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REVIEW ARTICLE

Unraveling the intriguing potential of protein-rich microbial biostimulants for horticultural crops

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Abstract

Nutritional security and minimizing the impact of farming practices on the environment are major challenges in modern farming systems. Currently, the horticulture sector is growing fast and moving towards sustainability and profitability. Indiscriminate and improper use of chemical inputs to ensure high yields of horticultural products could lead to significant contamination of soil and water bodies. Under these circumstances, farmers must optimize their input management to reduce pollution and preserve the economic margin by following sustainable production practices. The use of precision horticulture techniques is more sustainable than conventional to intensive farming methods. Among the various eco-friendly inputs, plant biostimulants are highly effective and can enhance plant growth and production as well as mitigate the adverse effects of abiotic stressors. Protein Hydrolysates (PHs) are a significant class of plant biostimulants based on amino acid and peptide mixtures. Because of their beneficial effects on crop performance, PHs has drawn increased amounts of attention recently. Compared with other biostimulants microbial biostimulants are more prevalent in crop production. A new approach is the formulation of a mixture of plant growth-promoting microorganisms/microbe-derived metabolites and protein hydrolysates as single biostimulants, to nourish the soil, plants and microbes. This review presents a thorough summary of recent research on the postulated modes of action of PHs and microbial biostimulants in horticultural crops. Furthermore, this study highlights the potential of protein hydrolysates and microbial biostimulants and the potential of the protein-rich microbial biostimulants to make horticulture more profitable and to safeguard the environment.

Keywords

horticulture; microbial biostimulants; protein hydrolysates; protein-rich microbial biostimulants

Introduction

Globally, horticultural systems must achieve a delicate balance between 2 demands: [1] increasing food production to feed an estimated 7 to 10 billion people by 2050 and [2] reducing the negative impacts of agriculture on the environment and public health. Population growth and anticipated rise in prosperity further stress the natural resources and agricultural ecosystems, which are already at risk of collapsing (1). It is essential to develop novel strategies for enhancing the sustainability of horticultural systems protecting the environment, maintaining farmers' livelihoods and providing the growing population with nutritious food.

The excessive use of synthetic herbicides and fertilizers in recent years is the real underlying cause of many ecological problems, leading to the deterioration of the environment and soil health. Farmers are under pressure to maintain profit margins while adopting sustainable production methods and optimizing fertilizer management to minimize nutrient losses and pollution (2).

The term "biostimulant" has garnered attention in the farming community for its role in promoting plant growth and soil health, further sustaining both plant and soil microbiota. A plant biostimulant refers to any organic/ inorganic substance or microorganism that can improve the metabolic processes of plants and lead to improvement in crop quality, abiotic stress tolerance and nutrition efficiency (3).

Among biostimulants, protein hydrolysates (PHs), derived from animal or plant sources are particularly noteworthy for their potential to enhance crop performance and production. PHs improve the productivity of roots and shoots in various horticultural crops and under stressful conditions, they have been shown to enhance plant resilience, particularly by stimulating antioxidant production within plants (4). Additionally, PHs may directly influence plants by altering carbon and nitrogen metabolism or indirectly through changes in the microbiome. Their application also promotes nutrient absorption, partly due to modified root architecture, increasing the availability of macro- and micronutrients. When applied either to the root (via drip irrigation or root drenching) or leaves (through foliar spray), PHs modify the microbial community within the phyllosphere or rhizosphere. The amino acids in PHs serve as a food source for billions of microorganisms (5, 6).

Microbial biostimulants include plant growth-promoting microorganisms (PGPM) that enhance nutrient uptake and solubilization as well as the synthesis of secondary metabolites, siderophores, hormones and organic acids. They promote rhizosphere microbial activity, improve crop quality and yield, fix nitrogen, enhance stress tolerance and solubilize phosphorus and potassium (7).

Although many studies have documented the potential benefits of using either beneficial microorganisms or PHs, no study has combined the two, except for some combinations of specific fungi with PHs (8, 9). This review aims to highlight the fascinating impacts of protein hydrolysates and microbial biostimulants on horticultural plant growth and development as well as the likely underlying mechanisms and the potential of combining these 2 categories.

Biostimulants and their categories

The term ‘biostimulant’ was first used by Zhang and Schmidt (10), who described it as ‘‘materials that, in minute quantities, promote plant growth’’. Du Jardin later provided a more comprehensible definition of biostimulant as ‘‘any substance or mixture of substances of natural origin or microorganisms applied to crops or soils to enhance nutrition efficiency, abiotic stress tolerance, and/or crop quality traits regardless of its nutrient content" (11). Plant biostimulants have been reported to alter the biological, biochemical and physical properties of soil, improve nutrient use efficiency and increase crop yields. It affects root growth and architecture and enhances plants' performance under abiotic stress. Based on their source and content, bio stimulants can be broadly divided into three primary types humic substances (HS), amino acid-containing products (AAP) and hormone-containing products (HCP), although they exist in a vast array of formulations and chemical combinations (10).

The biostimulant coalition of North America defines plant biostimulants as substances and/or microorganisms that, when used on plants or the rhizosphere, work to promote natural processes that improve crop quality, the ability to withstand abiotic stress, nutrient uptake and nutrient efficiency independent of its nutrients. Since biostimulants do not directly combat pests, they are exempted from pesticide regulations (12). Du Jardin carried out a bibliographic examination of plant biostimulants and classified them into 8 categories: humic substances, seaweed extracts, chitin and chitosan derivatives, complex organic materials, advantageous chemical components, inorganic salts (including phosphite), antitranspirants, free amino acids and other N -containing compounds. Fig. 1 provides the schematic representation of different plant biostimulants. A few key categories that include both chemicals and microbes are generally acknowledged by stakeholder groups, scientists and authorities as biostimulants. Beneficial microorganisms such as bacteria and fungi, particularly PGPMs are used as microbial sources for biostimulants. They may be endosymbiotic, rhizospheric or free-living (13). Fig.1. Schematic representation of different plant biostimulants and Table 1 depicts the effects of combined biostimulants in plant growth and their proposed mechanisms.

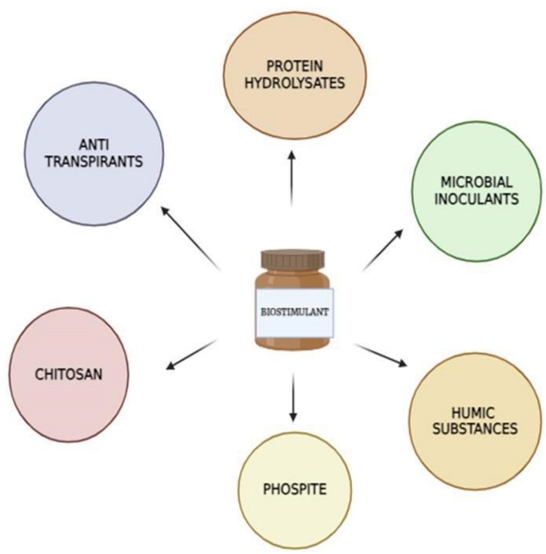

Fig. 1. Schematic representation of different plant biostimulants.

Table 1. Effects of combined biostimulants in plant growth and their proposed mechanisms.

Biostimulant combination	Benefits	Proposed mechanisms	Reference
Microbial (*Bacillus* so) and a non-microbial (natural extract) biostimulant	Increases yield in lettuce (*Lactuca sativa*) under salt stress conditions	Decrease in antioxidant response and an up-regulation of cytokinin biosynthesis genes and elicit a cytokinin-dependent response	(68)
Chitosan nanoparticles + protein hydrolysates from microalgae	Promotion of plant growth by 49.5 %, enhancing carotenoid, chlorophyll, flavonoid and phenolic compounds in leaves, along with increased lycopene content in tomato	Presence of higher concentrations of phenylalanine, lysine, proline and the polyamines in the combined formulation enhance plant growth and physiological functions, presence of arginine in the PH and NP enhanced the activity of ureases facilitating nitrogen assimilation, augmentation in vegetative growth, could subsequently lead to an orchestrated enhancement in the metabolic synthesis and accumulation of lycopene in the fruits.	(69)
Seaweed extract + microbial biostimulant	Increase in total fruit yields per plant, total fruit numbers per plant, whole-season fruit yield, root dry weight, total root surface, increased plant crown number, total leaf number lead to an average increase of 23 % in marketable yield and 20 % in total yield as compared with the no-biostimulant control in organic production for 2 years	The beneficial microorganisms from the biostimulant have assisted the strawberry plant roots in absorbing the seaweed extract from biostimulant more effectively and efficiently by stimulating root growth and improving root system architecture and the complex ingredients potentially promote the activity of extraneous microbes from the microbial biostimulant as well as the indigenous communities of soil organisms by providing carbon (C) and N sources	(70)
Humic acid + seaweed extract	Improve salt tolerance and increase growth as well as anti-oxidant enzymes that mitigate damage caused by salinity stress	Increased hormonal effect, modulation of chemical, physiological and metabolic activities, increase in SOD levels are the attributed reasons for the salt stress tolerance	(71)

Protein hydrolysates

PHs are blends of amino acids, polypeptides and oligopeptides that are produced when various protein sources undergo partial hydrolysis. They can be derived from a variety of plant and animal sources, some of which are by-products or trash from different industries. Most of the ingredients in PHs formulations are amino acids (AAs). They exist in 2 different forms: simple (free AAs) and complex peptides (4). Protein hydrolysates are a valuable source of nitrogen and other active macromolecules for use in organic farming. Waste products from animal waste, food and agricultural sectors are the primary raw materials utilized in PHs production. Their transformation into biostimulants is a component of a circular economy strategy that supports sustainable agriculture and environmental preservation.

Protein hydrolysates (PHs) have gained popularity as plant biostimulants as they enhance germination, production and quality of a range of horticultural crops. PH application might also lessen the detrimental consequences of abiotic plant stress brought on by salt tolerance, drought and heavy metals. Recent research has shown that PHs may directly affect plants by boosting the metabolism of carbon and nitrogen and their hormonal function. PHs may have indirect impacts by enhancing nutrient availability and increasing nutrient acquisition and efficiency (14), regulating the uptake of nitrogen by the main assimilation-processing enzymes and regulating the activity of tricarboxylic acid cycle enzymes, namely citrate synthase, isocitrate dehydrogenase and malate dehydrogenase. The application of PHs may improve the diversity and abundance of the plant microbiome, which will aid in the plant's ability to endure biotic and abiotic challenges and obtain more water and nutrients (15). They may be applied near the root or as foliar sprays and available in the form of granules, soluble powders and liquid extracts. The main processes for creating PHs include hydrolysis of various plant biomasses and animal wastes through thermal, enzymatic and chemical (acid and alkaline hydrolysis) methods. Depending on the method and degree of hydrolysis, different hydrolysates have different flavours, solubility, appearances and biochemical safety.

Chemical hydrolysis of proteins

Chemical hydrolysis for the production of protein hydrolysates can be categorized into acid and alkaline hydrolysis. Spray drying, pasteurization and evaporation are applied to the hydrolyzed product. In 1920, the French chemist H. Braconnot published the first report on the acid hydrolysis of a protein (gelatin) at high temperatures. It is an aggressive process in which hydrochloric and sulfuric acids are used to hydrolyze proteins at high temperatures (> 121 °C) and pressures (> 220.6 kPa) (16). Acid hydrolysis of proteins is less expensive and advantageous. However, as a result of this process, some amino acids are affected or modified as in the case of destruction of tryptophan, partial loss of methionine, conversion of glutamine into glutamate and asparagine to aspartate (17). Alkaline hydrolysis, on the other hand, is an easy procedure that involves adding alkaline substances such as potassium hydroxide, sodium hydroxide and calcium hydroxide at a steady temperature. All of the protein peptide bonds were broken during chemical hydrolysis, which resulted in a significant level of protein hydrolysis and amino acid degradation. Furthermore, during this process, other thermolabile substances, such as vitamins are also degraded. A disadvantage of chemical hydrolysis is the racemization process or the change of free amino acids from their L-form to their D-form. However, plants cannot directly employ D amino acids for their metabolism; protein hydrolysates from chemical hydrolysis may become less effective (18) and the pH of the soil may change depending on how much and how often protein hydrolysates are treated. When D amino acids are applied to the soil, they inhibit bacterial growth and biofilm formation (19). The influence on pHs can be more noticeable with higher application rates or more frequent treatments and mostly it will not cause any harm.

Enzyme hydrolysis

Currently strong acid hydrolysis is increasingly being replaced by safe, moderate techniques such as enzymatic hydrolysis. Enzymatic hydrolysis is the process that employs different kinds of enzymes that convert organic residues into high proteinous compounds. The hydrolyzed plant and animal proteins produce amino acids as by-products and a common procedure in the farming sector is the foliar application of amino acids (20). Enzymatic hydrolysis is frequently employed to produce exact hydrolysates that preserve the nutritional value of the source protein and produce fish protein hydrolysates of superior quality. The use of enzymes for fish hydrolysate production produced stronger bioactive peptides and dramatically decreased the amount of garbage disposed of in landfills by 79 % (21). Alcalase, papain, pepsin, trypsin, alpha-chymotrypsin, pancreatin, flavourzyme, pronase, neutrase, protamex, bromelain, cryotin F, protease N, protease A, orientase, thermolysin and validase are some of the proteolytic enzymes that are frequently employed to hydrolyze protein rich by products (22). Enzyme hydrolysis results in 3 main structural changes: a reduction in the mean molecular mass, an increase in the accessibility of hydrophobic regions and the release of ionizable groups (23). Two different methods of protein hydrolysate preparation are depicted in Fig. 2.

Diverse sources of protein hydrolysate

Plant-based biomass includes legume seeds (24), alfa hay (25), wet-milled corn (26) and vegetable by-products; animal residues include animal epithelial or connective tissues such as leather by-products, blood meal (27), fish byproducts (28), chicken feathers (29) and casein (30). In particular, PHs originating from vegetable by-products and the wet-milling of corn are becoming increasingly common and well-liked in the scientific community and business sectors since they may offer a cost-effective, environmentally beneficial and long-lasting solution to waste disposal issues (31). Animal-derived proteins obtained by acid hydrolysis are the major sources of PHs biostimulants; the remaining portion is acquired from the enzymatic hydrolysis of proteins originating from plants (14).

Plant-derived protein hydrolysates for horticultural plants

Plant-derived PHs are eco-friendly and more cost-effective because they are produced from agricultural by-products mainly through enzymatic hydrolysis. The majority of essential amino acids are found in soy protein, which makes soybean (*Glycine max* Merr.) an excellent source of protein. The application of soy-based protein hydrolysates (SPH13 and SPH18 at 10 g/L) by soil drenching on tomato plants ('Micro Tom') has led to notable improvements in growth and fruit production. SPH18 increased the expression of defence-related genes, indicating enhanced resistance to certain pathogens. SPH13 statistically increased plant diameter by 24 % and height by 28 %, while SPH18 increased plant diameter by 32 % and chlorophyll content by 10 %. Both SPHs increased the total number of fruits per plant by over 80 % with the increase in total fruit weight by 81 % and 60 % respectively in the case of SPH13 and SPH 18 over untreated plants. Statistical analysis was performed using One-Way ANOVA, post hoc LSD tests and Tukey's Ladder of Powers for data transformation (32).

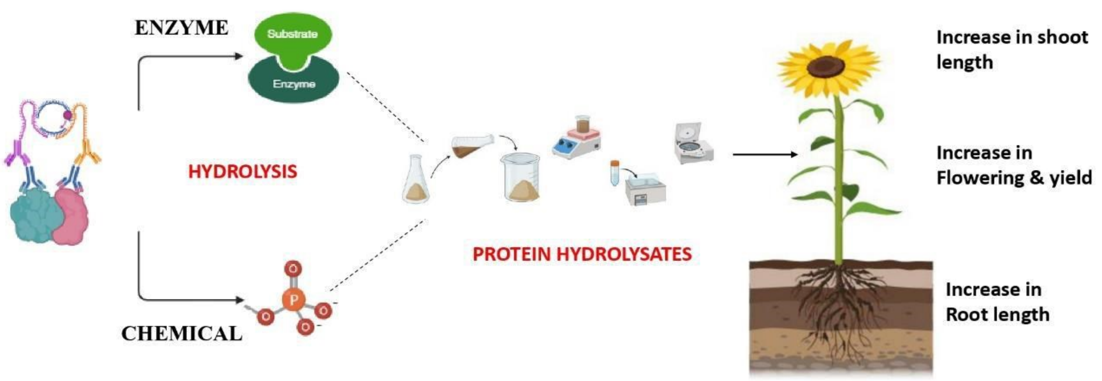

Fig. 2. Process of production of protein hydrolysates.

The use of protein hydrolysate-rich extracts from *Chondrus crispus*, a red seaweed, as a biostimulant has been explored in a study to improve plant growth and drought tolerance of tomatoes. The foliar application of *C. crispus* extract significantly increased shoot height by 20 %, 20 % and 12 % under well-watered conditions and by 4 %, 16 % and 13 % under drought conditions at 3 different time points (14th day, 16th day (recovery period), 21st day after biostimulant application). The treatment also led to a 70 % increase in shoot dry weight under controlled conditions. Application of the extract elevated the levels of abscisic acid (ABA) and proline, which are associated with drought tolerance. The gene Solyc02g084840, a drought marker, was upregulated by over 8-fold after drought stress, showing higher expression in treated plants. Statistical analysis was conducted using two-way ANOVA and Tukey's HSD test to determine the significance of the results (33).

Effects of 2 PHs biostimulants derived from alfalfa (AH) and red grape (RG), on the growth and nutraceutical properties of *Capsicum chinensis* L. were evaluated in a field study. Both biostimulants were applied at different doses (50 and 100 mL L^{-1}) and their impact was assessed at flowering and maturity stages. The application of AH at 50 mL L^{-1} significantly increased fresh leaf weight by 2.6 times, total fruit weight by 2.2 times and the number of fruits by 2.4 times compared to untreated plants and RG exerted the lesser influence on growth. Additionally, the biostimulants reported an increase in levels of nutraceutical compounds such as ascorbic acid and quercetin in leaves, chlorogenic acid and capsaicin in fruits and the statistical analysis was conducted using Bartlett's Test, Multiple-Way ANOVA test and Post hoc LSD test to determine the significance of the results (34).

Animal-derived protein hydrolysates for horticultural plants

Whey protein is becoming increasingly popular as a functional food ingredient. It is a by-product that is valued as a food ingredient with significant nutritional and functional qualities. It contains approximately 55 % of the nutrients found in milk and has a greater biological value than most other proteins due to its high sulfur-containing amino acid, which supports its antioxidant properties (35). Predigested proteins such as whey hydrolysate, which is primarily composed of dipeptides and tripeptides, are absorbed considerably faster than intact (non-hydrolyzed) proteins and free-form amino acids. Approximately 80 % of the total protein content in milk is made up of casein, a primary proteinaceous compound. Casein hydrolysate provides a mixture of amino acids starting from alanine to valine that is capable of promoting plant growth and stress (36).

Protein hydrolysate liquids or solids are now being made from waste products of fish processing. Fish protein hydrolysates are typically a product made from fish waste by-products under accelerated digestion by proteolytic enzymes. The fish proteins must be broken down by active proteolytic enzymes that are either above the temperature at which spoilage bacteria may live or outside of the pH range that would promote their growth. Controlling the hydrolysis process with the right proteolytic enzymes can result in the production of a wide range of products. Fish protein hydrolysate, which includes arginine, asparagine/aspartate, glutamine/glutamate, glycine, alanine and proline/hydroxyproline, is applied to various plants to promote their growth (37).

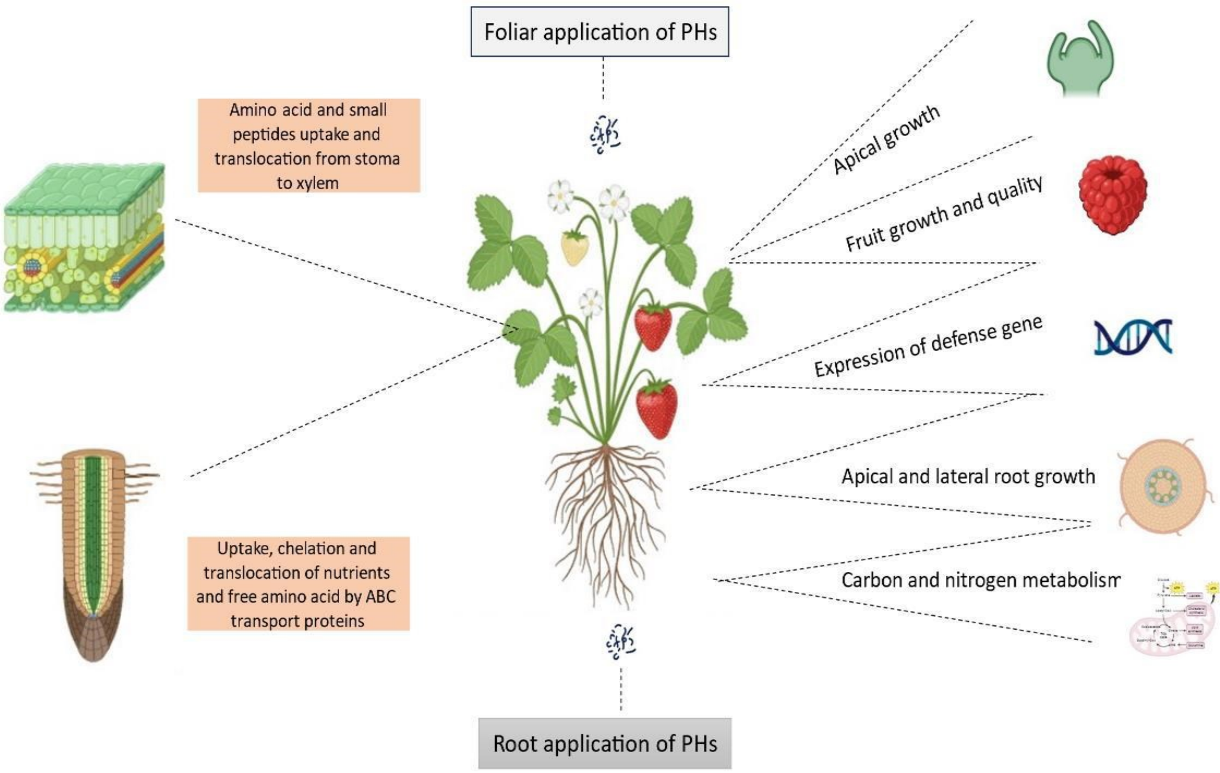

Fig. 3. Schematic representation of the different mechanisms of action of protein hydrolysate on fruits and vegetables.

Animal-derived PHs have a greater nitrogen concentration (9-16 %) than vegetal-derived PHs. Furthermore, since most of the raw materials needed in agriculture are obtained from plant-derived waste, using animal-derived PHs as a biostimulant is an economical method. Because animal-derived PHs can promote plant growth and lessen the impacts of environmental stressors, they have been used for more than 50 years (38). A comparative study on the effect of plant and animal-derived protein hydrolysate products on tomatoes showed the inhibition of plant growth and development upon topical application of animal-based PHs due to their induction of phytotoxicity. Conversely, no growth depression or phytotoxicity was noted for plant-derived amino acids. Animal-derived protein hydrolysates appear to include more free amino acids (particularly small-size amino acids like glycine and proline) and salts (such as NaCl) than plant-derived protein hydrolysates, which may explain the growth inhibition that results from them (39).

Recently, animal-derived protein hydrolysate use has been the subject of increasing concern over food safety, as evidenced by the recent ban on the application of substances to crop plants intended for human consumption in organic farming. Further restrictions could be enforced on animal-derived protein hydrolysate in food preparation for vegetarians or those adhering to religious dietary prohibitions against eating meat because it is necessary to prevent food from becoming contaminated with anything originating from animals (4). However, the assessment of the safety and effectiveness of organic fertilizer from animal-derived PHs showed that there was no possible damage to the ecology or public health when it was used in conventional and organic farming and depending on the source and method of production, average nitrogen content ranging between 10 and 29.9 % N. In contrast, numerous studies have shown more significant effects of animal-derived PHs due to their rapid absorption of short molecular weight peptides and free amino acids by all plant tissues (40). In addition to these studies, some of the results showing the impact of protein hydrolysates are shown in Table 2 and the Benefits of protein hydrolysates on plant growth and stress tolerance are depicted in Fig. 4.

Mechanism of action of protein hydrolysates on vegetable crops, fruits and ornamental plants

In horticultural plants, tolerance to abiotic stresses is an important trait because of the cash value of plants which is usually higher than field crops. These plants require more resources for farming since they provide a source of many nutrients, fibre, minerals and carbohydrates, which are essential for a healthy diet. Stress tolerance can also be induced by biostimulants or specific bioactive compounds upon application to vegetable crops when they truly need to be protected. PHs can improve the soil structure, water and mineral nutrient transport and usage efficiency, thereby increasing plant productivity as well as stress tolerance. Modification in root morphology by changing the architecture of roots in terms of length and total area also contributes to stress tolerance. (41). A study investigated the effects of a biostimulant complex (BC) consisting of fish hydrolysate, *Aloe vera* extract, and kelp on cannabis root architecture, phytohormone profile and nutrient uptake. The application of BC significantly enhanced root development, with a 1.85-fold increase in root tips ($P = 0.050$), a 1.94-fold increase in branch points ($P = 0.038$) and a 1.77-fold increase in total root length ($P = 0.046$). Significant increases in jasmonic acid and salicylic acid contents were noticed in roots. The treatment also increased the uptake of phosphorus ($P = 0.038$) and potassium ($P = 0.040$). Statistical analysis included the use of a general linear model (GLM) and Tukey's test for assessing treatment effects and principal component analysis (PCA) for profiling phytohormones (42). PHs can promote biomass and root development in a variety of crops (tomatoes, lilies and potted snapdragons). Generally, protein hydrolysates that contain anti-stress compounds, such as proline or glutamic acid, can be applied when stress occurs or during stress conditions. On the contrary, those that are involved in the activation of biosynthesis of bioactive compounds must be applied before stress occurs (43). The growth and development of

Table 2. Influence of protein hydrolysates on plant growth.

PROTEIN HYDROLYSATES	CROP	EFFECTS	REFERENCE
Chichen feather-derived protein hydrolysate	Tea	Increase in leaf chlorophyll content	(72)
Meal worm-derived protein hydrolysate	All crop	Improve root architecture	(73)
Animal-based protein hydrolysate	Basil	Increased primary and secondary metabolite content	(74)
Plant-derived protein hydrolysate	Leafy vegetable	Increasing nitrogen use efficiency	(75)
Pea protein hydrolysate	Courgette, melon, pumpkin, tomato snack paprika	Improve rhizosphere microbial community	(76)
Micro algae-derived protein hydrolysate	Tomato	Improve vegetative growth of tomato	(69)
Plant-derived protein hydrolysates	Lettuce	Enhancing the yield	(77)
Fish protein hydrolysates	Deep netted melon	Increase in fruit yield	(78)
Plant-derived Protein hydrolysates	Grapevine	Increase in growth, physiological parameters, fruit development and yield of grapevine	(79)
Plant based protein hydrolysates	Peppermint and spearmint	Improving yield	(50)
Legume derived protein hydrolysates	Capsicum	Mitigating drought stress	(80)

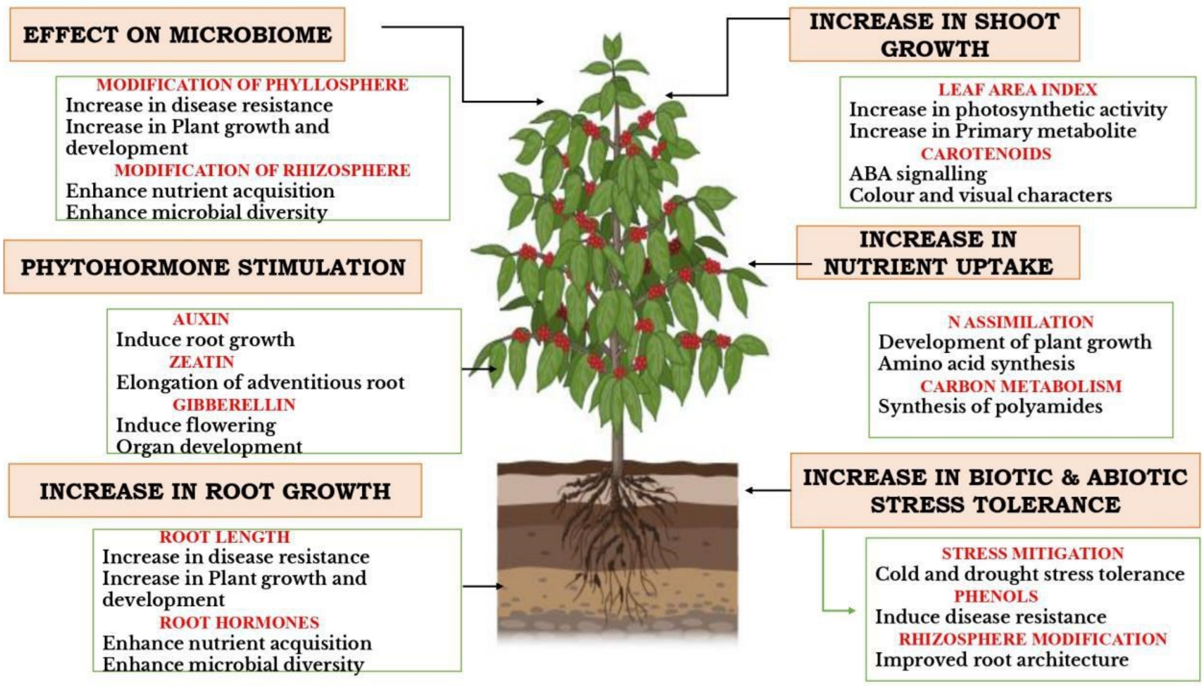

Fig. 4. Benefits of protein hydrolysates on plant growth and stress tolerance.

Brinjal and Chilli plants are improved in response to the exogenous application of animal-derived PHs (feather hydrolysate) due to the action of tryptophan which is the precursor of the phytohormone indole acetic acid. Feather hydrolysate contains IAA which is required for embryogenesis, tip dominance, cell plasticity, tissue elongation and root development. Cold induces damage to cell membranes with destabilization of the phospholipid layers (44). When animal-derived PHs (enzymatic hydrolysates obtained from animal haemoglobin) were applied to cold-stressed strawberry plants, the plants blossomed earlier, the survival rate increased and a noticeably greater volume of fruit was produced due to the proline content in the biostimulant, which has reduced the effect of abiotic stress (45). Further research revealed that PHs produced from alfalfa influence the transcriptional accumulation of important genes involved in primary carbon metabolism, such as fumarate dehydrogenase, malate dehydrogenase and phosphoenolpyruvate carboxylase lead to an increase in carbon skeleton synthesis, that may encourage plants to absorb nitrogen (46). These results showed the fine regulation of photosynthesis, primary carbon metabolism and N assimilation by PHs collectively contribute to greater plant biomass and growth. In tomatoes, expression of genes encoding glutamine-dependent asparagine synthetase, aspartate aminotransferase, nitrate reductase, glutamine synthetase and N-associated genes linked to amino acid synthesis and turnover (glutamate dehydrogenase, serine decarboxylase) and protein accumulation (translation initiation factors) was stimulated by an alfalfa-derived PH.

The biostimulant potential of sago bagasse hydrolysate (SBH), derived from the sago industry by-product was investigated. The plant growth-promoting effects of SBH were confirmed through seed germination and greenhouse experiments. SBH treatment enhanced seed germination, increased protein (3.13 %) and sugar (9.53 %) content and significantly boosted the activity of carbon-assimilating enzymes (malate dehydrogenase by 5.0 %, citrate synthase by 11.47 % and isocitrate dehydrogenase by 8.08 %). It also elevated nitrogen-assimilating enzymes, including nitrate reductase (15.14 %) and glutamate synthase (10.98 %). qPCR analysis showed that SBH upregulated genes involved in carbon and nitrogen assimilation, indicating its potential as a low-cost, eco-friendly biofertilizer to enhance agricultural productivity (47).

Impact of foliar application of 2 commercial protein hydrolysates (PHs)-Trainer® (plant-derived) and Isabion® (animal-derived)-on baby-leaf spinach grown under 3 nitrogen (N) levels: 2 mM (N2, deficient), 8 mM (N8, sub-optimal) and 14 mM (N14, optimal) were investigated. The focus was on optimizing N use and reducing nitrate accumulation. Trainer® increased spinach leaf fresh weight by 16 % at N14 (3.28 g/plant vs. 2.82 g/plant in control) and boosted chlorophyll content by 10 % (714.7 μg/g vs. 597.8 μg/g). Isabion® improved leaf weight by 19 % at N8 (1.95 g/plant vs. 1.85 g/plant) but led to higher nitrate levels at N14 (2677 mg/kg), still below the EU limit. Antioxidant profiles showed no significant changes (48).

Apart from regular cultivation systems, the influence of biostimulant application was studied under hydroponic cultivation. The application of protein hydrolysate to hydroponically grown tomato plants under nutrient stress or sub-optimal temperatures had a significant effect on the concentration of the endogenous chorismate-derived phytohormone salicylic acid and the concentration of auxin. An increase in auxin concentration was detected in the shoot portion, but in roots, a significant increase in salicylic acid concentration was observed, which resulted in a significant increase in the primary and lateral root growth of the tomato. These results suggested that biostimulant application stimulated the salicylic acid pathway in tomato plants to cope with the stress (49).

Investigations were made on the impact of adding a commercial protein hydrolysate supplement (Amino16®) to the nutrient solution of soilless-grown peppermint (Mentha × piperita L.) and spearmint (*Mentha spicata* L.) using a floating raft system. The research aimed to enhance the quality of the produced herbs by improving their nutritional, essential oil and polyphenolic content. The addition of protein hydrolysates at 0.50 % reduced plant height and root length in peppermint and spearmint without affecting the plant biomass. Nitrate content was significantly reduced, while chlorophyll content was greatly increased by 16.6 % in peppermint and 17.6 % in spearmint at 0.25 % PH application. In spearmint, total antioxidant capacity rose by 165.2 % and total phenols by 107 % at 0.50 % PH level, whereas no such increase was noticed with peppermint. Further essential oil content was increased by 26.5 % in peppermint and 26.3 % in spearmint at the same concentration. The statistical tool used in the study was a one-way analysis of variance (ANOVA). Significant differences among means were detected using the least significant differences (LSD) test at a probability level of $p < 0.05$. Assumptions of normality were tested using the Shapiro-Wilk test (50).

Floating raft systems are promising for microgreen cultivation and ease the application of biostimulants to roots. A study evaluated the influence of a protein hydrolysate solution of legume origin in the cultivation of *Daucus carota* L. and *Anethum graveolens* L. microgreens in a greenhouse using a floating raft system. In carrot microgreens, increases in anthocyanins (+461.7 %) and total phenols (+12.4 %) were observed and in dill (*Anethum graveolens* L.) increase in fresh yield (+13.5 %) and ascorbic acid (+17.2 %) content was noticed as a result of the use of protein hydrolysate in the nutrient solution; soluble proteins and total free amino acids increased by 18.5 % and 20.6 % respectively, in both species. The statistical tools used in the study include a two-way analysis of variance (ANOVA) and Duncan's multiple range test. Significant differences among means were identified using Duncan's multiple range test at a significance level of $p \leq 0.05$. Additionally, principal component analysis (PCA) was performed to analyze the relationship between different variables and treatments, providing insight into how the biostimulant and species influenced the overall traits of the microgreens (51).

The fastest-growing sector in the horticultural industry is the ornamental industry. Ornamental plants offer a very diverse range of products. The most important aspects of flower production to consider are flower growth, blooming characteristics and shelf life. Growing plants in containers with the use of artificial growth media offers a convenient marketing package, quicker transportation, quick crop turnover and an extended planting season is also a prevalent practice. Greenhouse floriculture uses the greatest number of resources and chemicals to increase flower yield and shelf life compared with crop cultivation (52). Flower dimensions and very long vase life (postharvest duration) are among the preferable quality traits that account for the great ornamental value of the flowers. The spraying of several chemicals is practised to increase the vase life of flowers. An alternate approach is the use of naturally derived biostimulants for enhancing ornamental flower production and post-harvest handling. The impact of protein hydrolysate application on flower quality and yield was discussed in *Chrysanthemum*, *Calendula officinalis*, *Petunia* x *hybrida* and prime Rose as below.

Among the top-selling and most famous cut flowers in the world, with a prominent position in the cut flower industry is *Chrysanthemum morifolium* (Asteraceae). In this study, the effects of three commercial biostimulants: two plant PHs (PH V1 and V2) and one animal PH (PH A) on the morphophysiological traits, ornamental quality and mineral composition of 2 cultivars of chrysanthemum (*Chrysanthemum morifolium* -Pinacolada and Radost) were evaluated. Only the plant-derived PHs (V1) treatments in both cultivars significantly increased the fresh plant biomass (by 18 %), stimulation of stem elongation and the apical flower diameter compared to the untreated control especially in Pinacolada. Concentrations of nitrate and P in the leaves and Ca in the flowers also increased significantly in comparison to the control (+43 %, +27 % and +28 % for nitrate, P and Ca respectively). In Radost, PH A and V2 applications caused a significant reduction in nitrate concentration in both leaves and flowers compared with the control. The study utilized two-way ANOVA and Tukey's HSD test to assess treatment effects and identify significant differences. Principal Component Analysis (PCA) was used to explore key factors driving variations among the treatments and cultivars (53).

In a study on *Calendula officinalis* (Pot Marigold), the plants were treated in a greenhouse with varying dosages of soy-protein hydrolysate (SPH) (0, 1, 2.5, 5 and 10 g/L) for 21 days. Foliar spraying (SPH-F) and soil drenching (SPH-S) were followed. SPH-F had no discernible effects on floral biomass, whereas SPH-S increased the biomass of both the flowers and the entire plant. The application of the lowest (1 and 2.5 g/L) SPH-S dosage had the greatest influence on floral biomass and the best treatment for increasing the average number of flowers per plant was SPH-S at 1 g/L (n = 14.30 for SPH-S 1 g/L and n = 8.00 for control). Both methods of application induced changes in the flower metabolome, by which hexadecanoyl- (16:0) and linoleoyl- (18:2) lysophosphatidylethanolamines, (known as plant growth regulators) were induced, together with dipeptides, diglycerides and saponins, while the amount of several flavonoids decreased (59). One-way ANOVA and Duncan's test were employed to analyse the plant growth and yield and two-way ANOVA, multiple comparisons and post-hac test were used for the metabolic studies (54).

The impact of an animal-based protein hydrolysate (PHs) biostimulant on petunias was assessed by comparing three doses (0, 0.1, 0.2 g L^{-1}) of application via foliar spray and root drenching. The highest dose (0.2 g L^{-1}) applied as a foliar spray significantly enhanced plant quality, yielding 161 flowers, 450 leaves, 1487 cm^2 leaf area and 35 g aboveground dry weight. It also improved nutrient content, root morphology and leaf gas exchange, with net photosynthesis at 22.9 µmol CO_2 m^{-2} s^{-1} and stomatal conductance at 0.42 m mol H_2O m^{-2} s^{-1}. The study employed two-way ANOVA to analyze the interaction effects between biostimulant doses and application methods, with Duncan's multiple-range test for mean separation at ($p \leq 0.05$) (55).

The study explored the effects of foliar applications of microalgae hydrolysates (*Scenedesmus* sp. and *Arthrospira platensis*) on the growth and nutrient status of *Petunia* × *hybrida*. Three treatments were applied: control (T1), *Arthrospira* (T2) and *Scenedesmus* (T3), with foliar spraying 5 times on different days after transplanting. The study found that *Scenedesmus* treatment significantly accelerated plant development, increased the root dry weight by 49 % and the number of leaves and shoots by 24 % and 20 % and flower numbers by 18 % respectively over untreated plants. *Arthrospira* also enhanced root dry weight by 35 % and the number of flowers by 66 %. The study utilized one-way ANOVA and Fisher's LSD test for statistical analysis (56).

The study investigated the effects of protein hydrolysate (PH) derived from fish (anchovy) by-products on the growth of primrose plants and analyzed root system architecture (RSA) using machine learning. PH treatments at 0.5, 1.0 and 1.5 g/L were applied via root drenching. Application of 1.5 g/L significantly increased dry weight (4.85 g) and leaf area (39.87 cm^2), while the 1.0 g/L treatment resulted in the highest chlorophyll content (34.35 SPAD units) and improved RSA parameters such as surface area, projected area and root volume. Machine learning models, including MLP, GP, RF and XGBoost, were used to predict RSA traits, with MLP and GP showing the highest accuracy (57).

An extensive and varied range of microbial taxa colonizes plants, providing them with the ability to absorb nutrients and water as well as to endure both biotic and abiotic stress. Plant-associated bacteria may find their optimal food source in the substrates that PHs, such as amino acids. Recent research that PH treatments alter plant microbiomes, lending credence to the theory that PHs may function, at least partially, by altering the makeup and motility of these microbial communities. (58,59). A study linked the application of biostimulants to changes in the composition of the soil microbial population and increased soil microbial activity in damaged soils leading to improved plant establishment (60).

Changes in the variety of the phyllosphere microbial population and an improvement in the growth and chlorophyll content of lettuce leaves were observed during the evaluation of a PHs-based biostimulant product derived from a legume and another product derived from extracts of tropical plants. Topical application of these products altered the composition of the microbial population and stimulated the growth of specific bacteria such as *Pantoea, Micrococcus*, *Acinetobacter* and *Bacillus*. These organisms were capable of producing IAA solubilizing phosphorous and acting against *Erwinia amylovora*. *Bacillus* strains from lettuce leaves showed substantial inhibitory effects on phytopathogens (61).

Microbial biostimulants

The Fertilizer Control Order (FCO) of 1985 in India classified biostimulants primarily into 2 categories: biofertilizers and organic fertilizers. Biofertilizers are products that contain living microorganisms in the form of carrier-based solids or liquids that are used in agriculture for fixing nitrogen, solubilizing phosphorus or mobilizing nutrients to improve soil and/or crop yield. This rule was updated in 2021 and now describes biostimulants as *« a substance or microorganism or a combination of both whose primary function when applied to plants, seeds or the rhizosphere is to stimulate physiological processes in plants and to enhance its nutrient uptake, growth, yield, nutrient efficiency, crop quality and tolerance to stress, regardless of its nutrient content, but does not include pesticides or plant growth regulators which are regulated under the Insecticide Act, 1968 (46 of 1968) » (62).*

All plant growth-promoting microorganisms (PGPMs), in addition to biofertilizers are regarded as microbial stimulants since they can affect plant physiological pathways through the action of a biostimulant. Plant metabolism responds differently to the application of PGPM and the compounds they create, which affects hormone levels, enzyme activity and the K^+/ Na^+ ratio. The physiological parameters related to photosynthetic efficiency, water usage efficiency and nutrient use efficiency are positively impacted by these metabolic responses (63).

To preserve soil fertility and lessen the loss of soil biodiversity, a more sustainable agriculturally productive system is becoming increasingly necessary. Microbial biostimulants can guarantee high-nutrient agricultural yields and mitigate the adverse effects of changes (64). Precise selection of consortia and helpful microbes are prerequisites for the increased usage of these products. Given the damage caused by current fertilization methods to the environment, optimizing plant-microbe nutritional interactions for more environmentally friendly agricultural systems is a major area of focus (65).

Additionally, plants can form symbioses with arbuscular mycorrhizal fungi (AMFs), which expand the surface area of the roots and in turn also increase the absorbance of nutrients. Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria and other taxa are among the highly diverse array of endophytic bacteria that make up PGPM. Because of their extensive range, the several genera improve plant growth and the most studied microorganisms include *Aeromonas, Arthrobacter, Azospirillum, Azotobacter, Bacillus, Clostridium, Enterobacter, Gluconacetobacter, Klebsiella, Pseudomonas, Rhizobium* and *Serratia.* Several other endophytic bacteria are widely used for drought mitigation and promoting plant growth (66). Influence of microbial biostimulants on plant growth is depicted in Table 3.

Table 3. Influence of microbial biostimulants on plant growth

Microorganisms	Strain	Crop	Effects	Reference
Azotobacter	*Azotobacter chroococcum*	Sorghum	Increase the germination of seeds	(81)
	Azotobacter chroococcum	Tomato	Increase in yield and fruit size	(82)
Azospirillum	*Azospirillum brasilens*	Hydroponic Pumpkin	Increases the female flower	(83)
	Azospirillum spp.	African marie gold	Increase the growth parameters	(84)
Bacillus	*Bacillus megaterium*	Onion	Increase in yield under field condition	(85)
	Bacillus spp.	Saffron	Defense against *Fusarium oxysporum*	(86)
Pseudomonas	*Pseudomona fluorescens*	Wheat and Maize	Increased Iron uptake and accumulation in grains	(87)
	Pseudomonas putida	*Arabidopsis*	Influence on root architecture system	(88)
Rhizobium	*Rhizobium* MAP7	Lettuce	Stimulate growth and pigemntation	(89)

The combined action of protein hydrolysates and microbial biostimulants and their future aspects.

Protein hydrolysates and microbial stimulants are the 2 important categories of biostimulants and the combination of both is an interesting approach to safeguarding plants from stressors and satisfying their nutritional needs. Very few studies have combined the 2 categories of biostimulants. The study explores the combined effects of plant-derived protein hydrolysate (PH) and arbuscular mycorrhizal fungi (AM) on eggplant yield and quality over 2 years. The results of the study showed the mutual effects of the biostimulant on yield, quality and nutritive parameters of the fruit as well as the nutrient uptake potential of the plants over the single category of biostimulant. AM forms a profuse network of hyphae, which can help plants take up more nutrients and water from the soil. Similarly, the response for the protein hydrolysates can be linked to the presence of carbohydrates, amino acids and phytohormones leading to enhanced mineral uptake and assimilation of nutrients. Enhancement in the nutrition of the fruit is explained by the modulation of the uptake and accumulation of minerals.

PH and AM combined application boosted qualitative fruit parameters, such as soluble salts (+16 %), chlorogenic acid (+4.6 %), potassium (+8.6 %), magnesium (+23.9 %) and anthocyanin concentrations. At the same time, the combination of biostimulants significantly reduced the glycoalkaloid content (-19.8 %) in fruits, making them more nutritious and also decreasing the browning potential over the control plants. Nitrogen use efficiency (+26.7 %) and uptake efficiency (+18.75 %) of the plants were also improved to higher extent than the control. All these observations showed the superiority of the beneficial impact of combination of biostimulant over control as well as the use of single biostimulant. The study used ANOVA (Analysis of Variance) for statistical analysis. Specifically, a two-way ANOVA was performed to assess the impact of biostimulant treatments and yearly variations (main factors) on plant growth, yield and quality traits. Tukey's HSD test at $p \leq 0.05$ was employed for mean separation and to determine significant differences between treatments (9).

The research investigated the synergistic effects of a microbial-based biostimulant tablet containing *Rhizophagus intraradices* and *Trichoderma atroviride* and a plant-derived protein hydrolysate (PH) on lettuce *Lactuca sativa* L. grown under saline and alkaline conditions. The experiment employed a 3 × 3 factorial design with treatments including different nutrient solutions (standard, saline and alkaline) and biostimulant applications (control, microbial biostimulant and microbial biostimulant combined with PH). Results demonstrated that the combined application of microbial based biostimulant and PH was more effective rather than microbial biostimulant application alone to mitigate the negative effect of stress on the growth of Lettuce under saline, alkaline and standard nutrient solution conditions. The percentage of yield increase in comparison to the control (i.e., no application) was higher under standard, saline and alkaline conditions with microbial tablet + PH application (62, 40 and 43 % respectively) than with the microbial biostimulant tablet (46, 25 and 18 % respectively) alone. Similarly increase in proline content was also reported. The better crop performance of combined biostimulant treated plants has been attributed to (i) higher nutrient uptake, (ii) better root system architecture, (iii) osmotic adjustment and (iv) increase of several secondary metabolites (e.g., flavonoids, terpenes and glucosinolates). The study used analysis of variance (ANOVA) to analyze the data, with significant differences between treatments identified using Duncan's multiple-range test at a significance level of ($P = 0.05$) (10).

This study examined how PHs and AMF affect the growth of citrus (Goutoucheng) seedings, physiological processes and the expression of genes that respond to stress (SOSs, TIPs and PIPs) in response to salt stress. This study shed light on the process by which PHs and AMF interact to adapt to citrus plants' salt tolerance. Citrus root AMF colonisation was stimulated by PH application. There was a 21 % rise in the overall colonisation rate of RP (AM + PH) as compared to the individual Ri (AM) treatment. Due to richness of polypeptides, oligopeptides and free amino acids, PHs provide sufficient nutrients for the growth of rhizosphere microorganisms including AMF and plants. Applying PHs can significantly enhanced the physicochemical characteristics of soil, resulting in the development of an ideal rhizosphere environment for the growth of soil microbes and subsequently raising their biomass. In comparison to the AM inoculation, the hyphae colonisation rate, overall colonisation rate and hyphal density in the combined treatment were significantly higher. These findings suggest that PHs facilitated AMF colonisation on citrus roots under salt stress, leading to the formation of more mycelial structures and the release of more GRSP (Glomalin related soil proteins). Citrus's resistance to salt stress was increased by the interaction between PHs and AMF as well as the symbiotic relationship between AMF and citrus. As eco-friendly biostimulants in crop production, PHs and AMF can improve resilience to environmental stress (67).

Combining protein hydrolysates and microbial biostimulants can be an alternative approach to sustainable horticulture practices. As protein hydrolysates are rich sources of amino acids and short peptides, they provide nourishment to plants and associated microbial communities. Combing both PHs and microbial biostimulants will certainly enhance plant growth by stimulating cell division and photosynthesis, the production of growth hormones, enzymes, and other bioactive substances. In addition to their ability to promote plant growth, the bioactive compounds in PHs can enhance plant stress tolerance and microbial biostimulants can induce systemic resistance in plants, making them more resilient to environmental stresses such as drought, salinity, alkalinity and pathogens. In soil health improvement, both can contribute to soil structure improvement and microbial activity. Thus, combining both PHs and microbial biostimulants can improve plant nutrient uptake, plant growth promotion and yield, stress mitigation and soil health improvement. Further research and development are also extremely important for the future of this combo. The best formulations, administration techniques and particularly interactions between various protein hydrolysate types and microbial biostimulants can all be explored in immediate future. This will optimize their benefits for different crops and farming systems and help them become more effective. The ways and means for combining these 2 as a single formulation need to have thorough standardization of the process. While combining these components in making biostimulants, their interaction in the product also needs to be studied well to avoid any adverse effects on each other. Certainly, there should not be any negative influence among microbial with protein hydrolysates, making suitable formulations will be a concern for such products. Concentrated liquid form/ granular form /water-soluble powder formulations are suggested for better delivery. These technologies established effectiveness, economic viability and compatibility with current agricultural techniques will probably have an impact on their future acceptance which should be focused more. Due to the advantages and attraction of each product by the consumers, the combined formulation will be a future biostimulant in the market.

In addition, certain cell-free microbial metabolites are also having biostimulant properties could also be considered. Some bacteria can secrete metabolites that improve soil health and promote plant growth. One of the advantageous metabolites of some bacteria is bacterial exopolysaccharides, which are said to improve the chemical and physical characteristics of the soil as well as the rhizosphere microflora. In economical aspects Protein hydrolysates, which can be from plant or animal sources or from the hydrolysis of proteins, are variable in price depending on how they are processed and from what source. Protein hydrolysates and microbial biostimulants combined may be a financially viable product if the benefits of the combined product are evident and exceed the increased costs of manufacturing. Market demand, skillful formulation and effective distribution are necessary for success. Determining the overall viability and profitability will need performing in-depth cost-benefit analysis and market research. Microbial biostimulants necessitate the cultivation and upkeep of advantageous microorganisms, which can be expensive. The complicated formulation procedures that may be required to combine these two items could raise the cost of production. On the other hand, economies of scale might lower costs per unit. Successful marketing of the combined product can be achieved by examining competitors and their offerings. If comparable items are profitable, that could serve as a guide for viability.Future research may also think about this microbial metabolite for combining with other stimulants to augment the benefits and Fig 5. depicts the combined formulation of protein hydrolysates and microbial biostimulants.

Conclusion

PHs has significant potential to improve not only the agronomic performance of several horticultural crop species but also their resistance to stressful conditions. Many reports point out the beneficial effect of PH application on plant physiological processes relating to yield and quality parameters, therefore PHs have greater potential to improve crop overall performance. Root and foliar applications of PHs from various sources have resulted in improved root growth and modified root architecture, improved nutrient acquisition via the production of enzymes such as nitrate reductase, glutamine synthetase and Fe (III)-chelate reductase and biotic and abiotic stress resistance. It also exhibits hormonal activities such as those of auxin, cytokinin and gibberellin which influence every part of the plant. PHs not only improve yield but also improve some quality parameters such as fruit size, visual quality, soluble solids and antioxidant content and also increase the phenolic

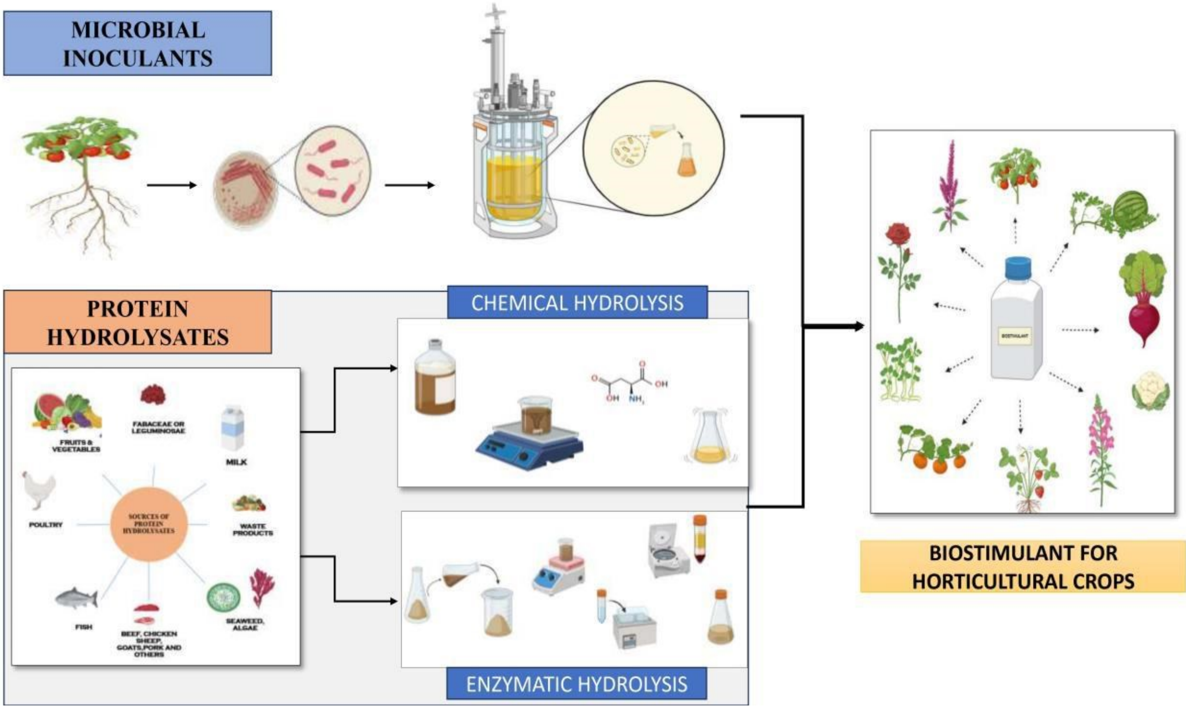

Fig. 5. Depicts the combined formulation of protein hydrolysates and microbial biostimulants.

content which acts as a defense compound against diseases. PH also promotes amino acid synthesis via the expression of genes encoding glutamine-dependent asparagine synthetase, aspartate aminotransferase, nitrate reductase and glutamine synthetase which are involved in N metabolism. These strains also exhibited high tolerance to elevated ROS. Recent studies have provided evidence that PHs can affect plant microbiomes and be involved in the modification of the root architecture. Furthermore, microbial biostimulants especially the PGPR, also play an important role in influencing the plants' growth and yield, mitigating biotic and abiotic stressors, and offering multiple benefits to the plants.

Several researchers are exploring the potential of microbial stimulants on one side and protein hydrolysates on the other for the benefit of crop plants and soil health and there is a lacuna on the positive side of combining these products in a single formulation. Protein hydrolysates have been shown to improve the phyllosphere and rhizosphere microbiota, which in turn has an indirect impact on crop growth and development (3). A few of the advantages of these products may come from modifications in the make-up and activities of these plant-associated communities, as recent research has shown that plant microbiomes are impacted by PHs. A literature survey suggested the combination of these two components as a single biostimulant product could have additive effects on crop and soil health, especially by increasing plant-microbial interactions and increasing plant yields. Combining microbial biostimulants and protein hydrolysates is a promising approach to raising agricultural productivity and sustainability. Future developments for this novel strategy will be largely determined by ongoing studies, technology breakthroughs and farmer uptake.

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Authors' contributions

K Kiruba wrote the framework of this review article; K Kumutha conceptualized the idea this review article and also performed manuscript correction and editing; KGS, PV, MLM and RA reviewed and helped in editing the article.

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