.)	Field & Greenhouse	Plant & animal-based	Enzymatic vs. Chemical, Foliar spray & Soil	Yield ↑, improved N assimilation, enzyme activity (nitrate reductase, Fe-chelate reductase)
Colla et al. (2016), Italy [9]	Basil (<i>Ocimum basilicum</i>)	Greenhouse	Plant-based	Enzymatic, Foliar	↑ chlorophyll; improved morphology; ↑ nutrient efficiency
Colla et al. (2017), Italy [5]	Multiple crops (review of PH action)	Review	Plant & animal-based	Foliar, Soil, Seed	described PHs effects on physiology, microbiome, nutrient uptake
Kocira (2015), Poland [8]	Soybean (<i>Glycine max</i>)	Field	Plant-based	Enzymatic, Foliar	↑ protein content in seeds; ↑ pods per plant; ↑ yield

Table 2: Cont.

Author (Year) & Country/ Chronological Order	Crop Species	Setting	PH Source (Plant/Animal)	Hydrolysis & Application Method	Outcomes Measured/Results
Rouphael et al. (2022), Italy [33]	Lettuce (<i>Lactuca</i> spp.)	Greenhouse	Plant & animal-based	Enzymatic vs. Chemical, Foliar, Roots	Improved salt tolerance; ↑ osmolytes, glucosinolates, antioxidants
Li et al. (2022), Belgium [10]	Multiple (47 PH field trials)	Field	Plant & animal-based	Mixed, Mixed	Yield ↑16.5% (average across studies)
Ávila-Pozo et al. (2023), Spain [34]	Pepper (<i>Capsicum</i> spp.)	Greenhouse	Animal-derived (slaughterhouse sludge)	Enzymatic hydrolysis; root + foliar spray	Yield ↑, ↑ nutritional quality
Engel et al. (2024), Brazil [35]	Soybean (<i>Glycine</i> spp.)	Greenhouse	Animal-based (collagen-derived hydrolysate)	Collagen hydrolysate processed via acid treatment + alkalinization; seed + foliar (0.20% HP; foliar at V3 & V5)	Outcomes included N metabolism pools (nitrate, amino acids, ureides), expression of N-assimilation-related genes, plus yield components (pods and grains)
Leporino et al. (2024), Italy [36]	Tomato (<i>Solanum</i> spp)	Greenhouse with repeated drought cycles	Plant-based (vegetal proteins from Malvaceae = PH1; Fabaceae = PH2)	Enzymatic hydrolysis; foliar spray (3 mL L ⁻¹)	PH1 ↑ recovery of digital biomass and 3D leaf area after each drought event; phenomics + metabolomics showed shifts in dipeptides/fatty acids and regulation of phenolic-related compounds
Rodegher et al. (2024), Italy [37]	Cucumber (<i>Cucumis sativus</i>)	Nutrient-solution study (Fe deficiency/resupply)	PH source not stated in abstract	Simultaneous FA + PH added to Fe-free nutrient solution; Fe resupply with FeCl ₃ ; hydrolysis type not stated	MIX ↑ leaf SPAD via endogenous Fe redistribution; with Fe resupply: ↑ dry root & shoot weight, ↑ root system; Strategy-I genes (CsFRO1, CsIRT1) remained elevated; FA-PH complex formation supported by biophysical analyses
Di Serio et al. (2025), Italy [38]	Baby leaf lettuce (<i>Lactuca sativa</i> L.)	Greenhouse	"Microalgal PH" from residual <i>Chlorella vulgaris</i> biomass	Protease-induced hydrolysis (trypsin = TPH; pepsin = PPH); foliar spray weekly until harvest	PHs improved agronomic/physiological/quality traits from germination to harvest; reported ↑ bioactive compounds (flavonoids/anthocyanins) and antioxidant activity

Table 2: Cont.

Author (Year) & Country/ Chronological Order	Crop Species	Setting	PH Source (Plant/Animal)	Hydrolysis & Application Method	Outcomes Measured/Results
Peli et al. (2025), Italy [39]	Table grape (<i>Vitis vinifera</i>)	Vineyard/field “in vineyards”	Plant-based (maize gluten-derived PH)	Enzymatic hydrolysis (wet-milling by-products) + soil drench at veraison	14 days post-application: ↑ anthocyanins, ↑ sugars, ↑ berry diameter while maintaining firmness; transcriptomics supported accelerated ripening processes and stress-resilience-related gene modulation
Naranjo et al. (2026), Spain [40]	Grapevine (<i>Vitis vinifera</i> ; cv. Syrah)	Greenhouse	“LEE” biostimulant from red wine lees (agro-industrial waste)	Enzymatic hydrolysis (subtilisin protease) to create LEE; foliar spray (LEE 0.1%); ozone fumigations 300 ppb (acute stress test)	LEE mitigated ozone damage: improved net photosynthesis, delayed fluorescence; transcriptomic evidence of altered stress/secondary metabolism responses

1.4 Amino Acid Effects on Plant Health

The amino acid composition of protein hydrolysates (PHs) plays a central role in their effectiveness. Amino acids contribute directly to plant health as nitrogen sources [25], signaling molecules [41], stress reducers [6], and precursors to phytohormones [7]. They also serve as a food source for microbes [5], linking plant nutrition to microbial activity in the rhizosphere. Of particular importance is the distinction between L-form and D-form amino acids: plants can only metabolize the L-form, which is preserved in enzymatically produced PHs but often lost during chemical hydrolysis due to racemization [9,18]. This makes enzymatic processes not only more sustainable but also more effective in producing bioactive compounds [4]. Comparative studies show that PHs derived from animal collagen via chemical hydrolysis have higher nitrogen and free amino acid content but fewer peptides and more undesirable residues, such as chlorine and sodium, whereas plant-based PHs produced enzymatically contain more peptides and a greater diversity of amino acids [4]. These differences underscore the importance of both source material and processing method in determining the agronomic potential of PHs. While the precise mechanisms remain incompletely understood, accumulating evidence points to peptide signaling and phytohormone-like responses as key contributors to plant growth and stress resilience [9,42]. Research consistently demonstrates that PHs improve nutrient uptake, reduce abiotic stress, stimulate root and shoot growth, and positively influence microbial communities in the phyllosphere and rhizosphere [11,43,44].

1.5 Protein Hydrolysate Impact on the Agroecosystem

1.5.1 Soil and Microbes

Protein hydrolysates influence agroecosystems by altering soil structure, microbial activity, and nutrient cycling. Within the rhizosphere, the soil zone is directly influenced by root activity. Thus, the PHs interact with highly active microbial communities that play a critical role in disease suppression, nutrient transfer, and plant establishment [43]. In soils, PHs have been shown to improve respiration and biological fertility [6,9] and to enhance nutrient mineralization [43]. While microbes naturally compete with plants

for amino acids and peptides, the application of PHs increases the pool of available compounds, reducing competition and supporting both microbial and plant needs [5]. By promoting beneficial microbial activity, PHs strengthen soil fertility and create more favorable conditions for plant growth and resilience.

1.5.2 Nutrients

Amino acids within PHs are integral to the soil nitrogen cycle, serving as both nitrogen sources and facilitators of nitrogen transfer within plants. When applied to soil, a portion of the amino acids is incorporated into microbial biomass, while the remainder supports plant nutrition [45]. The application of PHs can enhance nutrient mineralization and chelation of essential micronutrients such as Fe, Zn, Mn, and Cu, thereby improving uptake and translocation within plants [6,17,43]. Studies also show that PHs can influence progeny seed composition, increasing protein and oil content in crops such as cotton and soybeans [29,46,47]. Similar results were found in soybeans, where PH applications improved nitrogen metabolism and protein accumulation [48]. Earlier studies in corn and tomato also confirmed enhanced nitrogen and iron uptake through increased enzyme activities such as nitrate reductase and Fe (III)-chelate reductase [49,50].

1.5.3 Roots and Shoots

Applications of PHs have consistently been linked to improvements in root architecture, shoot biomass, and photosynthetic efficiency. Amino acids and peptides within PHs stimulate enzymatic activities, including nitrate reductase and glutamine synthetase, thereby enhancing nitrogen assimilation [50,51]. Root applications increase root length, branching, and density, while foliar applications improve chlorophyll content and photosynthetic capacity [11]. Collectively, these effects result in greater nutrient uptake efficiency and crop performance [3,5,52]. Field studies highlight yield increases averaging 16.5% across PH treatments [10], though results vary by crop species, environmental conditions, and application timing. These findings underscore the importance of tailoring PH application strategies to specific crops and conditions to maximize benefits [22].

1.6 Plant and Crop Stress Mitigation

Abiotic stress is a leading cause of yield loss in agriculture, and PHs are increasingly recognized for their ability to mitigate the impacts of stress [5,33]. Amino acids such as proline and glycine betaine act as osmoprotectants, enhancing plant tolerance to salinity, drought, and temperature extremes [53,54]. Protein hydrolysates also stimulate antioxidant responses, protecting cellular structures from oxidative stress [33]. In crops such as lettuce, PHs treatments have been shown to reduce sodium accumulation, increase osmolyte production, and improve photosynthetic performance under salinity stress [55]. Similar benefits have been reported for corn, where PHs improved potassium-to-sodium ratios and nitrogen metabolism, enabling plants to tolerate salinity stress [31]. Other studies confirm positive roles in heavy metal tolerance and heat resilience [56,57]. Hammad and Ali [32] investigated the physiological and biochemical effects of drought tolerance in wheat plants by applying amino acids and yeast extract. Overall, by promoting the accumulation of bioactive molecules and activating defensive pathways, PHs have been shown to enhance plant recovery and resilience, offering a valuable tool for farmers facing increasingly variable and extreme climatic conditions.

1.7 Knowledge Gaps and Future Direction

Despite significant progress, challenges remain in fully realizing the potential of PHs and other biostimulants. A persistent limitation is variability in composition across commercial products, which undermines reproducibility and farmer confidence and reinforces the need for standardized testing, transparent compositional reporting, and robust quality control (QC) [27,58].

Regulatory definitions also reinforce why “product identity” and “proven effect” must be demonstrated: for example, in the European Union (EU), a plant biostimulant is defined as a product that stimulates plant nutrition processes independently of nutrient content to improve nutrient use efficiency, abiotic stress tolerance, quality traits, and/or availability of confined nutrients in the rhizosphere, and the regulation also specifies contaminant limits and requires that a product achieves the effects claimed on the label [23]. Moving forward, research should therefore shift from reporting broad “PH effects” toward linking defined chemical features to reproducible biological outcomes across crops, environments, and management systems—an approach emphasized in PH-focused reviews that note PH composition depends strongly on raw materials and processing (including hydrolysis conditions) [4,26,27].

1.7.1 Standardize PH Characterization and Reporting

A primary knowledge gap is that many PH studies still do not report enough product detail to support replication or cross-study synthesis, despite repeated calls for more rigorous characterization [4,27]. At minimum, each PH product (and each batch) should be described by: raw material identity and provenance (plant/animal origin, tissue type, and byproduct context); hydrolysis metadata (enzymatic versus chemical, enzyme class if enzymatic, temperature/pH, reaction time, neutralization steps, and downstream filtration/drying); degree of hydrolysis (DH) reported with method and units (because DH shapes peptide size distribution and activity); molecular weight distribution using methods such as size-exclusion chromatography (SEC) or validated gel filtration approaches; amino acid profile distinguishing free amino acids from peptides to avoid conflating nitrogen nutrition with signaling-like effects; and a peptide “fingerprint” (for example via liquid chromatography–tandem mass spectrometry (LC–MS/MS) to support batch comparability. Analytical literature also highlights DH and molecular weight distribution as key parameters and provides approaches for their measurement (including liquid chromatography–mass spectrometry–based methods) [28,59].

1.7.2 Build Quality Control into Experimental Design

To improve reproducibility and farmer trust, experimental designs should explicitly address batch-to-batch variability rather than treating “brand name” as sufficient identity. This can be done by using two or more production batches of the same commercial PH where feasible, or at a minimum, retaining a sealed reference sample for later re-analysis and defining a priori QC release criteria (for example, DH range, SEC profile tolerance, and peptide fingerprint similarity threshold) [26,59].

For animal-derived PHs and waste/byproduct-derived materials, QC should also include safety-relevant checks aligned with the regulatory context (for example, heavy metals and other contaminants) and source-specific requirements; EU rules explicitly set contaminant limits for plant biostimulants, and separate EU requirements exist for certain animal byproduct-derived hydrolyzed proteins, including molecular-weight-related compliance that has driven validated analytical methods [23,28].

1.7.3 Strengthen Causal Inference with Better Controls

A recurring methodological weakness is inadequate separation of PH “biostimulant” effects from confounders such as added nitrogen, salts, and pH shifts. This is particularly important because regulatory and conceptual definitions emphasize effects independent of nutrient content [12,23]. Future trials should therefore routinely include nitrogen-equivalent controls (mineral nitrogen or defined amino acid mixtures matched to the total nitrogen applied via PH), electrical conductivity (EC) and pH controls (especially for foliar applications), and matrix controls when the PH formulation includes adjuvants. Where feasible, a well-characterized positive reference biostimulant can help benchmark effect sizes across experiments [26].

1.7.4 Optimize Dose, Timing, and Placement Using Response-Surface Approaches

Many studies still test one or two doses, limiting actionable agronomic guidance and masking non-linear responses. Future research should standardize a two-step optimization approach: (i) range-finding to detect phytotoxicity and identify plausible response windows, then (ii) formal dose–response testing using at least four doses and appropriate models (often non-linear). Designs should be expanded to factorial or response-surface experiments that test application route (foliar versus root/soil versus seed), timing (vegetative versus reproductive; pre-stress versus during stress), frequency (single versus repeated), and interactions with fertilization and irrigation regimes. PH reviews emphasize that outcomes depend on application method and context, reinforcing the need for systematic optimization rather than single-factor comparisons [26,27].

1.7.5 Move from “Effects” to Mechanisms with Testable Hypotheses

Mechanistic claims are often broad (for example, “improved stress tolerance”) without pathway-level specificity, and reviews of PHs identify both direct effects on plant metabolism and hormonal signaling and indirect effects mediated by the plant-associated microbiome, supporting the need for hypothesis-driven tests rather than endpoint-only studies [26,27]. Future work should pair performance data with mechanistic assays such as tracing uptake and fate using stable-isotope strategies (for example, nitrogen-15, ¹⁵N) to distinguish uptake/nutrition from signaling-like effects; standardized phytohormone profiling (for example, abscisic acid (ABA)), auxin-related metabolites, cytokinins, and ethylene-related metabolites) with consistent sampling times, and predefined marker-gene panels aligned to physiological endpoints.

Critically, mechanistic work should also operationalize “what is active” by fractionating PHs (for example, by molecular weight cut-offs) and testing intact products versus fractions. Recent studies show that different molecular fractions of PHs can drive distinct growth and quality responses in lettuce and other crops, supporting a fraction-based hypothesis-testing approach [60,61]. Molecular weight fractionation combined with metabolomics has also been used to connect specific fractions to auxin-like activity and broader metabolic reprogramming, providing a template for moving from correlation to mechanism [62].

Recent work shows that omics-based designs can already move biostimulant research beyond descriptive growth responses toward testable, pathway-level hypotheses. For example, an integrated transcriptomics (ribonucleic acid sequencing, RNA sequencing) and untargeted metabolomics study in tomato (*Solanum lycopersicum*) compared a Malvaceae-derived protein hydrolysate with a defined molecular fraction under limited nitrogen (N) availability, and reported distinct, treatment-dependent shifts in coordinated gene networks and metabolite classes consistent with improved resilience under nutrient limitation. Importantly, this approach illustrates a practical workflow for the field—fractionate the product, quantify the chemistry, then use multi-omics to connect specific fractions to reproducible

biological pathways—which is a key step toward explaining “why” and “when” a given protein hydrolysate works rather than only reporting that it works [63].

Complementary progress has also been achieved by pairing omics with time-resolved phenotyping to capture dynamic stress responses. In tomato exposed to repeated drought cycles, high-throughput phenotyping (phenomics) combined with metabolomics enabled discrimination among protein hydrolysate treatments based on recovery trajectories and identified metabolite signatures (including changes in dipeptides, fatty acids, and secondary-metabolism-related compounds) associated with improved post-stress performance. This type of design demonstrates how linking stress-recovery phenotypes with molecular fingerprints can generate concrete mechanistic hypotheses (for example, whether dipeptide accumulation reflects direct delivery versus stimulation of endogenous peptide metabolism) and can guide selection of sampling time points for downstream mechanistic validation [36].

1.7.6 Account for Soil and Microbiome Mediation

PH effects can be strongly mediated by soil chemistry and microbial communities, yet these factors are still often unmeasured. Reviews report that PH applications can alter microbial communities in the rhizosphere and phyllosphere and highlight the need for deeper investigation of these interactions [26,27]. Recent experimental work also shows that PH source (plant versus animal) and production context can shift rhizosphere microbiota and that responses vary by crop species [64]. Recommended approaches include paired sterile versus non-sterile systems (for example, axenic seedlings versus soil), microbiome profiling based on the 16S ribosomal ribonucleic acid (16S rRNA) gene for bacteria and the internal transcribed spacer (ITS) region for fungi, and minimum soil context reporting (texture, organic matter, pH, baseline EC, and nutrient status). Where feasible, PH effects should be tested across contrasting soils (for example, acidic versus calcareous; low versus high organic matter) as a planned experimental factor.

1.7.7 Prioritize Multi-Site, Multi-Season Field Validation with Agronomic Endpoints

To translate greenhouse findings into practice, field research should emphasize multi-location, multi-year trials using robust designs such as a randomized complete block design (RCBD) with adequate replication and environmental characterization. Meta-analysis evidence indicates that biostimulant yield effects vary with application method, crop type, climate, and soil properties, reinforcing the need for well-designed multi-site validation and for reporting effect sizes that can be synthesized across studies [65]. Trials should measure yield components and marketable quality traits (not only biomass), monitor stress-relevant indicators (for example, canopy temperature, phenology aligned with stress events, and chlorophyll status using the soil–plant analysis development (SPAD) chlorophyll index), and include agronomic efficiency metrics such as nitrogen use efficiency (NUE) where appropriate.

1.7.8 Improve Transparency and Synthesis

Finally, the field needs better comparability and stronger evidence synthesis. Studies should adopt a reporting checklist tailored to PH trials (PH identity card, control hierarchy, dose/timing details, environment and soil descriptors, and statistics), share raw data and metadata (weather, soil, management) in repositories, and use consistent agronomic metrics that facilitate meta-analysis and cross-study aggregation. Evidence syntheses show the value of harmonized reporting for drawing generalizable conclusions across diverse trials [59,65].

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Abbreviations

PH Protein Hydrolysates

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