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Modulation of Copper Uptake and Toxicity by Abiotic Stresses in *Matricaria chamomilla* Plants

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ABSTRACT: The impact of salinity (S) or nitrogen deficiency (−N) on copper (Cu) uptake and changes to metabolism were studied in the combined treatments after 7 days of exposure. S suppressed growth, water content, soluble proteins, and reducing sugars more negatively than −N. ROS (hydrogen peroxide and superoxide) were differentially but relatively slightly affected while peroxidase activities were strongly elevated mainly in Cu+NaCl variant. Total soluble phenols and individual phenolic acids (free and cell wall-bound fraction) were accumulated the most in Cu−N while, among free amino acids, proline sharply increased in Cu+NaCl; this suggests a compensatory mechanism between the syntheses of antioxidants aimed to maintain antioxidative protection because numerous root phenolic acids were even depressed by S. Salinity also suppressed accumulation of coumarin herniarin, but its glucosidic precursors ((*Z*)- and (*E*)-2-*ß*-D-glucopyranosyloxy-4-methoxycinnamic acids) increased. Activities of selected phenolic enzymes were rather suppressed by S after a given exposure period while lignin content increased, suggesting different time dynamics if S and −N variants are compared. Selected mineral nutrients (K, Fe, and partially Mg) were more reduced by S than by −N. Shoot and root Cu amounts were depressed by −N but elevated by S. Significance and possible role of observed metabolic changes in relation to Cu accumulation are discussed.

KEYWORDS: *chlorogenic acid, heavy metals, NaCl, oxidative stress, phenylalanine ammonia-lyase*

INTRODUCTION

Copper (Cu) is an essential plant micronutrient, being a cofactor of many enzymes, but it can be phytotoxic at high concentrations.[1−3] Owing to its wide use in fungicides, it may easily enter the environment, leading to damage of plants if present in excess. It is a more harmful element in comparison with redox-inactive metals such as cadmium owing to enhancement of reactive oxygen species (ROS) formation by the Fenton−Haber−Weiss reaction. Cu therefore shows higher toxicity to plant growth if applied in identical concentrations despite its low root-to-shoot translocation.[3−7]

Nitrogen (N) is a quantitatively and qualitatively crucial plant macronutrient owing to its essentiality for the biosynthesis of amino acids, proteins, and enzymes.[8−10] N deficiency has therefore an extensive impact on the growth and overall metabolism, and a shift from N-based to C-based compounds has been observed in different plant species including *Matricaria chamomilla* L. (chamomile).[11−13] Enhanced accumulation of phenolic metabolites is a typical sign of N deficiency and is thought to ameliorate toxic symptoms and metal uptake[14] depending on N form used.[15]

Soil salinity is an important abiotic stress limiting productivity and geographical distribution of plants. Excess of NaCl has an adverse effect on the uptake of water and nutrients, photosynthesis, and growth, and also stimulates reactive oxygen species (ROS) overproduction and oxidative stress symptoms.[16−18] Contrary to N deficiency, changes to phenolic metabolites in response to salinity are rather negligible[16] while accumulation of amino acids was frequently interpreted as a symptom of NaCl-induced damage; as found in barley roots, physiologically relevant concentrations of free amino acids might contribute to amelioration of saline stress by regulating K^+ transport across the plasma membrane, thus enabling maintenance of an optimal K^+/Na^+ ratio.[19]

Plants' evolution exposed them to numerous environmental changes leading to development of an array of protective mechanisms. In terms of antioxidative protection, antioxidative enzymes and low molecular antioxidants (ascorbate, glutathione, and phenols) are essential protective tools. Phenolic metabolites are among the most abundant compounds representing a substantial part of the plant organic matter, and therefore their responses to different stresses are not surprising. For example, they scavenge ROS directly or through

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enzymatic reactions[20] and are able to chelate/precipitate metals.[21,22] In the case of Cu excess, their exudation in the rhizosphere has been found[23] and strong quantitative changes in different plant species suggest an essential role in Cu detoxification.[6,7,24] Additionally, phenolic metabolites are important antioxidants in the human diet, and therefore research of their quantitative changes in medicinal plants could be of practical significance.

Limited extent of agricultural soil suggests cultivation of plants nonessential for food production on the soil with lower physicochemical quality. Elevated uptake of metals by agricultural and/or medicinal plants may be potentially dangerous for human health.[1] Mechanisms aimed to reduce plant metal uptake are therefore frequently studied. Despite a huge number of studies focused on the toxicity of Cu, N deficiency, or salinity, there still exist only a limited number of studies which also compared combined treatments.[5,17,18] However, only few parameters were analyzed in the mentioned papers.

Chamomile is a widely used medicinal plant, and our previous studies have shown higher sensitivity to Cu (in comparison with Cd) and, at the same time, higher induction of specific phenolic metabolites preferentially in the roots.[2,25] Cu dose for the present study was therefore selected with respect to previous studies aimed to achieve clear impact on metabolites but without damaging effect on the growth after a given exposure period (7 days). On the other hand, selected NaCl concentration reduced growth but had no extensive impact on the metabolites analyzed.[16] Chamomile metabolism is also strongly affected by N deficiency including higher production of phenolic metabolites but depletion of amino acids.[8,14] Because single stress impact is almost improbable in nature, we combined saline or N-deficient conditions with Cu excess, and Cu alone was used as the main control in addition to control (no additional Cu). Basic physiological responses, phenolic metabolites and enzymes, free amino acids, stress parameters, and selected mineral nutrients including Cu were assayed in detail. Many parameters in terms of combined treatments are reported here for the first time. Significance of phenols for the observed changes to Cu uptake is compared and discussed in NaCl and N-deficient treatments.

MATERIALS AND METHODS

Plant Culture and Experimental Design. Twenty-one day old seedlings of *Matricaria chamomilla* L. (tetraploid 'Lutea', Asteraceae) germinated in sand (with 3−4 first true leaves) were placed in Hoagland solution containing 205 mg of N L^{-1}.[11,14,16,25] Uniform plants were cultivated in dark plastic boxes with 5 L of continually aerated solution (25 plants per box). The experiment was performed in a growth chamber under controlled conditions: 12 h day (6:00 a.m. to 6:00 pm); photon flux density ∼210 μmol m^{-2} s^{-1} PAR at leaf level supplied by cool white fluorescent tubes TLD 36W/33 (Philips, France), 25/20 °C day/night temperature, and relative humidity ∼60%. Under these conditions, plants form basal leaf rosettes only and whole rosettes were analyzed for all parameters (=shoot). Solutions were renewed weekly to prevent nutrient depletion. Plants which had been cultivated in hydroponics for 5 weeks were used in the experiment. The above-mentioned full nitrogen (N) solution was used for three variants: (i) control (no additional chemicals), (ii) 60 μM Cu, and (iii) 60 μM Cu + 100 mM NaCl (control, Cu, and Cu+NaCl). For the experiment with N deficiency, N containing salts from the above-mentioned Hoagland solution were substituted by salts without N ($Ca(NO_3)_2{\cdot}4H_2O$−$CaCl_2{\cdot}2H_2O$, $NH_4H_2PO_4$−KH_2PO_4, KNO_3−K_2SO_4[8,14]) plus 60 μM Cu (Cu−N). Cu ions were added in the form of $CuCl_2{\cdot}2H_2O$ (Lachema Brno, Czech Republic). In addition, all variants contained 0.21 μM Cu as micronutrient and pH was checked to be 6.0 ± 0.1. Parallel controls (N deficiency alone and salinity alone) were not tested because they were published in our previous papers and are carefully discussed here. Plants were harvested after 7 days of exposure to treatments. Fresh and dry masses were estimated in order to determine the tissue water content [100 − (dry mass × 100/fresh mass)] allowing recalculation of parameters measured in fresh samples. These dried samples were analyzed for free amino acids, phenolic acids, coumarins, lignin, reducing sugars, and mineral nutrients including Cu. Plants for fresh mass-requiring parameters were powdered using liquid N_2 and extracted as described below. For enzymes, selected supernatants were boiled to destroy enzyme activity and to check that the observed reaction was enzymatic. Spectrophotometry was carried out with an Uvi Light XTD 2 (Secomam, ALES Cedex, France).

Assay of Nitrogenous Compounds. Soluble proteins were quantified according to Bradford[26] using 20 μL of supernatants from homogenates prepared using borate or phosphate buffer (see below), and nonsignificant differences were observed; bovine serum albumin was used as standard, and data were expressed as mg g^{-1} DW. Free amino acids were extracted with 80% aqueous ethanol, and analyses were performed on an HP 1100 liquid chromatograph (Hewlett-Packard, Waldbronn, Germany) with fluorometric detector FLD HP 1100 and using precolumn derivatization with *o*-phthalaldehyde and 9-fluorenylmethyl chloroformate.[11,14]

Measurement of Carbonaceous Compounds. Total soluble phenols were extracted with 80% methanol from fresh tissue and measured using the Folin−Ciocalteu method with gallic acid as standard; flavonoids (sum of flavonols) were estimated in the same supernatant using $AlCl_3$ procedure and quercetin as standard.[8,16] The selected cinnamic and benzoic acid derivatives were measured in 80% methanol extracts (free acids) and after alkaline hydrolysis of methanol-insoluble root residue (cell wall bound compounds). Extraction was done using the ether method and quantification using UPLC−MS/MS system (ACQUITY UPLC, Waters, Milford, MA, USA; Micromass Quattro micro, Waters MS Technologies, Manchester, U.K.) as described in detail previously.[27,28] Amount of coumarin-related compounds [(*Z*)- and (*E*)-2-*β*-D-glucopyranosyloxy-4-methoxycinnamic acids (GMCAs) and herniarin] were estimated by gradient HPLC. Dried shoots were homogenized with sea sand and extracted with 80% methanol. Herniarin (Extrasynthese France) was used for the quantification, and herniarin precursors (*Z*)- and (*E*)-GMCAs were prepared as described in the previous study.[29] Root lignin content was estimated by the thioglycolic acid reaction after purification of homogenized tissue with 1% Triton X-100, 1 M NaCl, and acetone.[25] Reducing sugars were extracted with deionized water from dried material by homogenization with inert sand using mortar and pestle and determined colorimetrically using reaction with arsenomolybdate according to Somogyi−Nelson's method; standard curve was prepared with different concentrations of glucose (0.57−2.85 mg/L).[30]

Enzymatic Activities. Activity of phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) was determined as the production of (*E*)-cinnamic acid from phenylalanine using the HPLC method[31] with a slightly modified protocol in homogenates prepared using sodium borate buffer (pH 8.7), and PAL activity was expressed as nmol of (*E*)-cinnamic acid formed per minute and mg of protein.[8] To determine activities of shikimate dehydrogenase (SKDH, EC 1.1.1.25), cinnamyl alcohol dehydrogenase (CAD, EC 1.1.1.195), polyphenol oxidase (PPO, EC 1.10.3.2), guaiacol peroxidase (GPX, EC 1.11.1.7), and ascorbate peroxidase (APX, EC 1.11.1.11), samples were homogenized in 50 mM potassium phosphate buffer (pH 7.0) containing 5 mM polyvinyl polypyrrolidone at 4 °C and assessed as the production of NADPH (SKDH and CAD, 340 nm), oxidation of catechol (PPO, 420 nm), guaiacol (GPX, 470 nm), and ascorbate (APX, 290 nm). Calculations were done as described earlier.[8,32]

Assay of ROS. Accumulation of hydrogen peroxide (using $TiCl_4$ method) and superoxide (using hydroxylamine method) were measured in potassium phosphate buffer homogenates.[8,32]

Quantification of Cu and Selected Mineral Nutrients. Samples for quantification of metals were prepared as described elsewhere.[14,16,32] Briefly, dry material was mineralized in HNO_3 and H_2O_2 mixture and then evaporated to dryness at 90 °C. Dry residue was dissolved in 5% HNO_3. All measurements were carried out using an atomic absorption spectrometer AA30 (Varian Ltd., Mulgrave, Australia) and the air−acetylene flame. Samples for quantification of "intraroot" Cu were washed in 10 mM $CaCl_2$ at 4 °C for 30 min in order to remove metals adsorbed at the root surface, and all other mineral nutrients were quantified in these samples. For quantification of total root Cu, samples were washed with deionized water only (and Ca content was determined in these samples). Nitrogen content was estimated by the Kjeldahl method.[8,14]

Statistical Analyses. One box containing 25 plants was used for each treatment, thus the whole experiment included 4 boxes. Two independent repetitions of the whole experiment were performed in order to check reproducibility, and nonsignificant differences were observed. Data were evaluated using one-way ANOVA followed by Tukey's test (MINITAB Release 11, Minitab Inc., State College, PA) at $P < 0.05$. Number of replications (n) in tables/figures denotes individual plants measured for each parameter.

RESULTS AND DISCUSSION

Uptake of metals is affected by a wide range of factors, starting with plant species and ontogenetic stage. In terms of N nutrition, it has been observed that nitrate-fed chamomile plants exposed to N deficiency contained less Cd and Ni but ammonium-fed plants exposed to N deficiency did not.[14] We studied nitrate deficiency in the present study (because widely used Hoagland solution contains 14 mM nitrate from 14.6 mM of total N), and decrease of Cu in Cu−N therefore confirms essentiality of nitrate in the uptake of metals tested to date. In agreement, a study using Cd hyperaccumulator *Thlaspi caerulescens* showed that nitrate-fed plants contained more Cd than those ammonium-supplied.[15] Nitrate is the main N form absorbed by plant roots; therefore study of its effect on metal uptake is more physiologically and ecologically relevant. On the other hand, salinity enhanced Cu uptake, most considerably in the roots (Figure 1). We note that total and "intraroot" Cu content (desorption of surface-adsorbed Cu by $CaCl_2$, data not shown) did not differ in any variant, indicating various binding patterns in comparison with Cd.[32] Enhancement of toxic symptoms in the Cu+NaCl variant could therefore be directly related to elevated Cu uptake. Contrary to our data, cyanobacterium *Spirulina* showed a decrease in Cu amount if Cu+NaCl and Cu alone are compared,[17] indicating an uptake mechanism different from that in chamomile. In accordance with our data, N deficiency (comparison of 1× and 0.1× Hoagland strength where reduction of N content is also involved) decreased shoot Cu content in higher Cu dose (4 ppm) while, at lower Cu doses (0.5 and 1 ppm), even stimulation of Cu uptake was observed.[5]

Negative impacts of abiotic stresses are usually manifested through the appearance of chlorosis or necrosis, but we did not observe these symptoms in the present experiment. On the other hand, positive and negative changes to biomass production were found in Cu−N root and Cu+NaCl variant, respectively (Table 1). Copper alone had no impact because we used plants with bigger biomass[2] aimed to reduce drastic impact on the growth in combined treatments. N deficiency and salinity showed similar response at the shoot level (depression of growth) indicating Cu-independent action. In accordance with our data, salinity slightly enhanced Cu-induced depletion to growth in cyanobacteria *Spirulina* sp.,[17] but this species showed higher tolerance in combined treatments

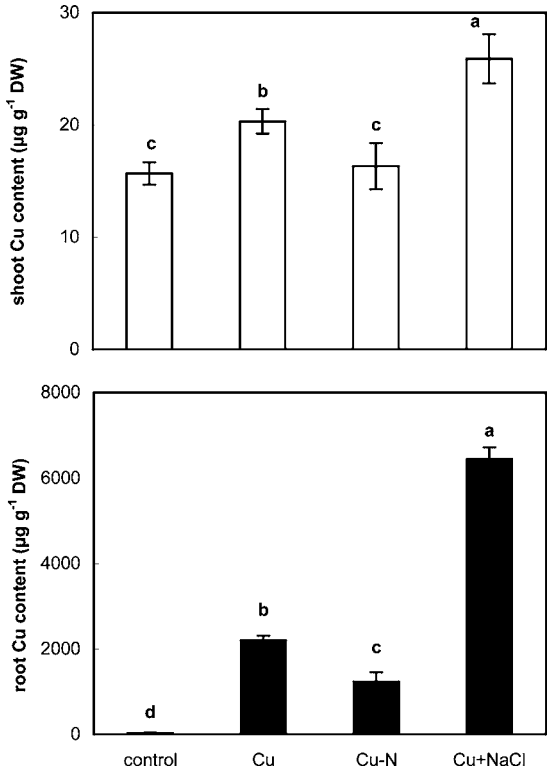

Figure 1. Copper accumulation in *Matricaria chamomilla* plants after 7 days of exposure to different treatments. Data are means ± SDs (n = 3). Bars represent standard deviation. Values for either shoots or roots followed by the same letter(s) are not significantly different according to Tukey's test ($P < 0.05$).

compared to chamomile analyzed here, probably owing to ecological/evolutionary differences. At the root level, increase in biomass in response to N deficiency (Cu−N, Table 1) fits well with our previous report where time dependence of this phenomenon was observed aimed to intensify N uptake from deficient environment.[8] A study on *Brassica pekinensis* also showed increase in root biomass if normal and 10-times diluted Hoagland solution (containing also lower amount of nitrogen) in combination with various Cu doses were compared.[5] In terms of effect of N deficiency on plant growth, it was recently shown that transgenic tobacco plants (contrary to wild type plants) did not show a decline in leaf/root ratio, indicating that rate-limiting enzyme of cytokinin synthesis may improve plant growth under N starvation.[9] Other physiological parameters (tissue water content and soluble proteins) were affected more negatively in Cu+NaCl treatment being in accordance with growth changes (Table 1). On the other hand, reducing sugars (glucose equivalents) were the most accumulated in Cu and Cu−N variants, indicating a shift of equilibrium between photosynthesis and respiration (Table 1), as previously observed in Cd but not in Ni excess.[14] On the other hand, salinity stimulated alternative oxidase in *Pisum* plants,[33] thus explaining less-visible increase in shoot sugars in comparison with N deficiency (Table 1). Overall, growth and basic physiological parameters are clearly differentially affected by salinity and nitrogen deficiency with the former factor enhancing metal-induced negative symptoms as visible also at the level of other parameters analyzed in the present study.

Reactive oxygen species are overproduced in response to various stresses including heavy metals, nitrogen deficiency, and

Table 1. Selected Physiological and Biochemical Parameters in *Matricaria chamomilla* Plants Exposed to Different Treatments over 7 Days[a]

	control	Cu	Cu−N	Cu+NaCl
		Shoot		
dry biomass (mg plant^{-1})	205.9 ± 14.5 a	201.3 ± 19.8 a	166.8 ± 11.6 b	118.4 ± 14.7 c
tissue water content (%)	91.1 ± 0.37 a	90.0 ± 0.32 b	89.9 ± 0.51 b	86.9 ± 0.73 c
soluble proteins (mg g^{-1} DW)	97.1 ± 6.34 a	96.3 ± 7.66 a	101.8 ± 4.09 a	79.4 ± 3.86 b
hydrogen peroxide (μmol g^{-1} DW)	12.5 ± 1.94 c	15.1 ± 3.63 bc	19.6 ± 1.70 b	26.7 ± 2.58 a
superoxide (μg g^{-1} DW)	14.3 ± 2.68 c	30.4 ± 3.71 b	39.5 ± 1.48 a	29.8 ± 3.07 b
ascorbate peroxidase (APX)	50.3 ± 4.43 a	51.0 ± 3.73 a	49.8 ± 2.98 a	37.6 ± 3.11 b
guaiacol peroxidase (GPX)	0.19 ± 0.03 c	0.20 ± 0.04 c	0.28 ± 0.03 b	0.37 ± 0.04 a
reducing sugars (mg g^{-1} DW)	19.7 ± 0.41 d	31.8 ± 0.52 b	34.5 ± 0.86 a	24.4 ± 0.18 c
		Root		
dry biomass (mg plant^{-1})	55.8 ± 3.61 b	57.4 ± 6.53 b	79.8 ± 6.64 a	35.6 ± 4.06 c
tissue water content (%)	95.0 ± 0.31 a	92.5 ± 0.35 c	93.5 ± 0.33 b	90.6 ± 0.24 d
soluble proteins (mg g^{-1} DW)	41.7 ± 3.71 a	28.3 ± 2.04 b	29.7 ± 4.10 b	13.2 ± 1.45 c
hydrogen peroxide (μmol g^{-1} DW)	19.8 ± 1.89 b	21.9 ± 2.28 b	27.8 ± 1.31 a	19.3 ± 2.13 b
superoxide (μg g^{-1} DW)	23.7 ± 3.62 b	62.5 ± 4.03 a	66.5 ± 4.35 a	28.1 ± 2.60 b
ascorbate peroxidase (APX)	88.3 ± 5.74 c	656.1 ± 23.2 b	583.9 ± 37.1 b	2431.7 ± 241.6 a
guaiacol peroxidase (GPX)	0.49 ± 0.08 d	1.40 ± 0.15 c	2.18 ± 0.44 b	3.35 ± 0.37 a
shikimate dehydrogenase (SKDH)	64.6 ± 2.61 c	97.2 ± 4.83 b	132.5 ± 18.7 a	16.1 ± 2.85 d
cinnamylalcohol dehydrogenase (CAD)	59.5 ± 4.64 b	346.7 ± 35.8 a	337.4 ± 28.7 a	80.9 ± 10.3 b
polyphenol oxidase (PPO)	0.88 ± 0.03 d	2.05 ± 0.09 c	2.52 ± 0.11 b	4.36 ± 0.12 a
lignin (mg g^{-1} DW)	26.1 ± 2.40 d	42.4 ± 1.83 b	33.9 ± 2.66 c	51.8 ± 2.16 a

[a] n = 15 for water content and dry biomass, and n = 3 for all other parameters. Copper (Cu) was applied in 60 μM and salinity (NaCl) in 100 mM concentrations. −N means nitrogen deficiency, and all other variants (control, Cu, and Cu+NaCl) were realized using full N Hoagland solution (see Materials and Methods for details). Units are μmol min^{-1} mg^{-1} of protein (GPX), nmol min^{-1} mg^{-1} of protein (APX, SKDH, CAD), and ΔA min^{-1} mg^{-1} protein of (PPO). Data are means ± SDs. Values within rows, followed by the same letter(s), are not significantly different according to Tukey's test ($P < 0.05$).

salinity also in chamomile.[3,11,16] Owing to fast metabolism of ROS, their actual level is affected by a balance between generation and removal by antioxidative enzymes as well as nonenzymatic antioxidants. Moreover, ROS may serve as the signals regulating biosynthesis of a variety of secondary metabolites.[11] Because we assayed ROS-related parameters after 7 days of exposure to treatments, direct correlations between the level of ROS (hydrogen peroxide and superoxide, Table 1), peroxidase activity and plant growth (Table 1) are not expected. In accordance, low level of H_2O_2 in the roots of Cu+NaCl fits well with the highest activity of both peroxidases in this treatment while shoots showed rather stimulation of GPX activity owing to the highest H_2O_2 level (Table 1). Previous study has shown that salinity under identical conditions (concentration and exposure time) had negligible impact on H_2O_2 level being correlated with low stimulation of peroxidases;[16] in the present study we observed stronger stimulation of GPX in both shoots and roots of Cu+NaCl than in Cu alone: this fact is probably related to elevated Cu uptake as discussed above. In accordance with our findings, combined Cu and NaCl treatment increased oxidative symptoms (malondialdehyde level) in rosemary leaves compared to Cu alone.[18] Nitrogen deficiency is a specific kind of stress because it primarily affects growth and overall metabolism through depletion of amino acids and proteins.[11] For this reason, changes to enzymatic activities including antioxidative enzymes must be expressed per mg of protein because they differ from per g FW calculations (see [8] for details). In accordance, mainly GPX activity was higher in the Cu−N variant than in Cu alone despite similar content of proteins (Table 1). Notwithstanding this, proteins were not so strongly reduced in the Cu−N variant as would be expected from our previous studies, and this phenomenon has also been observed in Cd−N and Ni−N treatments (even stimulation in comparison with N deficiency alone[14]). It is assumed that metal stress in combination with N deficiency generates pressure to protective mechanism aimed to maintain antioxidative protection; therefore balance between proteolysis and proteosynthesis is shifted toward enhancement of biosynthesis of antioxidative enzymes. Further studies will be focused on proteomic analyses of these events. N deficiency typically stimulates increase in ROS which may cross-talk with other radical (such as nitric oxide) leading to modulation of metabolism.[11] Because changes to ROS (H_2O_2) level showed an opposite trend under N deficiency in a time dynamic study if shoots and roots were analyzed,[8] higher amount of selected ROS could indicate a signaling role, e.g., for enhancement of phenolic metabolites.

Cu excess has usually a negative impact on plant nitrogen metabolism as observed in a sensitive population of *Silene vulgaris* which exhibited inhibition of nitrate uptake and protein synthesis.[34] This can be a reason for an increase in the sum of free amino acids and depletion of soluble proteins mainly in the roots (Tables 1 and 2). Changes to content of amino acids were more expressive in the roots which are directly in the contact with the cultivation solutions. In addition, N deficiency and salinity differ in terms of their impact on amino acid profile in chamomile mainly in the root tissue,[14,16] and the same we observed in combined treatments with similar intensity (Table 2). However, the shoot amino acid profile in the Cu−N variant was partially surprising because an expected depletion in response to N deficiency alone[14] was not found; this indicates that Cu excess is able to suppress N deficiency-induced depletion of amino acids probably owing to maintaining a pool for the biosynthesis of protective proteins because shoot

Table 2. Accumulation of Free Amino Acids (μmol g^{-1} DW; n = 4) in *Matricaria chamomilla* Plants Exposed to Different Treatments over 7 Days[a]

	control	Cu	Cu−N	Cu+NaCl
		Shoot		
aspartic acid	2.81 ± 0.18 b	2.73 ± 0.22 b	2.44 ± 0.35 b	4.46 ± 0.11 a
glutamic acid	1.52 ± 0.25 a	1.19 ± 0.21 ab	1.30 ± 0.28 ab	0.77 ± 0.06 b
serine	5.12 ± 0.17 a	5.21 ± 0.25 a	4.53 ± 0.26 b	4.08 ± 0.19 b
histidine	0.37 ± 0.03 c	0.47 ± 0.04 b	0.38 ± 0.01 c	0.62 ± 0.02 a
glycine	1.12 ± 0.11 a	1.05 ± 0.15 ab	0.83 ± 0.05 b	0.92 ± 0.09 ab
threonine	1.57 ± 0.07 b	1.69 ± 0.10 b	1.76 ± 0.15 ab	1.98 ± 0.11 a
arginine	1.22 ± 0.13 b	0.84 ± 0.09 c	1.61 ± 0.10 a	1.03 ± 0.14 bc
alanine	13.5 ± 1.39 a	14.2 ± 1.35 a	13.4 ± 1.64 a	8.94 ± 0.61 b
tyrosine	0.46 ± 0.04 a	0.48 ± 0.03 a	0.53 ± 0.05 a	0.54 ± 0.04 a
cysteine	0.47 ± 0.06 b	0.65 ± 0.05 a	0.26 ± 0.06 c	0.26 ± 0.03 c
valine	1.24 ± 0.08 a	1.13 ± 0.14 a	0.83 ± 0.03 b	1.07 ± 0.04 a
methionine	0.12 ± 0.01 b	0.15 ± 0.01 b	0.16 ± 0.01 b	0.27 ± 0.03 a
phenylalanine	0.62 ± 0.03 b	0.64 ± 0.05 b	0.61 ± 0.04 b	0.93 ± 0.14 a
isoleucine	1.74 ± 0.03 d	2.15 ± 0.11 c	2.64 ± 0.18 b	3.89 ± 0.26 a
leucine	0.75 ± 0.03 b	0.76 ± 0.06 ab	0.72 ± 0.04 b	0.88 ± 0.07 a
lysine	0.61 ± 0.03 a	0.62 ± 0.04 a	0.57 ± 0.03 a	0.62 ± 0.05 a
proline	8.12 ± 0.87 c	15.2 ± 1.47 b	13.7 ± 2.32 b	48.3 ± 3.73 a
sum	41.4 ± 2.65 c	53.6 ± 3.78 b	46.4 ± 2.29 bc	79.5 ± 4.75 a
		Root		
aspartic acid	2.33 ± 0.25 a	1.44 ± 0.11 b	0.57 ± 0.04 c	0.63 ± 0.03 c
glutamic acid	1.15 ± 0.13 a	0.97 ± 0.11 a	1.10 ± 0.07 a	0.45 ± 0.04 b
serine	3.14 ± 0.25 a	3.17 ± 0.43 a	1.65 ± 0.11 b	1.32 ± 0.23 b
histidine	0.63 ± 0.03 a	0.42 ± 0.04 b	0.51 ± 0.03 b	0.45 ± 0.03 b
glycine	1.36 ± 0.29 bc	3.24 ± 0.26 a	0.73 ± 0.04 c	2.06 ± 0.24 b
threonine	1.14 ± 0.09 b	2.26 ± 0.18 a	0.92 ± 0.05 b	0.72 ± 0.03 c
arginine	0.95 ± 0.04 b	1.06 ± 0.11 ab	0.44 ± 0.02 c	1.31 ± 0.15 a
alanine	12.0 ± 1.30 a	13.7 ± 1.04 a	7.42 ± 0.44 b	6.63 ± 0.25 c
tyrosine	0.35 ± 0.02 a	0.33 ± 0.03 a	0.22 ± 0.02 b	0.25 ± 0.03 b
cysteine	0.23 ± 0.02 a	0.24 ± 0.04 a	0.07 ± 0.01 b	0.20 ± 0.03 a
valine	0.75 ± 0.05 b	1.47 ± 0.12 a	0.68 ± 0.04 b	0.95 ± 0.13 ab
methionine	0.19 ± 0.02 a	0.21 ± 0.03 a	0.15 ± 0.02 ab	0.10 ± 0.01 b
phenylalanine	0.44 ± 0.03 a	0.39 ± 0.02 a	0.24 ± 0.04 b	0.23 ± 0.02 b
isoleucine	0.97 ± 0.13 a	0.85 ± 0.11 a	0.76 ± 0.07 a	0.74 ± 0.08 a
leucine	1.75 ± 0.16 a	1.53 ± 0.21 a	1.65 ± 0.18 a	1.13 ± 0.09 b
lysine	0.24 ± 0.02 a	0.21 ± 0.02 a	0.13 ± 0.03 b	0.04 ± 0.002 c
proline	5.51 ± 0.36 b	7.52 ± 0.42 b	2.44 ± 0.30 c	27.8 ± 2.47 a
sum	33.2 ± 2.07 c	38.6 ± 1.02 b	19.7 ± 0.81 d	45.1 ± 2.33 a

[a]Other details are as in Table 1.

soluble proteins were not altered in Cu−N (cf. Tables 1 and 2). Besides, N deficiency-induced depletion of amino acids is regulated by cytokinin synthesis because transgenic tobacco showed different responses of respective enzymes in comparison with wild-type plants.[10] Among individual compounds, proline showed the most pronounced quantitative responses. This amino acid is thought to protect cells against various stress impacts including metals, and its ability to scavenge ROS is also known.[35] We previously observed its increase in NaCl-exposed chamomile roots but not in shoots,[16] while N deficiency alone reduced its accumulation in both plant parts.[14] The strong increase we observed here mainly in Cu+NaCl shoots (Table 2) indicates salinity-enhanced Cu toxicity owing to increase in shoot Cu amount and potential antioxidative role of this particular amino acid. In fact, we previously observed proline increase in plants with applied inhibitor of phenolic metabolism,[24,36] and compensatory mechanism in order to maintain regulation of ROS level is assumed. Contrary to our results, cyanobacterium *Spirulina* exposed to Cu+NaCl (1 mg + 0.4 mol/L) showed only slight increase in proline level,[17] indicating differences between vascular and nonvascular organisms. Among aromatic amino acids, content of Phe and Tyr was depleted by N deficiency and salinity preferentially in the roots while shikimate dehydrogenase (an enzyme involved in the pathway leading to aromatic amino acids) was differentially affected in these two treatments, suggesting involvement of other than biosynthetic pathways in the regulation of aromatic amino acid levels. Cysteine is a specific amino acid able to increase shoot Cu uptake in maize,[37] and we observed correlation between shoot Cys and Cu increase in diploid chamomile,[24] but the present study did not show any direct correlation in N deficient or saline conditions (Table 2).

Phenolic metabolites form a substantial part of plant organic matter and are greatly varied in their chemical structure. Therefore their quantitative responses to different stress impacts including excess of metals are not surprising.[6,7] Notwithstanding this, their role in many aspects of metal toxicity is seriously underestimated. We previously observed

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impact of phenols on Cd, Ni, and Cu uptake using inhibitor of phenolic metabolism.[24,36] In terms of individual stress impacts presented here, salinity alone had a negligible impact on shoot phenols and max. doubled individual root phenols[16] while N deficiency alone increased both shoot and root phenols.[14] In the present study, both N deficiency and salinity augmented Cu-induced enhancement of total phenols and flavonols mainly in the roots (Figure 2), being more visible in Cu−N treatment.

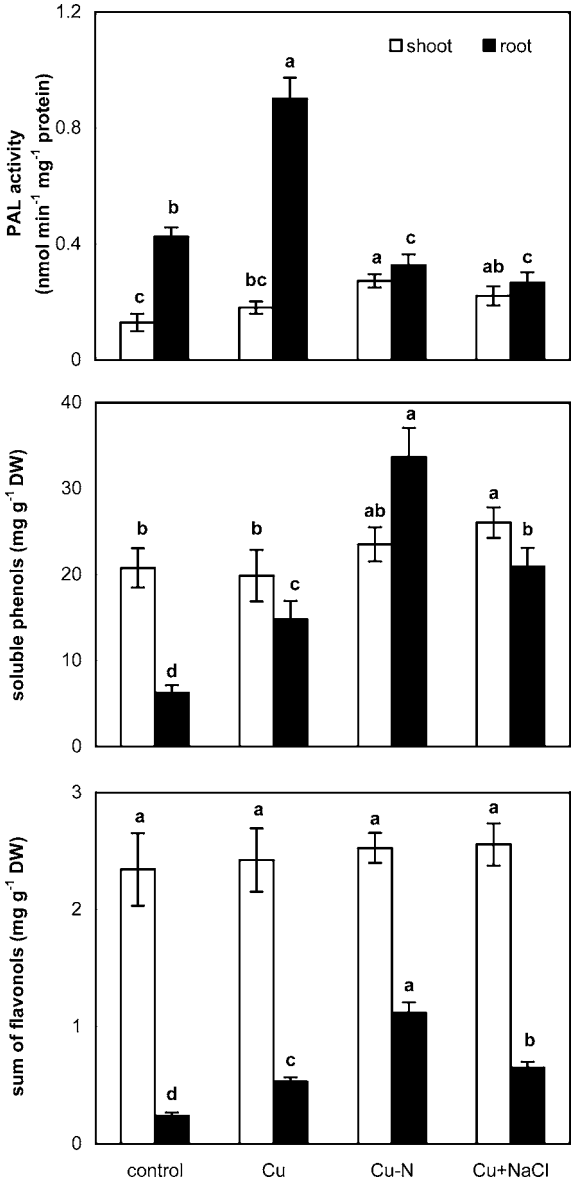

Figure 2. Phenolic metabolism in *Matricaria chamomilla* plants after 7 days of exposure to different treatments (see Table 1 for details). Data are means ± SDs (n = 4). PAL: phenylalanine ammonia-lyase. Other details are as in Figure 1.

It terms of signalization, this phenomenon may be evoked by elevated hydrogen peroxide content preferentially in Cu−N variant because this compound has significant impact on the regulation of phenolic metabolism in plants (ref 14 and the references therein). In accordance, individual phenolic acids were also elevated the most in the Cu−N variant in both fractions (Table 3). However, it remains unclear why total soluble phenols were not elevated in the shoots of the Cu−N variant if their additive increase was observed in Cd−N and Ni−N plants.[14] One possible explanation is that Cu is a redox-active metal with very slow root-to-shoot translocation and thus exudation of particular phenols, which is usually observed under Cu excess,[23] could take a place under N deficiency in combination with Cu excess because tissue Cu content was also depleted (Figure 1). Phenolic acids are also effective antioxidants, e.g., against Cu-induced protein carbonylation,[38] and this fact could be important in the Cu+NaCl variant where proteins were considerably reduced and shoot Cu content increased. On the other hand, decrease in individual phenols in the free and cell wall bound fraction of roots if Cu+NaCl and Cu alone are compared indicates different responses of photosynthetic and non-photosynthetic tissue to salinity. In accordance with our findings, rosemary leaves revealed a slight increase in total phenols in combined Cu+salinity treatments.[18] Less-visible stimulation of total phenols or even depletion of some phenolic acids in Cu+NaCl variant despite elevated Cu uptake could be compensated by strong enhancement of proline accumulation in order to maintain oxidative balance (cf. Tables 2 and 3). Enhanced lignin accumulation is a typical sign of metal excess being mainly evoked by Cu excess.[25] Additionally, lignification is controlled by ROS/NO level under Cu excess;[39] therefore the highest lignin content in the Cu+NaCl variant (Table 1) fits well with the most negative growth responses and confirms elevated oxidative stress. On the other hand, depleted activities of shikimate dehydrogenase and cinnamylalcohol dehydrogenase (Table 1) in the roots of the Cu+NaCl variant are in accordance with depletion of aromatic amino acids and selected root phenolic acids, indicating that phenols are not main NaCl-induced antioxidative compounds. Comparison of CAD activity vs lignin content in Cu+NaCl indicates different time dynamics and fast deposition of lignin (see ref 25 for details). Polyphenol oxidase was the only phenolic enzyme which revealed strong stimulation in Cu +NaCl (Table 1), supporting the assumption that salinity may deplete phenols through their oxidation. Phenylalanine ammonia-lyase, a pivotal step of phenolic biosynthesis, was slightly elevated in the Cu−N variant in shoots but depleted in both Cu−N and Cu+NaCl treatments in roots (Figure 2). This confirms essentiality of phenols rather in the shoots after the prolonged exposure we used (7 days) and earlier stimulation at the root level with respect to observed increase in root phenols as naturally expected due to direct contact of roots with culture solution (see, e.g., different time dynamics of PAL under Cd and Cu stress[25]). Coumarins are quantitatively important shoot-localized chamomile metabolites.[29,36] We note that herniarin and its precursors [(*Z*)- and (*E*)-2-*β*-D-glucopyranosyloxy-4-methoxycinnamic acids (GMCAs)] do not react with Folin−Ciocalteu reagent used for total soluble phenols assay probably owing to the absence of free hydroxyl group attached to the aromatic ring. It was shown that foliar Cu application led to decrease in GMCAs and increase in herniarin referred to as "stress-related" changes[29] mediated by enzymatic glucose elimination from GMCA and spontaneous cyclization to herniarin. Further studies with hydroponically applied Cu or inhibition of phenolic metabolism[2,36] did not show a similar effect, and either partial or parallel increase or decrease in both GMCAs and herniarin was observed. These data strongly suggest that foliar Cu application simply directly disrupts biosynthesis owing to toxic concentration, and all other data indicate coordinated regulation of biosynthesis. Our present data are in accordance with this conclusion in all three variants

Table 3. Accumulation of Free Phenolic Acids and Cell Wall Bound Phenolic Acids ($\mu g\ g^{-1}$ DW; $n = 3$) in *Matricaria chamomilla* Plants Exposed to Different Treatments over 7 Days[a]

	control	Cu	Cu−N	Cu+NaCl
		Shoot		
gallic	4.65 ± 0.26 b	3.58 ± 0.11 c	3.79 ± 0.36 c	6.23 ± 0.37 a
protocatechuic	17.4 ± 1.54 b	20.9 ± 3.02 b	30.3 ± 4.28 a	33.1 ± 2.29 a
*p*OHbenzoic	5.41 ± 0.33 b	8.70 ± 0.57 ab	12.1 ± 2.02 a	11.5 ± 2.17 a
vanillic	93.8 ± 6.39 d	158.2 ± 11.6 c	274.4 ± 12.1 a	201.7 ± 12.4 b
salicylic	1.62 ± 0.22 b	2.73 ± 0.17 a	2.22 ± 0.30 a	1.50 ± 0.12 b
chlorogenic	16.7 ± 2.62 c	28.0 ± 2.84 c	85.3 ± 8.90 b	125.3 ± 8.57 a
caffeic	1.49 ± 0.21 b	1.61 ± 0.17 b	2.53 ± 0.19 a	0.61 ± 0.06 c
p-coumaric	2.38 ± 0.19 c	5.38 ± 0.24 b	11.3 ± 1.89 a	2.51 ± 0.08 c
ferulic	nd	nd	nd	nd
sum	143.2 ± 5.72 d	229.2 ± 7.54 c	422.1 ± 8.00 a	382.5 ± 16.8 b
		Root		
gallic	nd	nd	nd	nd
protocatechuic	4.33 ± 0.28 c	5.43 ± 0.17 b	8.44 ± 0.40 a	7.69 ± 0.30 a
*p*OHbenzoic	3.21 ± 0.25 c	2.76 ± 0.21 c	7.11 ± 0.29 a	4.59 ± 0.25 b
vanillic	nd	nd	nd	nd
salicylic	2.11 ± 0.23 c	4.38 ± 0.23 a	3.59 ± 0.36 b	3.32 ± 0.08 b
chlorogenic	4.88 ± 0.33 d	55.4 ± 4.95 b	82.5 ± 7.27 a	18.6 ± 3.08 c
caffeic	5.47 ± 0.40 c	18.1 ± 2.27 b	29.8 ± 3.86 a	8.49 ± 0.81 c
p-coumaric	0.54 ± 0.04 b	1.35 ± 0.26 a	1.50 ± 0.12 a	0.35 ± 0.04 b
ferulic	1.71 ± 0.12 d	4.57 ± 0.27 b	5.49 ± 0.18 a	2.45 ± 0.19 c
sum	22.3 ± 0.50 d	92.1 ± 2.68 b	138.5 ± 9.91 a	45.5 ± 2.72 c
		Root−alk.hyd.		
gallic	nd	nd	nd	nd
protocatechuic	4.71 ± 0.29 c	8.17 ± 0.37 b	4.89 ± 0.52 c	14.3 ± 2.32 a
*p*OHbenzoic	2.55 ± 0.21 a	2.60 ± 0.26 a	2.35 ± 0.30 a	2.45 ± 0.45 a
vanillic	nd	nd	nd	nd
salicylic	0.51 ± 0.05 a	0.22 ± 0.03 c	0.40 ± 0.04 ab	0.30 ± 0.04 bc
chlorogenic	nd	nd	nd	nd
caffeic	125.1 ± 8.62 d	230.5 ± 14.2 b	305.8 ± 24.3 a	187.1 ± 14.2 c
p-coumaric	2.35 ± 0.17 c	5.61 ± 0.57 a	3.39 ± 0.28 b	2.37 ± 0.24 c
ferulic	33.5 ± 2.44 b	49.5 ± 4.83 a	46.2 ± 3.97 a	30.3 ± 4.63 b
sum	168.8 ± 8.02 d	296.6 ± 11.0 b	363.2 ± 14.7 a	236.9 ± 14.6 c
		Coumarins		
Z-GMCA	2.18 ± 0.16 c	3.07 ± 0.24 b	3.63 ± 0.13 b	4.57 ± 0.27 a
E-GMCA	6.12 ± 0.32 c	10.7 ± 0.83 b	13.8 ± 1.61 ab	15.9 ± 1.19 a
GMCAs	8.30 ± 0.60 c	13.8 ± 0.92 b	17.5 ± 1.55 a	20.6 ± 1.17 a
herniarin	1.20 ± 0.14 b	1.67 ± 0.15 a	1.41 ± 0.19 ab	0.83 ± 0.09 c

[a]Other details are as in Table 1. *p*OHbenzoic, *p*-hydroxybenozic acid; alk.hyd., alkaline hydrolysis of methanol-insoluble root residue (cell wall bound compounds); *Z*- and *E*-GMCA, (*Z*)- and (*E*)-2-*ß*-D-glucopyranosyloxy-4-methoxycinnamic acids.

(Cu, Cu−N, and Cu+NaCl) because GMCAs were elevated in comparison with control (indicating no negative effect on biosynthesis). On the other hand, herniarin content was elevated only by Cu alone (in comparison with control) and even depleted in the Cu+NaCl variant (Table 3), suggesting negative impact on GMCA−herniarin conversion (β-glucosidase activity). Previous study also showed that metals (Cd and Ni) evoked further enhancement of GMCAs accumulation under N deficiency,[14] indicating significance of these metabolites in chamomile response to metals stress and nutritional starvation.

Excess of metals usually reduces the accumulation of essential mineral nutrients. This fact has also been observed under Cu excess, and higher intensity in comparison with Cd confirms stronger toxicity of Cu.[32] On the other hand N deficiency had almost no impact on the amount of minerals[14] while salinity showed a more dramatic effect.[16] In the present study, Cu caused a similar effect as previously observed (Table 4). N deficiency (Cu−N variant) ameliorated decrease in K^+ ions in comparison with Cu but augmented decrease in root Ca and Fe as previously observed in Cd−N and Ni−N treatments.[14] Salinity alone decrease the amount of Ca, Fe, and Mg in both shoots and roots in the previous study,[16] and the present study revealed a more negative impact on individual minerals in comparison with N deficiency (Table 4). On the other hand, shoot and root Na content was ca. +10 mg/g DW higher in Cu +NaCl (Table 4) in comparison with an earlier study conducted under identical conditions,[16] indicating enhancement of Na^+ uptake by coapplication with Cu. Nitrogen was the only mineral nutrient parameter which was less affected by salinity than by N deficiency (Table 4). The main reason for this observation is different trend if sum of amino acids (increase in Cu+NaCl owing to strong elevation of proline content) and soluble proteins are compared because N content was quantified by the type of Kjeldahl method which detects organic ammonium nitrogen only.

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Table 4. Quantitative Changes of Selected Mineral Nutrients in *Matricaria chamomilla* Plants Exposed to Different Treatments over 7 Days[a]

	control	Cu	Cu−N	Cu+NaCl
		Shoot		
N (mg g^{-1} DW)	56.0 ± 1.12 a	48.9 ± 1.40 b	37.3 ± 1.16 d	43.4 ± 1.40 c
K (mg g^{-1} DW)	103.2 ± 2.95 a	86.9 ± 2.66 b	89.9 ± 2.72 b	75.6 ± 3.84 c
Na (mg g^{-1} DW)	4.35 ± 0.29 b	4.22 ± 0.34 b	4.23 ± 0.22 b	31.5 ± 1.95 a
Ca (mg g^{-1} DW)	4.62 ± 0.23 a	4.36 ± 0.12 ab	4.04 ± 0.14 b	4.01 ± 0.12 b
Mg (mg g^{-1} DW)	2.36 ± 0.18 a	2.56 ± 0.23 a	2.36 ± 0.14 a	2.21 ± 0.19 a
Fe (mg g^{-1} DW)	0.25 ± 0.012 b	0.26 ± 0.018 b	0.32 ± 0.021 a	0.23 ± 0.020 b
Zn (μg g^{-1} DW)	70.6 ± 5.72 a	63.3 ± 4.16 ab	64.7 ± 3.35 ab	55.3 ± 4.68 b
		Root		
N (mg g^{-1} DW)	46.1 ± 1.16 a	46.4 ± 1.12 a	25.3 ± 1.96 c	41.0 ± 0.85 b
K (mg g^{-1} DW)	97.4 ± 4.79 a	63.6 ± 4.68 c	74.8 ± 4.12 b	26.7 ± 1.58 d
Na (mg g^{-1} DW)	5.11 ± 0.34 b	5.34 ± 0.21 b	5.18 ± 0.32 b	37.8 ± 2.23 a
Ca (mg g^{-1} DW)	5.29 ± 0.36 a	5.64 ± 0.15 a	4.02 ± 0.16 b	4.04 ± 0.20 b
Mg (mg g^{-1} DW)	1.53 ± 0.15 b	2.06 ± 0.19 a	1.46 ± 0.07 b	1.09 ± 0.16 c
Fe (mg g^{-1} DW)	9.47 ± 0.23 b	12.1 ± 1.22 a	9.05 ± 0.88 b	7.45 ± 0.64 c
Zn (μg g^{-1} DW)	162.6 ± 16.7 a	115.8 ± 10.6 b	144.3 ± 11.2 ab	109.6 ± 17.1 b

[a]Other details are as in Table 1.

In conclusion, N deficiency and salinity showed different impact on Cu toxicity. Absence of nitrate in the culture solution decreased Cu uptake but also amounts of free amino acids, while phenolic metabolites were considerably accumulated. Salinity almost exclusively enhanced toxic symptoms which could be partially ameliorated by excessive accumulation of proline, while individual phenolic metabolites were even depressed. Although the salinity dose we used is classified as medium stress and chamomile could be tolerant to lower doses, enhancement of Cu uptake indicates that chamomile should rather be cultured on the soils with lower level of nitrogen than on saline soils cocontaminated with metals.

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Notes

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