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ORIGINAL RESEARCH

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Casein as protein and hydrolysate: Biostimulant or nitrogen source for *Nicotiana tabacum* plants grown in vitro?

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Abstract

In contrast to inorganic nitrogen (N) assimilation, the role of organic N forms, such as proteins and peptides, as sources of N and their impact on plant metabolism remains unclear. Simultaneously, organic biostimulants are used as priming agents to improve plant defense response. Here, we analysed the metabolic response of tobacco plants grown in vitro with casein hydrolysate or protein. As the sole source of N, casein hydrolysate enabled tobacco growth, while protein casein was used only to a limited extent. Free amino acids were detected in the roots of tobacco plants grown with protein casein but not in the plants grown with no source of N. Combining hydrolysate with inorganic N had beneficial effects on growth, root N uptake and protein content. The metabolism of casein-supplemented plants shifted to aromatic (Trp), branched-chain (Ile, Leu, Val) and basic (Arg, His, Lys) amino acids, suggesting their preferential uptake and/or alterations in their metabolic pathways. Complementarily, proteomic analysis of tobacco roots identified peptidase C1A and peptidase S10 families as potential key players in casein degradation and response to N starvation. Moreover, amidases were significantly upregulated, most likely for their role in ammonia release and impact on auxin synthesis. In phytohormonal analysis, both forms of casein influenced phenylacetic acid and cytokinin contents, suggesting a root system response to scarce N availability. In turn, metabolomics highlighted the stimulation of some plant defense mechanisms under such growth conditions, that is, the high concentrations of secondary metabolites (e.g., ferulic acid) and heat shock proteins.

1 | INTRODUCTION

In plant nutrition, nitrogen (N) is crucial for the synthesis of amino acids, proteins, nucleic acids, and phytohormones, among other compounds. Soil N is available to plants in the form of nitrates (NO_3^-) and, to a lesser extent, ammonium ions (NH_4^+) (The et al., 2021). However, high-quality soil also contains a high proportion of organic N compounds, such as proteinogenic and non-proteinogenic amino acids, small peptides, nucleotides, and their polymers, including proteins larger than 100 kDa (Nasholm et al., 2009; Warren, 2014). These compounds are metabolized by soil microorganisms, but plants also have mechanisms for their utilization (Adamczyk et al., 2008; Adamczyk et al., 2010; Lonhienne, Trusov, Young,

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Rentsch, et al., 2014; Paungfoo-Lonhienne et al., 2008; Paungfoo-Lonhienne et al., 2012; Stoelken et al., 2010).

In contrast to plant inorganic N assimilation, organic N sources for plant growth have only recently been researched in the context of sustainable agriculture and environmental protection (Caruso et al., 2019; Franklin et al., 2017; Tegeder & Masclaux-Daubresse, 2018; Yan et al., 2020). The findings of such studies have indicated that the depolymerization of high-molecular-weight N compounds by extracellular proteases into small peptides and amino acids is the rate-limiting step in the N-cycle whereby proteins become available to plants (Warren, 2021). Plants produce many proteases that cleave multiple substrates and act in various processes (including development, homeostasis, growth, symbiosis and especially in plant defense and disease resistance), and their activity is tightly regulated accordingly (Godson & van der Hoorn, 2021; van der Hoorn & Klemencic, 2021). Similarly, during organic N uptake, proteases may act through four routes: (1) proteases are released from the roots to the external medium, and the resulting products diffuse back, (2) proteases inside root cells cleave their substrates after protein endocytosis, and (3) proteolysis occurs in root surface cells (with cell wall-bound proteases) or (4) in the apoplast, to which proteins diffuse through pores (with plasma membrane or inward-facing cell wall proteases) (Adamczyk, 2021; Greenfield et al., 2020; Kohli et al., 2012).

N uptake into cells and cellular compartments depends on integral membrane transporter proteins, as shown by the wide range of transporters for inorganic and organic N compounds with different substrate affinities and specificities identified in roots (Tegeder & Masclaux-Daubresse, 2018). All plant species can acquire intact amino acids, albeit with significant differences in their effectiveness, even at the subspecies level (Adamczyk et al., 2010; Guo et al., 2020). Studies conducted in *Arabidopsis thaliana*, angled lobelia, and wheat have confirmed the uptake of di−/tripeptides and their utilization as the sole N source (Hill et al., 2011; Komarova et al., 2008; Soper et al., 2011). Feeding experiments using a single amino acid supplementation showed its subsequent accumulation *in planta*, thereby inhibiting different metabolic pathways and negatively affecting plant growth and development (Pratelli & Pilot, 2014). On the other hand, some studies showed that up-taken amino acids and intact peptides can be rapidly metabolized (Persson et al., 2006; Warren, 2009).

Combined, these findings suggest that peptides and protein hydrolysates, representing a mixture of amino acids, could be a realistic source of N for plant growth. Organic manures (plant and animal wastes) as a source of plant nutrients have been readily used for centuries in agriculture to increase soil fertility, that is, increasing organic C and N content, improving soil physical properties such as water holding capacity (Song et al., 2018; Zaman et al., 2017). For example, the application of green manure was shown to enhance soil enzyme activities as well as soil fungal composition, and support plant growth through increasing available soil inorganic N (Asghar & Kataoka, 2021). Therefore, for sustainable agriculture, it is crucial to bring insight into what extent peptide-based nutrition could act directly (N source) or indirectly (overall soil quality) in the plant growth support.

When supplied as the sole N source in sterile media without symbiotic microorganisms, the intact proteins casein, bovine albumin, and green fluorescent protein enabled plant growth, as shown in wheat, hakea, and *Arabidopsis* plants, which were able to exude proteases, acquire proteins, and use them as N source (Adamczyk et al., 2008; Adamczyk et al., 2010; Lonhienne, Trusov, Young, Rentsch, et al., 2014; Paungfoo-Lonhienne et al., 2008; Paungfoo-Lonhienne et al., 2012; Stoelken et al., 2010). In line with these results, we have previously shown that casein hydrolysate also enables tobacco plant growth as the sole source of N (Synkova et al., 2016). Also, the application of protein hydrolysates was shown to alleviate the negative effects of abiotic plant stress due to salinity, drought and heavy metals. These so-called biostimulants may directly stimulate C and N metabolism as well as interfere with phytohormonal levels and indirectly support soil microorganism activity, that is, increase availability of nutrients (Colla et al., 2017).

Considering the above, the main aim of our study was to assess the impact of protein, peptides and amino acids as N sources on the metabolism of tobacco plants by analysing the extent to which casein protein and hydrolysate may support their growth, alternate their metabolism, and initiate the plant defense response. We employed proteomics, metabolomics, phytohormonal and free amino acid determinations to analyse plants grown with casein protein or hydrolysate with those provided with an inorganic N source and their combinations, evaluating differences between plants supplemented with inorganic and organic N sources and under osmotic stress simulated by polyethylene glycol (PEG). In addition, we aimed to identify the underlying metabolic pathways by following the phytohormonal content that strictly contributes to metabolic regulation.

2 | MATERIALS AND METHODS

2.1 | Plant material and growth conditions

Nicotiana tabacum L. cv. Petit Havana SR-1 seeds were surface-sterilized in 70% (v/v) ethanol for 1 min and in 1.5% (v/v) sodium hypochlorite and 0.5% (v/v) Tween 20 for 10 min and then rinsed with sterile deionized water 3 times. Subsequently, the tobacco seeds were grown in vitro for 3 weeks in a Petri dish containing 0.8% agar sterilized Murashige-Skoog medium (Murashige and Skoog 1962). Lastly, the plants were transferred into ventilated Magenta boxes, one plant per box, with 50 mL of the Murashige-Skoog medium with a modified N source and concentration (Tables 1 and S1) for 4 weeks. The plants were grown in a growth room under a 16/8 h light/dark period, ca. 100 μmol (photon) m^{-2} s^{-1} irradiance, 22/18°C day/night temperatures, and 80% relative humidity. Our experimental model included nine plant groups (Table 1), which had the same content as the Murashige-Skoog medium in nutrients, vitamins, agar and so forth, (Table S1) except for N source and PEG 6000 as a simulation of drought (Ahmad et al., 2020). For organic N source, casein protein and casein hydrolysate were purchased from Sigma-Aldrich and their N content was measured by elementary analysis (Thermo Finningan Flash FA 1112 CHNS/O analyzer). From each biologically independent series, we prepared leaf, stem, and root samples, which were immediately frozen in liquid nitrogen and kept at −80°C until further analysis. Leaves, stems and roots were collected and photographed. The apparent leaf area was calculated using ImageJ software (Schneider et al., 2012).

TABLE 1 Composition of N content in the culture medium for nine experimental groups of tobacco plants.

No.	Plant group	N source	N source concentration (g L^{-1})	Final N concentration [mg(N)/L]	PEG (%)
1	high-IN	KNO_3 NH_4NO_3	1.9 1.65	840	0
2	IN	KNO_3	0.38	50	0
3	no-IN	–	0	0	0
4	H-CAS	Casein hydrolysate	0.5	70	0
5	P-CAS	Casein protein	0.5	70	0
6	IN + H-CAS	KNO_3 Casein hydrolysate	0.38 0.5	120	0
7	IN + P-CAS	KNO_3 Casein protein	0.38 0.5	120	0
8	IN + PEG	KNO_3	0.38	50	5
9	H-CAS + PEG	Casein hydrolysate	0.5	70	5

Note: Whereas the content of necessary nutrients, vitamins and agar was the same for all groups as in the standard Murashige–Skoog medium (Table S1), the N source and its concentration was different as indicated. Also, the final N concentration in the medium was calculated and measured for both casein hydrolysate and protein.

Abbreviations: (1) High-IN, plants grown in standard Murashige-Skoog medium (high inorganic N content); (2) IN, plants grown with a moderate inorganic N content; (3) No-N, plants grown without any source of N; (4) H-CAS, plants grown with casein hydrolysate as the sole source of N; (5) P-CAS, plants grown with protein casein as the sole source of N; (6) IN + H-CAS, plants grown with both moderate inorganic N content and casein hydrolysate; (7) IN + P-CAS, plants grown with both moderate inorganic N content and protein casein; (8) IN + PEG, plants grown with moderate inorganic N content with 5% PEG-6000; and (9) H-CAS + PEG, plants grown with casein hydrolysate as the sole source of N with 5% PEG-6000.

The presence of putative common microbial contamination, for example, from the laboratory environment, was tested in the medium, roots and leaves. Root samples grounded in a 10-fold amount of 10 mM $MgCl_2$ and cultivation medium were spread on 20 mL of sterile King's medium (20 g L^{-1} peptone; 8.6 mM K_2HPO_4; 6.1 mM $MgSO_4 \cdot 7H_2O$; 18.9 g L^{-1} glycerol; 15 g L^{-1} agar) in a Petri dish. The samples were incubated at 26°C for 72 h.

2.2 | Carbon and nitrogen content

The C and N content of tobacco leaves and roots was determined on Thermo Finnigan Flash FA 1112 CHNS/O analyzer. In addition, the N content was validated in both casein protein and hydrolysate.

2.3 | Root N acquisition

Root N acquisition was calculated as total plant N content determined by elemental analysis (see chapter above) divided by the root fresh weight (FW) (Franklin et al., 2017).

2.4 | Phytohormone analysis

Phytohormones in the tobacco leaves were determined as described previously (Belonoznikova et al., 2020; Prerostova et al., 2020). They were extracted and separated on a reverse-phase cation exchange SPE column (Oasis-MCX, Waters) into the acid fraction (auxins, abscisic acid [ABA], salicylic acid [SA], jasmonic acid [JA]) by elution with methanol and into the basic fraction (cytokinins [CKs] and 1-aminocyclopropane-1-carboxylic acid [ACC]) by elution with 0.35 M NH_4OH in 60% methanol (Belonoznikova et al., 2020, Prerostova et al., 2020). The fractions were analysed on a C18 high performance liquid chromatography (HPLC) column (Ultimate 3000, Dionex) coupled to a 3200 Q TRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems). The phytohormones were quantified using the isotope dilution method with multilevel calibration curves ($r^2 > 0.99$), and the data were processed in the Analyst 1.5 software package (Applied Biosystems).

2.5 | Protein concentration

Protein concentration was measured spectrophotometrically at 595 and 450 nm using Protein Assay solution (Bio-Rad) with bovine serum albumin as the standard (Bradford, 1976).

2.6 | Determination of free amino acids

Free amino acids in plant material and in casein were determined by capillary electrophoresis with a contactless conductivity detector to separate 20 proteinogenic amino acids in an acidic background electrolyte (Hodek et al., 2017). All electrophoretic experiments were conducted in a fused-silica capillary (Polymicro Technologies) using a G7100A Capillary Electrophoresis System (Agilent Technologies) with a contactless conductivity detector. The detector consisted of two 4-mm-long cylindrical electrodes with a 1-mm insulation gap. The inner diameter of the electrodes was 400 μm.

2.7 | Proteomic analysis

Plant roots were used for proteomic analysis according to Cerna et al. (2017). Their proteins were identified and quantified by liquid chromatography coupled to mass spectrometry (LC–MS) on a Dionex Ultimate 3000 RSLCnano system (Thermo) and maXis Impact UHR TOF impact mass spectrometer. The protein quantitation was performed on maXis UHR TOF impact and/or TSQ Quantiva triple-stage quadrupole (Thermo) mass spectrometers. In brief, proteins were extracted using a gel-free protocol combining acetone/ trichloroacetic acid and phenol extraction with digestion in a solution with immobilized trypsin beads (Promega). The resulting peptides were desalted, dried, and dissolved in 0.5% (v/v) formic acid in 5% (v/v) acetonitrile and then analysed online by nanoflow C18 reverse-phase liquid chromatography using a 15 cm Zorbax 300SB C18 (Agilent). The protein profiles were compared in MS mode (Profile Analysis 2.1, Bruker) and differentially abundant peptides were then matched with the library. Data were processed by ProteinScape 3.1 (Bruker) with Mascot Server 2.4 (Matrix Science), assessing regulation in biological replicates with Skyline 3.1 (MacCoss Lab Software).

2.8 | Metabolomic analysis

Tobacco root metabolites were extracted and analysed on Q Exactive GC Orbitrap GC-tandem mass spectrometer and Trace 1300 Gas chromatograph (Thermo Fisher Scientific) as described previously (Saiz-Fernandez et al., 2022). The glutathione and ascorbate contents in tobacco leaf and root were measured by HPLC (ECOM, s.r.o.) on a reverse phase column Nucleosil 120-5-C18 (Watrex) (Hola et al., 2017).

2.9 | Statistical analysis

All experiments were repeated in three biologically independent experiments (newly cultivated plants under the same conditions). In total, at least 270 plants (i.e., 9 groups, each consisting of 10 plants, 3 independent experiments) were analysed. Each measurement was performed in at least triplicates. Data were analysed by One Way ANOVA (Analysis of Variance, Holm-Sidak method) and *t*-test; differences were considered significant at $p \leq 0.05$ in SigmaPlot 12.0 and GraphPad Prism 8.0. All measured experimental data in tobacco plants were subjected to principal component analysis (PCA), which was performed using the Seaborn library for making statistical graphics in Python (Waskom, 2021).

3 | RESULTS

The experiment was designed to address three different model sources of nitrogen and abiotic stressor (Figure S1, Table 1): (1) the presence of a source of inorganic N (high-IN, IN, no-N); (2) the presence of casein (as protein or hydrolysate, i.e., P-CAS or H-CAS); (3) combinations of inorganic N and casein (IN + H-CAS, IN + P-CAS); and (4) combinations with PEG as drought stress simulation (H-CAS + PEG, IN + PEG). Also, plant tissues and media were tested for the presence of common microbial contamination. The test did not reveal any visible microbial contamination (data not shown), indicating that the observed effects on plant growth were not a result of microbial metabolism.

3.1 | Plant growth, root N uptake and phytohormonal changes correlate with N availability

After 4 weeks of in vitro growth, P-CAS plants were comparable with no-N plants displaying chlorosis and reduced growth, while the FW of H-CAS plants was significantly higher (4.8- and 5-fold higher than no-N group in leaves and roots, respectively). Casein hydrolysate not only supported H-CAS plant growth but also significantly led to the lowest shoot/root ratio (Figure 1B).

Combining casein, in both forms (IN + H-CAS and IN + P-CAS), with inorganic N as an additional component in the medium positively stimulated plant growth and increased the shoots/roots (Figures 1A,B and S5). The high plant biomass of the high-IN was comparable with the IN + H-CAS and IN + P-CAS (Figure S5). In particular, casein hydrolysate likely stimulated root N acquisition because IN + H-CAS plants showed one of the highest values of this parameter (1.8-fold higher than IN-plants; Figure 1D).

In order to characterize, at least partly, the ability of the various N sources to meet the N demands of tobacco plants, we determined the total C and N contents in leaves and roots (Figures 1E and S2). All plants displayed similar root N content, except for high-IN plants, which showed the highest N amount (Figure 1F). In addition, H-CAS plants had a significantly higher leaf N content than P-CAS and no-N plants and a C/N ratio comparable to that of IN plants (Figure 1E,F). The IN + H-CAS group showed a higher leaf N content than IN plants, while IN + P-CAS showed similar N content than IN plants (Figure 1D). The C content in roots was slightly higher in the H-CAS and P-CAS than in high N (Figure S2).

The H-CAS plants showed a significantly higher content of indole-3-acetamide (IAM), a precursor of indole-3-acetic acid (IAA), in comparison with all other plants (Figure 2B). However, the content of IAA was rather comparable between H-CAS and high-IN (Figure 2A). In turn, the content of 2-oxoindole-3-acetic acid (OxIAA), a degradation product of IAA, was also higher in these plants if compared to high-IN (Figure 2B), which may indicate enhanced auxin metabolism. The content of phenylacetic acid (PAA), a form of auxin, was higher in the H-CAS plants

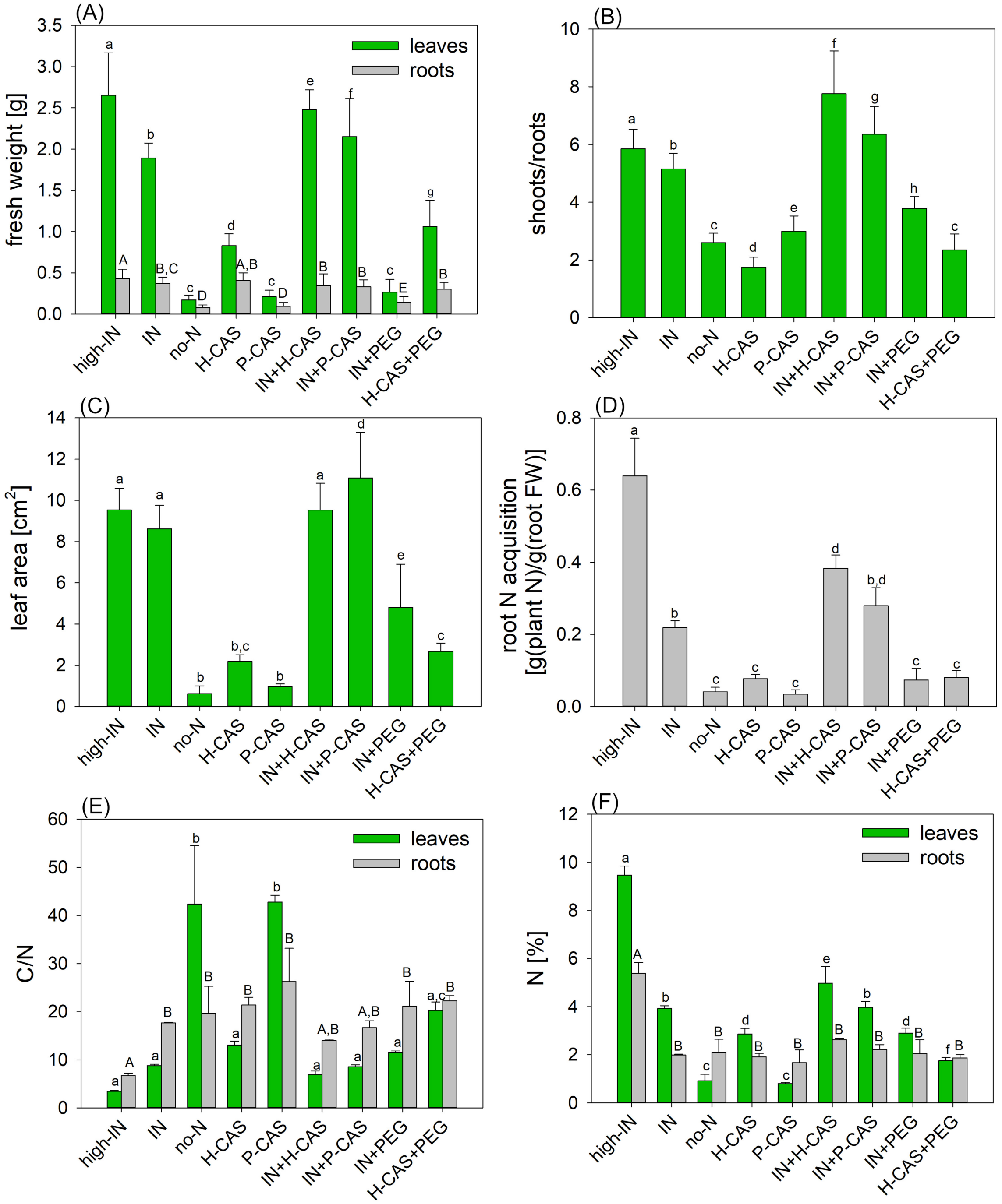

FIGURE 1 Fresh weight (A), shoots/roots ratio (B), leaf area (C), root acquisition (D), C/N ratio (E) and total N content (F) of plants grown in vitro with various N sources. Values shown represent the mean ± SD ($n = 3$ biological replicates, each being a pool of 10 seedlings per treatment). Different letters next to each bar denote significant differences ($p \leq 0.05$) among the plant groups according to ANOVA (Holm-Sidak). Root N acquisition was calculated as total plant N content divided by root fresh weight. The same letters next to a bar, which corresponds to a particular N nutrition, indicate no statistically significant differences between the experimental plant groups. Leaf area was calculated in ImageJ version 1.53k. FW, fresh weight.

(Figure 2A) than in the high-IN. In addition to auxins, plant growth also requires CKs, which are phytohormones also related to N metabolism and signalling. The reduction in active CKs in H-CAS and P-CAS plants likely indicates a link to root growth (Figures 1B and 2C). Elevation of CK precursors by H-CAS may indicate a positive casein effect on shoot growth, especially in combination with inorganic N in comparison with P-CAS and no-N.

Also, some changes in SA and JA content were detected (Figure 2E,F). JA levels were similar across the experimental groups,

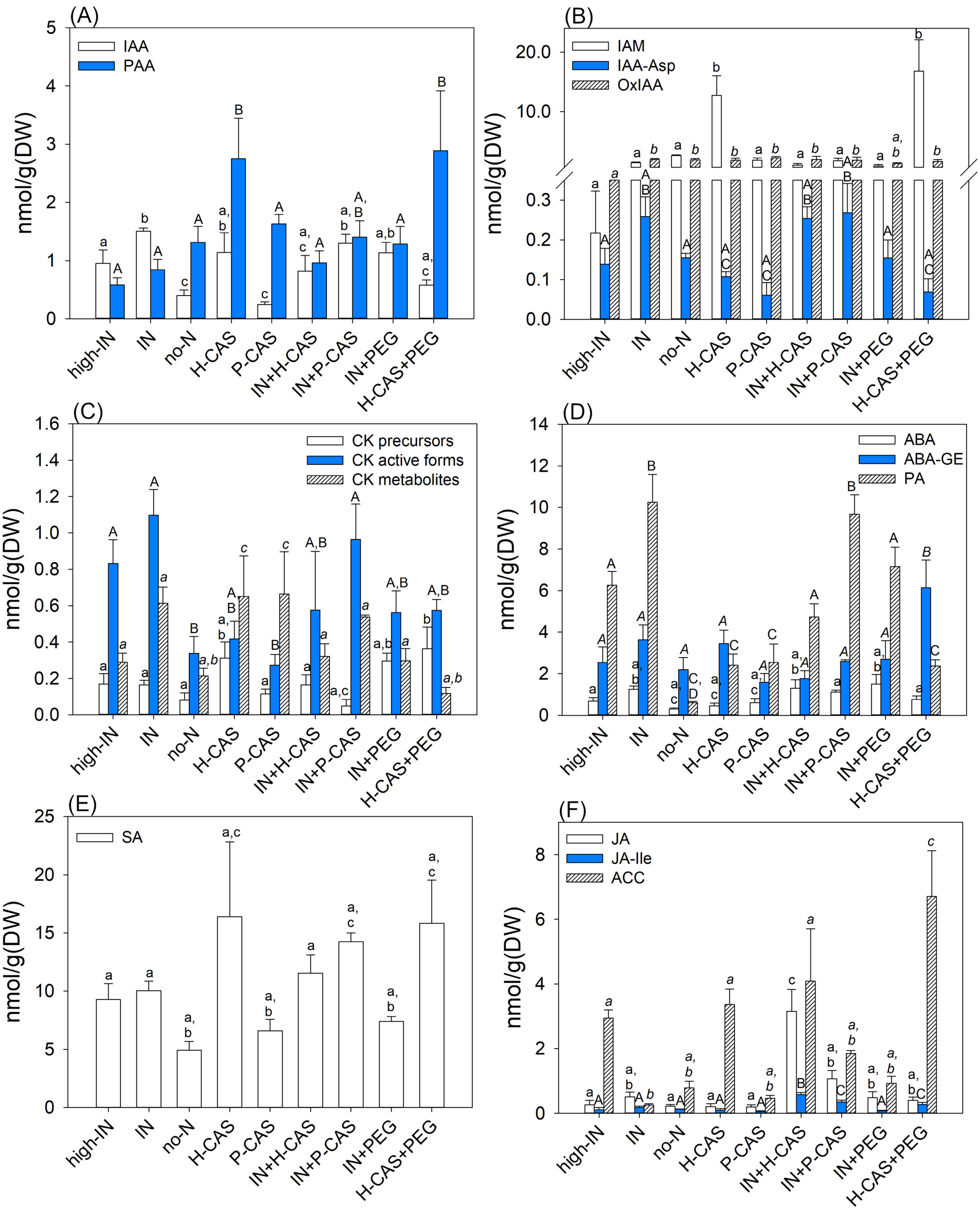

FIGURE 2 Analysis of phytohormones in tobacco leaves. Active auxins (A), precursors and deactivation products of auxins (B), cytokinins (C), abscisic acid and phaseic acid (D), salicylic acid (E), and jasmonic acid and jasmonate-isoleucine, aminocyclopropane-carboxylic acid (ethylene precursor) (F). The different letters for each phytohormone (standard, capitals, italics) above each bar denote significant differences ($p \le 0.05$) between groups according to one way-ANOVA (Holm-Sidak). ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; CK, cytokinin; DW, dry weight; IAA, indole-3-acetic acid; IAA-Asp, IAA-aspartate; IAM, indole-3-acetamide; JA, jasmonic acid; JA-Ile, JA-isoleucine; OxIAA, 2-oxoindole-3-acetic acid; PA, phaseic acid; PAA, phenylacetic acid; SA, salicylic acid.

except for IN + H-CAS plants, which showed a relatively high JA content (Figure 2F). By contrast, SA was slightly higher in the H-CAS plants than in the high-IN.

The ethylene (ET) precursor ACC was higher in not only IN + H-CAS and H-CAS but also in high-IN leaves in comparison with other plant groups (Figure 2F).

3.2 | Free aliphatic branched-chain and basic amino acids accumulate in the plants grown with casein

N availability is a major determinant of plant growth and development. H-CAS and IN leaves showed similar protein concentrations,

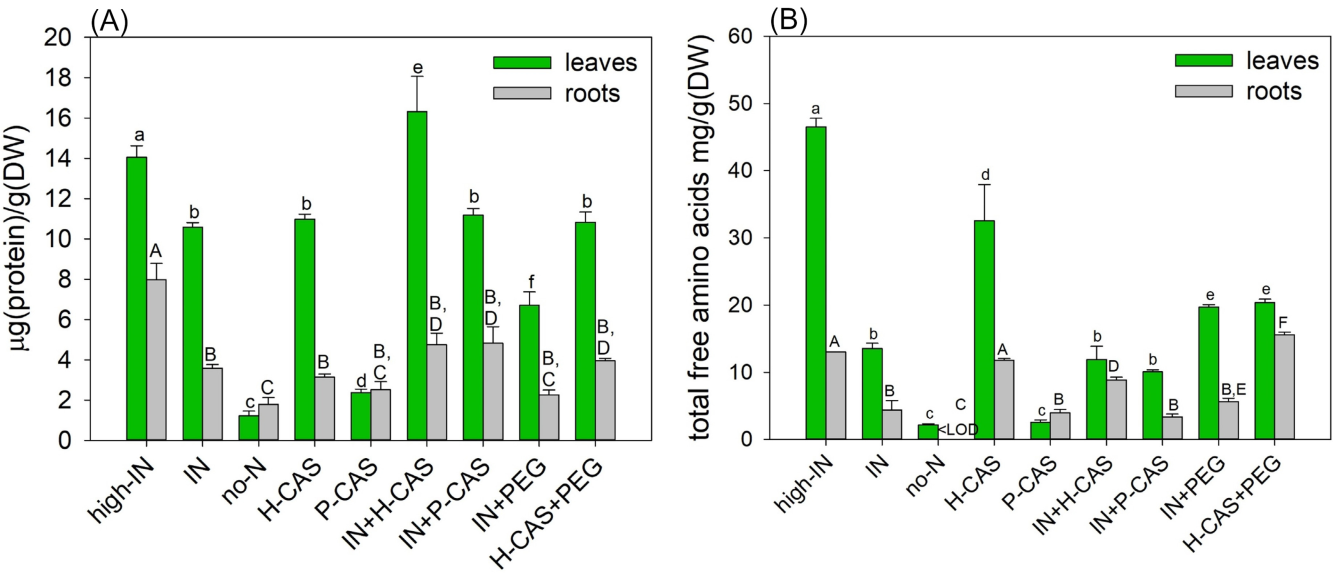

FIGURE 3 The content of proteins (A) and total free amino acids (B) in the tobacco roots and leaves. Values shown represent the mean ± SD ($n = 3$ biological replicates, each being a pool of 10 seedlings per treatment). Different letters next to each bar denote significant differences ($p \leq 0.05$) among the plant groups according to ANOVA (Holm-Sidak). The same letters next to a bar indicate no statistically significant differences between the experimental plant groups. DW, dry weight.

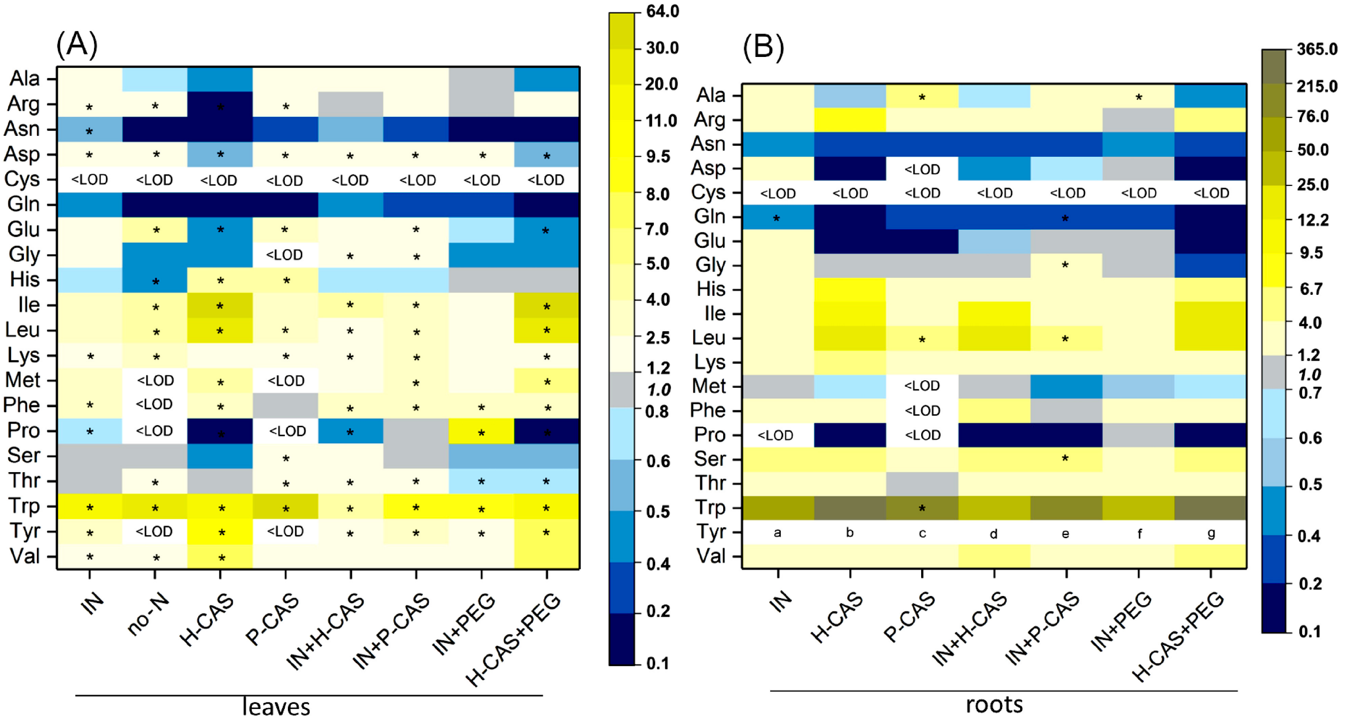

FIGURE 4 Free amino acid content in tobacco leaves (A) and roots (B). The content of free amino acids in the no-N roots was under the limit of detection. The content of individual free amino acids was expressed in % of the total amount of free amino acids in dry weight and compared with the high-IN group. Values shown represent the mean of 3 biological series, each being a pool of 10 seedlings per treatment. Asterisk denotes a significant difference ($p \leq 0.05$) against high-IN according to t-test. DW, dry weight; <LOD, under limit of detection.

but IN + H-CAS leaves had the highest values (Figure 3A). The protein content in P-CAS and H-CAS roots was comparable with IN roots.

The highest total free amino acid content was found in high-N leaves, closely followed by H-CAS leaves; these two plant groups also showed similar root concentrations of total free amino acids

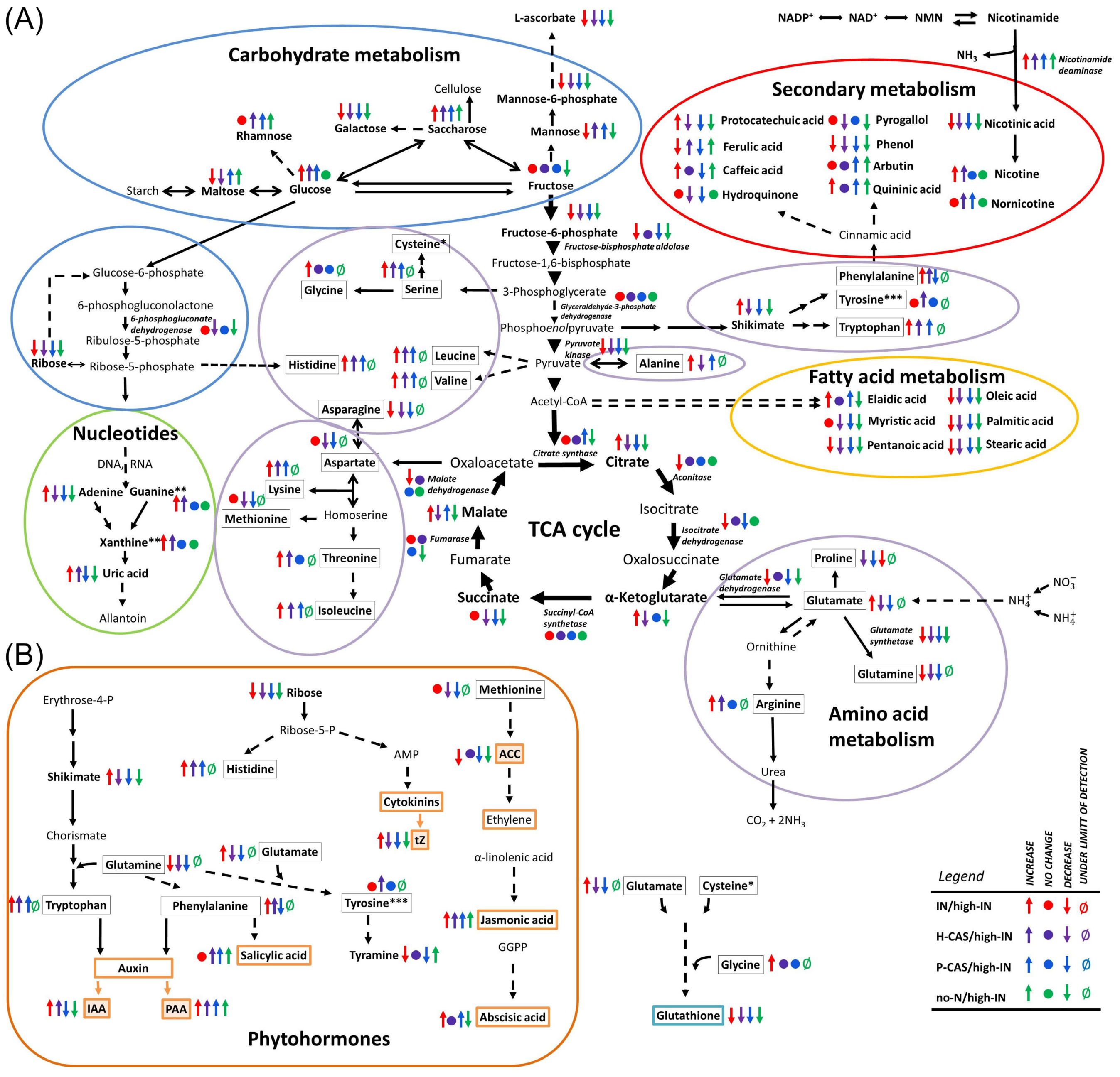

FIGURE 5 Proteomic and metabolomic analysis of tobacco roots. (A) Brief overview of tobacco metabolism. (B) Phytohormones (orange) and glutathione (light blue) are emphasized. The arrow direction indicates an increase (↑) or decrease (↓) greater than 20% in the representation of IN (red), H-CAS (purple), P-CAS (blue), no-N (green) roots when compared to the high-IN roots. The circle (○) indicates that there was no change between the group and high-IN. The symbol ∅ indicates that the value was under the limit of detection. No free amino acids were detected in the no-N roots. *Cys was under the limit of detection for all experimental groups. **Guanine as well as xanthine were not detected in high-IN, P-CAS and no-N roots. ***Tyr was not detected in high-IN, IN and P-CAS roots. No free amino acids were detected in the no-N roots. ACC, 1-aminocyclopropane-1-carboxylic acid (ethylene precursor); GGPP, geranylgeranyl diphosphate; IAA, indole-3-acetic acid; PAA, phenylacetic acid (weak auxin); tZ, trans-zeatin (active cytokinin).

(Figure 3B). Concurrently, the amount of free amino acids was below the limit of detection in no-N roots (Figure 3B).

Casein hydrolysate is rich in Leu, Lys, Val, Phe, Arg and Ile (sorted from the most abundant), which correlates with the high content of Ile, Leu, Phe and Val of H-CAS, IN + H-CAS and H-CAS + PEG roots (Figures 4 and S4). Even P-CAS plants showed a higher root content of Ile, Leu and Lys if compared to high-IN (Figure 4). Tyrosine was not detected in the high-IN, IN, and P-CAS roots, while the tyrosine concentration in the dry weight (DW) of roots of H-CAS was 648 ± 0 μg/g (DW), 176 ± 15 μg/g (DW) in IN + H-CAS and 9.7 ± 0 μg/g (DW) in IN + P-CAS.

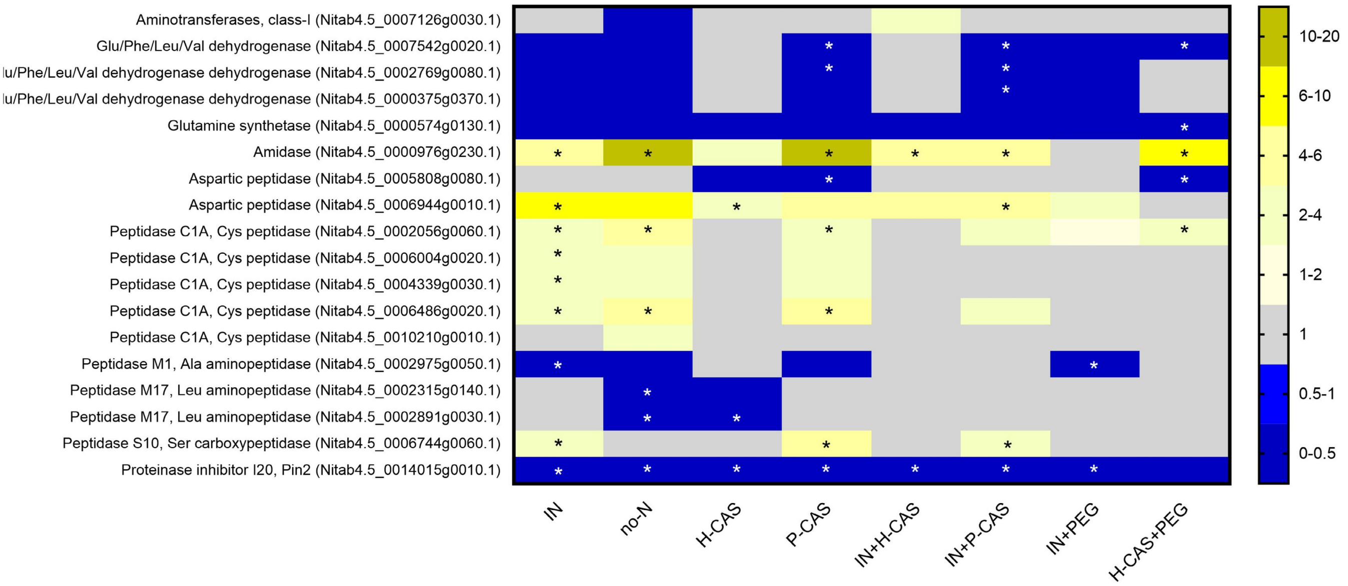

FIGURE 6 Proteomic analysis of tobacco roots: enzymes involved in N metabolism. Values shown represent the ratio against the high IN roots. Values represent the mean of 3 biological replicates, each being a pool of 10 seedlings per treatment. The asterisk (*) shows a statistically significant change in representation if compared to the high-IN roots at *t*-test <0.05.

Overall, we identified a shift in the free amino acid profile of H-CAS plants. The results showed a higher content of predominantly aromatic, aliphatic branched-chain and basic amino acids (Figure 4). In addition to the amino acids mentioned above, the Trp content was surprisingly high in the roots of plants supplied with organic N and even higher than in IN plants. As for the leaves, Trp was high in H-CAS and no-N groups (Figure 4). His residues do not abound in casein either (Figure S4). Yet, in addition to the higher content of His in H-CAS and P-CAS leaves, H-CAS and H-CAS + PEG roots also showed high His content in comparison with the high-IN (Figure 4). The high His content in P-CAS leaves (but not in roots) correlated with reduced ACC content (Figures 2F and 5A). Arg concentration was higher in both H-CAS and P-CAS roots than in high-IN (Figure 4B). The content of each single free amino acid per DW of leaves or roots can be found in Tables S2 and S3.

3.3 | Targeting proteases responsible for casein degradation

The presented results confirmed that protein casein can be utilized by plants. Next, proteome analysis was employed to identify enzymes that could be involved in the process. Ammonium ions are incorporated into amino acids via the glutamine synthetase-glutamate synthase pathway. Glutamine synthetase, which catalyzes Glu amidation to Gln, was downregulated in all experimental groups when compared to high-IN plants (Figures 5 and 6). This downregulation likely reflects the low content of Gln in those experimental groups (Figure 4). Glutamate dehydrogenase did not differ significantly between H-CAS and high-N roots (Figures 5 and 6), and its abundance was lower in the P-CAS and no-N groups compared to high-IN.

Amidases, conversely, seemed to be heavily involved in providing plants with NH_3 for metabolism. The amidase representation was significantly higher in P-CAS and no-N roots compared to high-IN (Figure 6).

In tobacco roots, we identified representatives of different classes of proteases, such as aspartic peptidase, peptidase C1A, peptidase M1, peptidase M17 and peptidase S10 (Figure 6). The abundance of aspartic peptidase was higher in most experimental groups in comparison with high-IN (Figure 6). The content of peptidase S10, a serine carboxypeptidase, was higher in P-CAS and IN + P-CAS but not H-CAS roots if compared to high-IN (Figure 6). However, the representation of this protease was also higher in the IN group than in the high-IN. The content of cysteine peptidase C1A was higher in the no-N and P-CAS roots than in high-IN (Figure 6).

3.4 | Accumulation of saccharides and fatty acid utilization

In comparison with high-IN roots, H-CAS and P-CAS roots showed a shift in the abundance of transported saccharide saccharose, and transported sugar alcohols, including arabitol and inositol. Differences were found also in monosaccharides (such as glucose, fructose, arabinose, rhamnose) and in disaccharides (including melibiose and isomaltose). However, the contents of key monosaccharide phosphate derivatives (fructose-6-phosphate and mannose-6-phosphate) were lower than in the high-IN (Figures 5, 7 and 8). The content of most

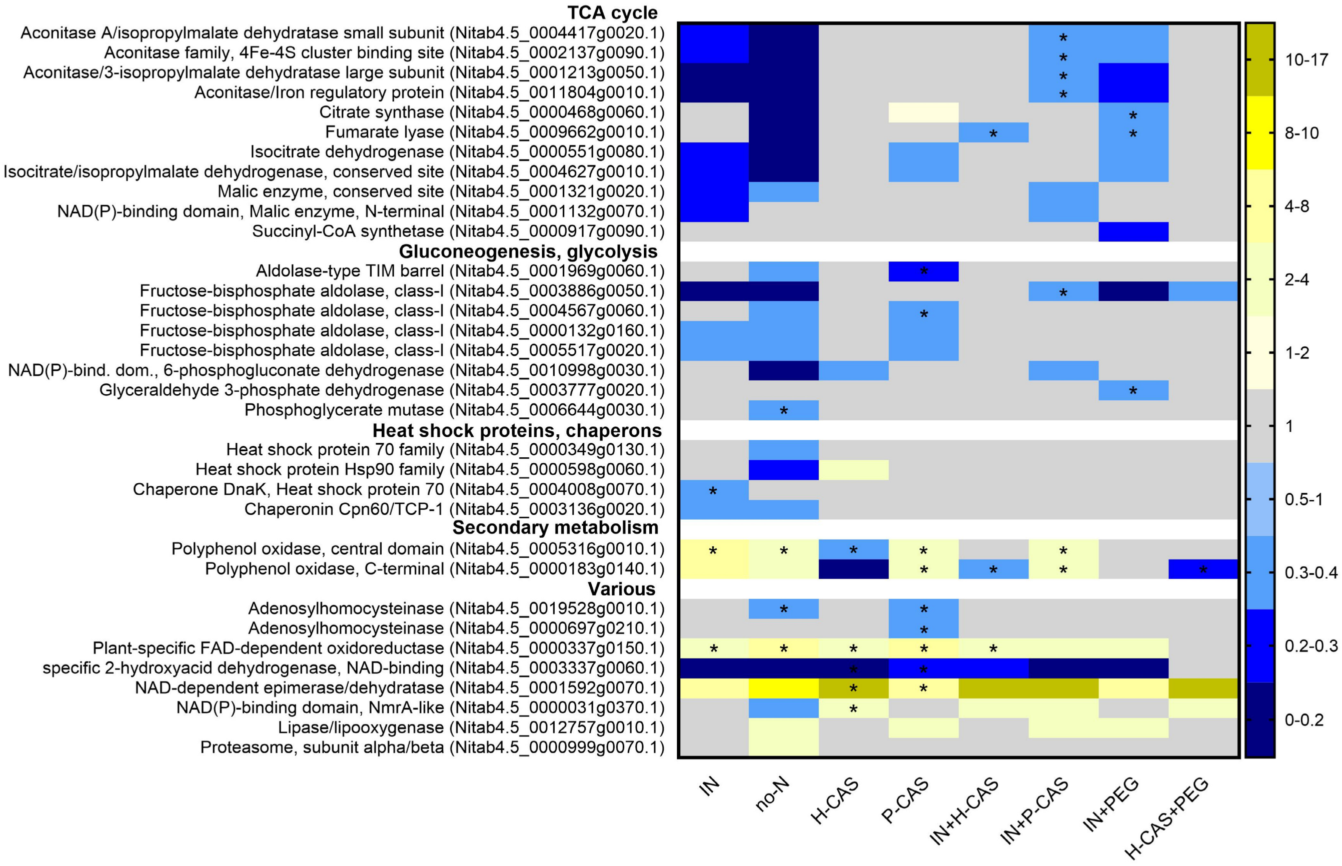

FIGURE 7 Proteomic analysis of tobacco roots: primary and secondary metabolism pathways. Values shown represent the ratio against the high IN roots. Values represent the mean of 3 biological replicates, each being a pool of 10 seedlings per treatment. The asterisk (*) shows a statistically significant change in representation if compared to high-IN roots at *t*-test <0.05.

fatty acids in the H-CAS and P-CAS roots was also lower than in the high-IN (Figures 5 and 8). The representation of most metabolites of the tricarboxylic acid (TCA) cycle (citrate, α-ketoglutarate, succinate, and malate) was lower in H-CAS roots than in high-IN roots (Figures 7 and 8).

3.5 | Response of plant defense system to N availability

In tobacco roots, we analysed the content of secondary metabolites and other compounds and proteins that may be responsible for plant response to N availability. The content of ferulic acid was significantly higher in H-CAS and no-N roots (Figure 8) than in high-IN. Ferulic acid is biosynthesized from caffeic acid, whose content was comparable between high-IN and H-CAS roots (Figure 8). Protocatechuic acid was found in high concentration in IN, IN + P-CAS and IN + H-CAS roots (Figure 8). Nicotinic acid was significantly higher in high-N plants than in all other experimental groups (Figures 5 and 8). A significantly higher amount of nornicotine was detected in both H-CAS and P-CAS roots than in the high-IN. We observed a higher ascorbate content in no-N leaves than in both H-CAS and P-CAS leaves (Figures 5 and S6). However, the glutathione concentration was significantly higher in P-CAS than in no-N and H-CAS leaves (Figures 5 and S6).

3.6 | Response to drought stress

In our experiment, osmotic stress was imposed by adding PEG to the medium. The IN + PEG and H-CAS + PEG showed lower values of all physiological parameters than the high-IN (Figure 1). On the other hand, the IN + PEG and H-CAS + PEG roots showed higher C content than high-IN (Figure S2). In the phytohormonal analysis, the ABA concentration was only slightly higher in IN + PEG and H-CAS + PEG plants than high-IN (Figure 2D). H-CAS + PEG plants had the highest content of ABA glucose ester (ABA-GE; Figure 2D). In turn, the plants supplied with casein hydrolysate, either alone (H-CAS) or in combination with inorganic N (IN + H-CAS) and PEG (H-CAS + PEG), showed a lower content of phaseic acid (PA), an ABA catabolite, than all plants grown with inorganic N (Figure 2D). The H-CAS + PEG plants showed a high content of PAA (Figure 2A), IAM (Figure 2B,E) and even ACC (Figure 2F) as the H-CAS did.

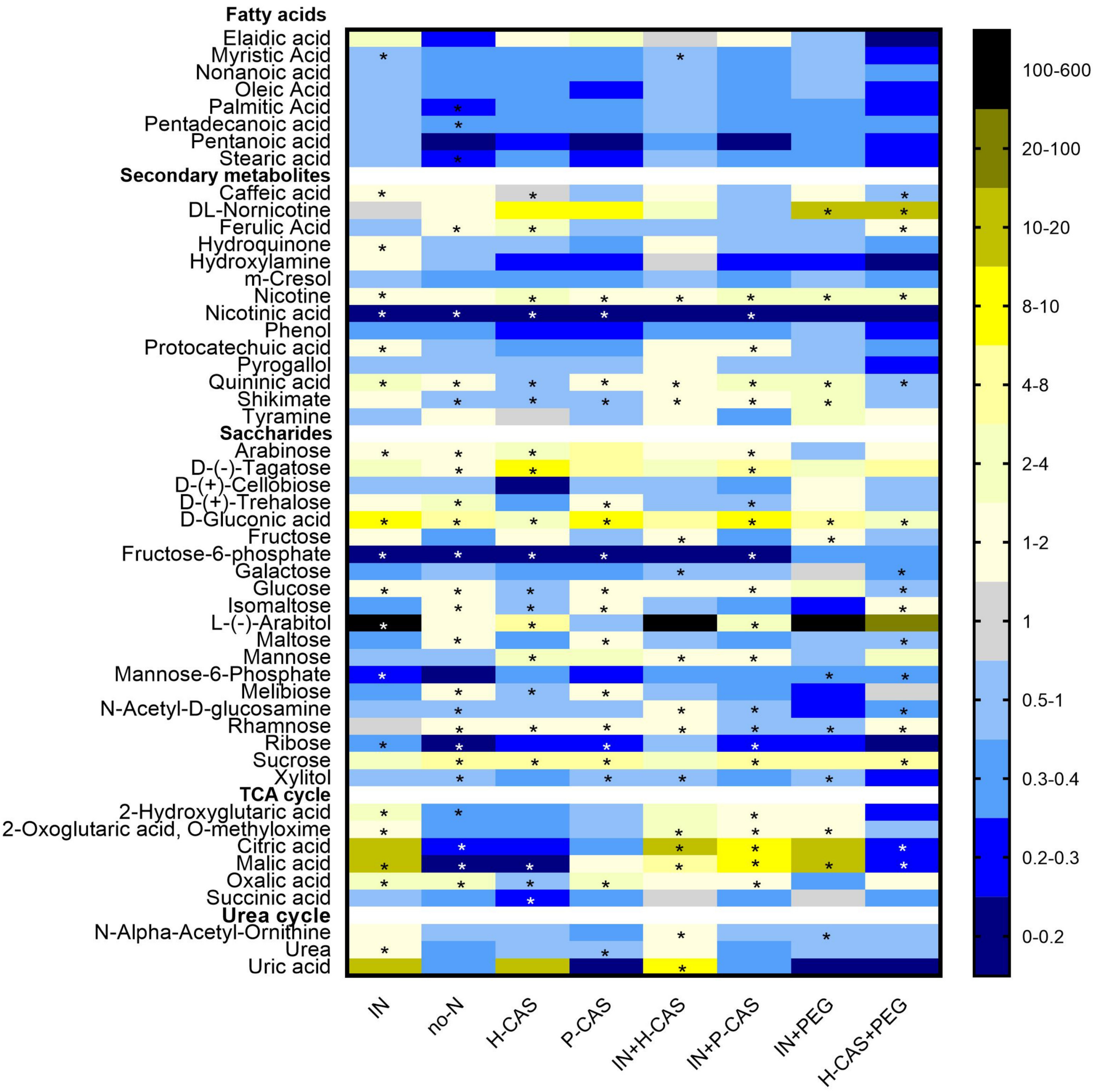

FIGURE 8 Metabolomic analysis of tobacco roots. Values shown represent the ratio against the high-IN roots. Values represent the mean of 3 biological replicates, each being a pool of 10 seedlings per treatment. The asterisk (*) shows a statistically significant change in representation if compared to high-IN roots at *t*-test <0.05.

The free amino acid profile of H-CAS + PEG and H-CAS plants was rather similar (Figure 4). However, the IN + PEG leaves had a higher free amino acid content than IN leaves (Figure 3), especially due to enhanced Pro and Trp (Figure 4). Yet, exposure to drought in the form of PEG in the medium led to a significantly lower Pro content of H-CAS + PEG leaves and roots than in the high-IN (Figure 4). Tyrosine was detected in the roots of IN + PEG (21 ± 19 μg/g DW) as well as H-CAS + PEG (1164 ± 17 μg/g DW).

As for the secondary metabolites, the content of ferulic acid was significantly higher in H-CAS, H-CAS + PEG and no-N roots than in the high-IN (Figure 8). The content of nicotine was very high in H-CAS and H-CAS + PEG roots (Figures 5 and 8). Also, H-CAS + PEG and IN + PEG plants showed a significantly higher amount of nornicotine than the high-IN (Figure 8). IN + PEG plants showed high concentrations of some secondary metabolites, including nicotine, quininic acid, and shikimate (Figure 5, Figure 8).

4 | DISCUSSION

4.1 | Response of plant growth and phytohormone levels to organic N

Protein hydrolysates are getting at the centre of scientific attention as 'promising biostimulants' because several positive effects on plant growth and pathogen resistance have been reported (Caruso et al., 2019; Colla et al., 2014; Colla et al., 2015; Colla et al., 2017; Lachhab et al., 2014). In general, selecting the source and appropriate amount of N is a difficult decision because its utilization by plants depends on the availability of N, the C/N ratio, and environmental conditions that control N mineralization, its subsequent transformation and eventually its availability to plants. In a natural system, N compounds change dynamically, mainly due to the influence of the soil microbiome. To study the effect of different forms of N on plant metabolism, we chose sterile culture medium conditions. In addition to NO_3^- and NH_4^+ uptake or symbiosis with soil microorganisms, plants can also utilize organic N in the form of amino acids, oligopeptides and even proteins, such as bovine serum albumin, green fluorescent protein or casein (Adamczyk et al., 2008; Adamczyk et al., 2010; Lonhienne, Trusov, Young, Rentsch, et al., 2014; Lonhienne, Trusov, Young, Schmidt, & Paungfoo-Lonhienne, 2014; Paungfoo-Lonhienne et al., 2008; Paungfoo-Lonhienne et al., 2012; Stoelken et al., 2010; Synkova et al., 2016). Here, casein was chosen as a model protein for its open, flexible, and mobile conformation resulting from the high proline level, which contributes to the low number of α-helix and β-sheet in its secondary structure and accounts for its susceptibility to proteolysis (Otter, 2003).

Plants treated with some protein hydrolysates were associated with modifications of root architecture such as density, length, and number of lateral roots (Colla et al., 2017). The IN + H-CAS group stands out in terms of growth parameters (such as shoots/roots ratio, leaf area), protein and N content, reasonable C/N ratio and root N acquisition in comparison with an inorganic N source (IN group) (Figures 1 and 2). In the H-CAS and P-CAS plants, the shoot growth was reduced at the expense of a larger root system, which is probably related to N foraging by preferentially developing lateral roots in N-rich areas, thereby maximizing N acquisition. This phenomenon is related to changes in CKs level, which are phytohormones affecting growth, nutrient sensing and N metabolism (Pavlu et al., 2018). The low content of CK active forms and slightly higher content of CK precursors in H-CAS leaves compared to IN and high-IN groups (Figure 2) are probably related to the CK effect on supporting root growth (Pavlu et al., 2018). Some commercially available products based on protein hydrolysates were shown to influence the levels of auxin and gibberellins, thus promoting root and shoot growth (Colla et al., 2017). The plants grown with casein hydrolysate showed high concentrations of IAA (auxin) as well as its precursor IAM, which may be linked to the Trp elevated content (Figure 4), a crucial metabolite of biosynthetic pathways (Figure 2A,B). By contrast, ABA, SA and JA modulate the metabolism to activate defense reactions (Rubio et al., 2009). The levels of ABA are related to water balance and discussed in the last chapter about drought stress induced by PEG. SA has been shown to improve plant tolerance to various abiotic stress forms such as metal, salinity, osmotic, drought, and heat stress (Khan et al., 2015). Here, the slightly higher SA levels in H-CAS and IN + P-CAS may be related to the higher supply of its precursors, such as Phe maybe as a response to exogenous protein or peptides in the medium (Figures 2E and 4).

4.2 | Role of free amino acids in plant in response to casein

Casein hydrolysate represents a mixture of amino acids that could be readily absorbed; thus, some free amino acids in plants may originate directly from the medium. A direct protein uptake could be possible (Paungfoo-Lonhienne et al., 2008), but it is more likely that casein is first digested by proteases (Greenfield et al., 2020). The high concentration of free amino acids in both H-CAS leaves and roots likely indicates that tobacco roots readily absorb single amino acids from casein hydrolysate in the medium (Figure 3B). Especially aromatic (Trp), basic amino acids (His, Arg, Lys) and free aliphatic branched-chain (Ile, Leu, Val) are accumulated in the plants grown with casein (Figure 4). Considering the low Trp content in casein hydrolysate (Figure S4), de novo Trp biosynthesis through the shikimate pathway should occur in the H-CAS plants (Figures 4 and 6). Casein has a His content similar to that of other proteins such as serum albumin, gelatin and whey (Gorissen et al., 2018). Excluding the aromatic amino acids, His is the most energetically expensive amino acid to synthesize but, in contrast to the other essential amino acids, our knowledge of His catabolism is still incipient (Galili et al., 2016). Therefore, we suggest that His was taken up by H-CAS and IN + H-CAS plants from the cultivation medium. LYSINE HISTIDINE TRANSPORTER 2 was identified in yeast as a high-affinity transporter for both Lys and His and as an ACC transporter in *A. thaliana*, wherein His supplementation reduced the ACC-induced root growth inhibition (Choi et al., 2019). Therefore, the high His content of H-CAS plants may have affected ET biosynthesis. Also, the higher His content was correlated with the lower content of ribose, a precursor of the initial metabolite of His biosynthesis (ribose-5-phosphate) when compared to high-IN (Figure 5). As the amino acid with the highest N/C ratio, Arg, abundant in H-CAS roots, may serve as a storage and transport compound. Moreover, Arg could be the precursor of nitric oxide and polyamines putrescine, spermidine and spermine, involved in developmental regulation processes and responses to stress (Winter et al., 2015). Another compound with a high N/C ratio is uric acid, biosynthesized from xanthine, which was stored in H-CAS roots (Figures 5 and 8).

We did find a significant impact on the free amino acid pool in plants supplemented with both casein hydrolysate and protein. However, the observed patterns did not show any clear correlation with the casein amino acid composition. This indicates that the supplied amino acids were utilized as a source of energy or converted. The proportions of individual free amino acids differ from those bound in proteins (Hildebrandt et al., 2015). In contrast, a high nitrate supply results in the accumulation of Lys, Leu, Phe and Trp besides Gln and Glu (Urbanczyk-Wochniak & Fernie, 2005). In general, low nitrate availability leads to a lower content of many amino acids, especially Glu and Gln (Hildebrandt et al., 2015; Pratelli & Pilot, 2014). The IN plants showed a rather similar trend in the content of Lys, Leu, Phe and Trp with the plants supplemented with both forms of casein, thus it indicates their targeted synthesis rather than merely amino acid uptake from casein the medium (Figure 4).

4.3 | Enzymes involved in N utilization

Root proteases play an important role in endocytosis or root exudate-mediated nutrient (organic N) uptake (Adamczyk et al., 2008; Adamczyk et al., 2010; Godlewski & Adamczyk, 2007; Kohli et al., 2012).

Amidases produce carboxylic acids and either amine or ammonia compounds derived from the C-N amide bond (Moya-Cuevas et al., 2021), using Gln and Asn to yield NH_4^+. In scarce N availability, amidases are also involved in Arg metabolism via 4-guanidino butyramide hydrolysis to NH_4^+; both no-N and P-CAS roots showed a high representation of amidase (Figure 6). PLANT AMIDASE-1, a specific indole-3-acetamide amidohydrolase, synthesizes IAA from IAM (Moya-Cuevas et al., 2021). Amidases also function as enzymes involved in the concentration of regulatory substances, such as N-acylethanolamines. It seems that in the P-CAS plants, amidases could play a role in N utilization by releasing ammonia from organic amides and in plant growth by modulating auxin synthesis (Figure 6).

Cysteine peptidases C1A and serine carboxypeptidases S10 classes seem to play a key role in casein utilization and response to N shortage (Figure 6). The (papain-) peptidases C1A (family C1, clan CA), grouped as various cathepsins, are the most abundant among plant peptidases and participate in various processes, including protein degradation during senescence, accumulation and mobilization of proteins and play a key role in defense responses against pathogens (Cambra et al., 2012; Diaz & Martinez, 2013). The content of peptidase C1A (Cys peptidase) was higher in no-N and P-CAS roots than in the high-IN, likely as a response to N starvation and utilization of the plant's own proteins (Figure 6). Proteases could be secreted to search for a possible source of N. However, the representation of peptidase C1A was also higher in the IN roots compared to high-IN (Figure 6). Some serine carboxypeptidases are involved in the re-mobilization of N resources during seed germination, wound stress, and leaf senescence and may also be involved in various signalling events important for plant growth and development (Bienert et al., 2012). Another candidate protease involved in protein degradation in the medium is peptidase S10, a Ser carboxypeptidase (Figure 6). The representation of peptidase S10 was higher in P-CAS and IN + P-CAS but not H-CAS roots when compared to the high-IN. However, the content of this protease was also higher in the IN group than in the high-IN. Therefore, we must consider other possible functions. Also, a higher representation of aspartic peptidase was detected in P-CAS, IN + P-CAS, and no-N roots than in the high-IN (Figure 6). This higher content could indicate the involvement of aspartate peptidase in obtaining N from proteins and peptides, but this higher value was also found in the IN and H-CAS plants, which at least partly suggests another function. Plant aspartic proteases are involved in both biotic and abiotic stress responses. For example, plants that accumulated more aspartic proteases were more tolerant to drought and induced a better systemic defense response (Figueiredo et al., 2018).

4.4 | Exogenous protein: A putative signal to promote plants' resilience?

Some proteases identified in our analysis (e.g., peptidases C1A; Figure 6) are involved in plant defense systems (Schaller et al., 2018; van der Hoorn & Klemencic, 2021). Considering these findings, an intriguing question comes to mind. Could the exogenous protein in the medium be interpreted as a potential threat signal by plants? Proteases would be then released in a defense response, and the subsequent acquisition of N would be a side effect. For example, plants grown with BSA as the source of N were significantly more resistant to fungal root pathogen *Cylindrocladium* sp. Furthermore, the increase of root biomass induced by protein in the medium may likely improve plant vitality (Lonhienne, Trusov, Young, Rentsch, et al., 2014; Lonhienne, Trusov, Young, Schmidt, & Paungfoo-Lonhienne, 2014). As another case in point, Paungfoo-Lonhienne et al. (2010) discovered that *Arabidopsis* and tomato plants were able to take up non-pathogenic *Escherchia coli* and *Saccharomyces cerevisiae* into root cells and to digest and use these microbes as a nutrient source (Paungfoo-Lonhienne et al., 2010). Nowadays, various biostimulants containing protein hydrolysates are being used as priming agents to improve crop tolerance to abiotic stressors in sustainable agriculture (Agliassa et al., 2021). The application of an animal-based protein hydrolysate under water stress increased the endogenous content of auxin, CK and JA in treated tomato plants, helping plant growth under drought conditions (Casadesus et al., 2019). Protein hydrolysate application mitigated the negative effects of drought and heat, for example, increased antioxidant content and decreased accumulation of hydrogen peroxide in tomato plants (Francesca et al., 2022). Foliar application of various vegetal-derived protein hydrolysates has also been shown to modulate tomato plant growth, affecting the root system and likely interfering with signalling processes (Ceccarelli et al., 2021). Similarly, applying protein hydrolysates on maize has a root growth-promoting effect, increases K, Zn, Cu and Mn accumulation, changes hormonal levels and modifies the expression of a high number of genes involved in responses to oxidative stress (Santi et al., 2017).

Under stress conditions, the content of plant secondary metabolites may increase at the expense of growth. Plant phenolic compounds are powerful antioxidants that can mediate reactive oxygen species (ROS) scavenging *in planta* (Samec et al., 2021; Tupec et al., 2017). Several studies have reported that application of protein hydrolysates led to enhanced contents of secondary metabolites and antioxidants such as *p*-coumaric acid, chlorogenic acid, and ascorbate in pepper and tomato fruits (Caruso et al., 2019; Colla et al., 2017;

Ertani et al., 2016). We identified several secondary metabolites in tobacco roots (Figures 5 and 7). Ferulic acid, which content was higher in H-CAS and no-N roots in comparison with the high-IN, serves as an intermediate in lignin synthesis and contributes to crosslinking lignin and polysaccharides, thereby increasing the rigidity of the cell wall (Iiyama et al., 1994). For example, in tobacco plants exposed to heat and viral stress, ferulic acid concentration was induced by both factors (Hyskova, Belonoznikova, Doricova, et al., 2021). Protocatechuic acid, a possible by-product of the shikimate pathway (Hyskova, Belonoznikova, Smeringaiova, et al., 2021), was significantly higher in IN, IN + P-CAS roots than in high-IN (Figure 8). In vitro tobacco cultures, protocatechuic acid mimicked auxin by stimulating cell proliferation and callus induction (Mucciarelli et al., 2000). Thus, protocatechuic acid could be associated with a positive influence on the growth of the root system (Figure 1A). In *Arabidopsis*, ascorbate metabolism is enhanced under N starvation, while the glutathione pool remains mostly unchanged (Iwagami et al., 2022). Here, we suppose that the high glutathione concentration in P-CAS leaves (Figures 5 and S6) may be most likely responding to protein in the medium via interactions with other signalling molecules and phytohormones or membrane protection (Hasanuzzaman et al., 2017). In tobacco roots, we and others also identified heat-shock proteins that play an important role in plant metabolism and stress tolerance (Anaraki et al., 2018; Berka et al., 2022; Doubnerova Hyskova et al., 2014; Hyskova, Belonoznikova, Doricova, et al., 2021). The roots of H-CAS showed a higher representation of heat shock protein Hsp90 than high-IN, while the values remained unchanged for all other groups (Figure 7).

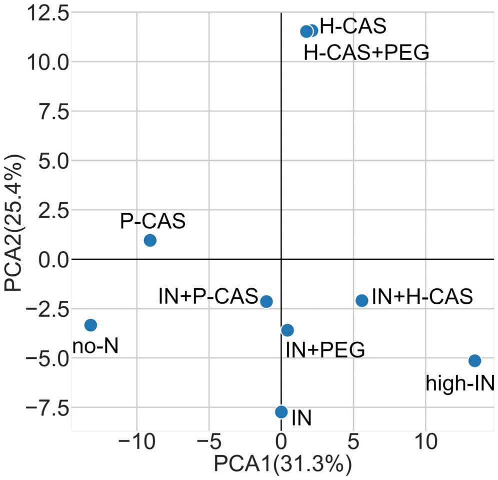

FIGURE 9 Principal component analysis resulting from all measured experimental data in tobacco plants. The horizontal axis indicates the score in the first principal component (PCA1), and the vertical axis indicates the score in the second principal component (PCA2). R^2 value was calculated as 0.5, that is, moderate reliability.

4.5 | Drought stress

In plants, drought stress represses photosynthesis and growth while inducing oxidative stress via ROS accumulation, and stomatal closure controlled by ABA (Doubnerova Hyskova et al., 2014). Organic fertilizers in the form of nano-vermicompost have beneficial effects on drought-stressed tomato plants (Ahanger et al., 2021). In *Capsicum annuum*, applying a plant protein hydrolysate to the soil resulted in a quick recovery after drought, most likely due to its priming effect, for example, antioxidant compounds accumulation, high catalase activity and increased aboveground growth (Agliassa et al., 2021). Here, the growth parameters, total N and protein content in leaves and N acquisition in roots were lower in the IN + PEG plants than in the IN plants, while the growth parameters of H-CAS were mostly similar to those of H-CAS + PEG plants (Figures 1, 2, and 4). Lower N availability or drought (simulated by PEG) implies an increase in root biomass relative to shoots (Figure 1). Surprisingly, the plants with the lowest N content (no-IN and P-CAS) were found to have the highest DW content, that is, the least water content (Figure S3). Water management and N assimilation in plants are influenced by many factors, including uptake (e.g., number of aquaporins) and transpiration (Araus et al., 2020; Ding et al., 2018). Thus, no-IN and P-CAS plants appear to have the lowest amount of aquaporins in their roots. They most likely have limited transpiration due to reduced stomatal conductance due to low N availability (Araus et al., 2020), similar to IN + PEG and H-CAS + PEG plants that close stomata because of lack of water. In comparison with the plants exposed to PEG, the no-IN and P-CAS plants most likely have a lower content of osmotically active compounds, they showed low total free amino acid content (Figure 3) and proline was under the limit of detection (Figure 4). Pro is known to be induced under drought and functions as an osmoprotectant and ROS scavenger (Lahuta et al., 2022). These results suggest that other metabolites, such as arabitol and gluconic acid, are being used instead of proline (Figure 8). The phytohormone ABA acts as a potential nutrient starvation stress-protecting hormone (Rubio et al., 2009). Considering that ABA regulation, responding to abiotic stress, is partly controlled by its catabolism, more specifically by its oxidation to PA or formation of ABA-glucose ester (Marusig & Tombesi, 2020), our results suggest that casein hydrolysate directly or indirectly downregulates ABA oxidation to PA (Figure 2D). The significantly high amount of nornicotine in H-CAS, P-CAS, H-CAS + PEG and IN + PEG roots (Figure 8) may be related to the endurance of these groups to abiotic forms of stress (N shortage, drought). Based on our results, it seems that the chosen concentration of PEG caused only moderate stress in the plants. Another explanation could be the positive effect of casein hydrolysate on plant stress tolerance, which is indicated by higher leaf protein content (Figure 3A).

Based on the PCA analysis (Figure 9), casein hydrolysate as N source seems to protect the plants from drought stress simulated by PEG as all the measured parameters positively correlate between H-CAS and H-CAS + PEG. The negative correlation between no-N and H-CAS plants further corroborates casein hydrolysate as a reasonable source of N. The closeness of IN + P-CAS and IN + PEG may insinuate the presence of casein protein as a signal to promote plants' resilience.

5 | CONCLUSIONS

Casein hydrolysate can serve as the sole source of N that enables tobacco growth, while casein protein may be utilized as source of N only to a limited extent. Both forms of casein had a strong effect on the concentrations of phenylacetic acid, a type of auxin, and on cytokinins, indicating adjustments of the root system to N availability. Tobacco roots grown with protein contained free amino acids most likely derived from casein. By contrast, in plants grown with no source of N, the concentration of free amino acids was below the limit of detection. Branched-chain (Ile, Leu, Val) and basic amino acids (Arg, His, Lys) accumulated in the plants grown with both forms of casein and thus may be preferably taken up. Proteases are most likely key components to protein and peptide utilization. Proteomics of tobacco roots revealed that peptidase C1A and peptidase S10 families may play a key role in protein casein degradation and amidases response to N starvation by releasing ammonia from organic amides and modulating auxin synthesis. The high content of secondary metabolites such as ferulic acid, nicotine and nornicotine together with phytohormones SA and ET precursor (ACC) in the roots of plants supplemented with casein hydrolysate may point out to the priming effect of this form of biostimulant.

Combined, these findings indicate that casein hydrolysate enables tobacco plant in vitro growth as the sole source of N by engaging peptide and amino acid uptake and modulating the metabolism. Moreover, the phytohormonal levels were affected and defense responses activated, so that a certain type of biostimulation could occur.

AUTHOR CONTRIBUTIONS

Kateřina Bělonožníková performed experiments, analysed the data and co-wrote the manuscript; Martin Černý performed experiments, analysed the data and co-wrote the manuscript; Veronika Hýsková analysed the data and co-wrote the manuscript; Helena Synková designed the project and edited the manuscript; Roland Valcke secured funding, supervised the project and edited the manuscript; Ondřej Hodek, Tomáš Křížek, Daniel Kavan, Radomíra Vaňková, Petre Dobrev and Daniel Haisel performed experiments; Helena Ryšlavá wrote the manuscript, designed and supervised the project.

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DATA AVAILABILITY STATEMENT

All data are available in the manuscript or Supplementary files. The raw data that support the findings of this study are available from the corresponding author upon reasonable request.

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