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UPTAKE, TRANSPORT AND ASSIMILATION

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Rhizosphere pH and cation-anion balance determine the exudation of nitrification inhibitor 3-*epi*-brachialactone suggesting release via secondary transport

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Abstract

Biological nitrification inhibition (BNI) of *Brachiaria humidicola* has been attributed to nitrification-inhibiting fusicoccanes, most prominently 3-*epi*-brachialactone. However, its release mechanism from *B. humidicola* roots remains elusive. Two hydroponic experiments were performed to investigate the role of rhizosphere pH and nutritional N form in regulating 3-*epi*-brachialactone release by *B. humidicola* and verify the underlying release pathway. Low rhizosphere pH and NH_4^+ nutrition promoted 3-*epi*-brachialactone exudation. However, the substitution of NH_4^+ by K^+ revealed that the NH_4^+ effect was not founded in a direct physiological response to the N form but was related to the cation-anion balance during nutrient uptake. Release of 3-*epi*-brachialactone correlated with the transmembrane proton gradient ΔpH and NH_4^+ uptake (R^2 = 0.92 for high ~6.8 and R^2 = 0.84 for low ~4.2 trap solution pH). This corroborated the release of 3-*epi*-brachialactone through secondary transport, with the proton motive force (ΔP) defining transport rates across the plasma membrane. It was concluded that 3-*epi*-brachialactone release cannot be conceptualized as a regulated response to soil pH or NH_4^+ availability, but merely as the result of associated changes in ΔP.

1 | INTRODUCTION

Biological nitrification inhibition (BNI) is defined as the plant-mediated control of soil nitrification via the release of nitrification inhibitors (Subbarao et al., 2006). This phenomenon has been described in situ for the forage grasses *Brachiaria humidicola* and *Hyparrhenia diplandra* as well as for the grain crop *Sorghum bicolor* (Lata et al., 2004; Sarr et al., 2019; Sylvester-Bradley et al., 1988; Tesfamariam et al., 2014).

Furthermore, BNI has been proposed for a series of graminaceous crops due to their ex situ proven ability to produce and release antimicrobial compounds with nitrification-inhibiting activity, e.g. *Leymus racemosus*, *Triticum aestivum*, and *Oryza sativa* (O'Sullivan et al., 2016; Subbarao, Tomohiro, et al., 2007; Sun et al., 2016). In several cases, nitrification-inhibiting compounds have been successfully isolated and identified. Prominent examples for *B. humidicola* are low-molecular-weight (LMW) terpenoids such as brachialactone (Subbarao et al., 2009) and its recently described isomer/derivative 3-*epi*-brachialactone and 3,18-epoxy-9-hydroxy-4,7-*seco*-brachialactone, both showing a significantly higher BNI activity than brachialactone (Egenolf et al., 2020). Methyl 3-(4-hydroxyphenyl) propionate, sakuranetin, and sorgoleone were found in *S. bicolor* (Subbarao et al., 2013; Tesfamariam et al., 2014; Zakir et al., 2008), while 1,9-decandiol was characterized for *O. sativa* (Sun et al., 2016).

The release mechanism of nitrification inhibitors (NI), however, is still a matter of debate. One acknowledged mechanism is the stimulation of NI release by low rhizosphere pH and NH_4^+ nutrition, as was proven in hydroponic experiments. This has been described for brachialactone in *B. humidicola* (Subbarao et al., 2009; Subbarao, Wang, et al., 2007), polar NIs released by *S. bicolor* (Di et al., 2018), unidentified NIs from *L. racemosus* (Subbarao, Wang, et al., 2007) and to a certain extent also for 1,9-decandiol in *O. sativa* (Pariasca Tanaka et al., 2010). These results were perceived as evidence for NIs being secreted via a regulated process, e.g. active transport or facilitated diffusion (Subbarao, Wang, et al., 2007; Zhu et al., 2012). Contrastingly, Souri and Neumann (2018) attributed the increase of NI release to membrane damage associated with low pH and, hence, suggested an unregulated, passive release mechanism as the principle release pathway.

Active transport is defined as an energy-driven primary or secondary transport, whereas passive exudation refers to diffusion along a concentration gradient, either through the plasma-membrane (non-polar compounds) or via channel proteins (polar compounds) (Marschner, 1995). Furthermore, exudation is considered as regulated if the plant controls the release rate and directly responds to ecological triggers. In the case of *S. bicolor*, Di et al. (2018) explained the stimulating effect of NH_4^+ on NI release by a signaling cascade, where NH_4^+ uptake leads to an increase in plasmalemma (PM) H^+ATPase activity triggering the opening of anion channels, which is finally responsible for NI release. This theory thus suggests a regulated, facilitated passive transport as release pathway. However, it is widely recognized that NH_4^+-dominated nutrition generally leads to the activation of PM-H^+ATPase (Alvarez-Pizarro et al., 2014; Schubert & Yan, 1997). Furthermore, the subsequent signal cascade and the proposed NI channels remain merely speculative.

Taking this controversy as an impetus, the objective of this study was to investigate the ecophysiological conditions and release pathway regulating the exudation of 3-*epi*-brachialactone, the most bioactive brachialactone isomer described so far for *B. humidicola*. We hypothesized that (1) low pH and NH_4^+ nutrition both stimulate the exudation rates of 3-*epi*-brachialactone. Accordingly, we hypothesized that (2) these stimulating effects are specific to 3-*epi*-brachialactone exudation, whereby its release is a regulated process and that (3) NH_4^+ supply constitutes a prerequisite for 3-*epi*-brachialactone accumulation in planta and release into the rhizosphere of *B. humidicola*.

2 | MATERIALS AND METHODS

2.1 | Experimental setup

Two consecutive hydroponic experiments were performed. The hydroponic system itself was designed in a way to ensure root cell plasma membrane integrity during exudate collection, e.g. by using only complete/balanced trap solutions, cross-validating primary metabolite release/loss (sugars, amino acids) and restricting the exudate collection time to a maximum of 6 h to avoid severe pH drops in the trap solution.

Experiment 1 was conceptualized to address hypotheses 1 and 2. It was designed as a two-factorial experiment with four replications, with "*pH*" (4.2 vs. 6.8) and "*Trap solution*" (NH_4^+ vs. NO_3^-) as experimental factors. Exudation rates of 3-*epi*-brachialactone, total sugar (TS), and amino acids (AA) were treated as response variables.

Experiment 2 was conceptualized to address hypothesis 3. It was designed as a two-factorial experiment with three replications. Experimental factors consisted in "*Preculture*" and "*Trap solution*." "*Preculture*" refers to the N source (NH_4^+ or NO_3^-) offered to the plants during a pre-culture period of 10 days prior to exudate collection, a time span considered sufficient for de novo synthesis of young root tissue, active in nutrient uptake and metabolite release. "*Trap solution*" refers to the nutrient solution offered during exudate collection and consisted of a NH_4^+ and a NO_3^- treatment plus an additional K^+ treatment (without N source). The latter treatment was included to separate charge balance effects from physiological responses directly related to N nutrition.

2.2 | Hydroponic system and trap solutions used for the experiments

Brachiaria humidicola cv. CIAT 679 plants (commercial name "Tully," ranked as high BNI cultivar) were propagated and raised during 6 weeks in a growth chamber-based hydroponic system with a day length of 12 h (6:00–18:00 h), light intensity of 525 W m^{-2}, air humidity of 75% and day/night temperatures of 30/20°C. The nutrient solution contained [μM]: NH_4NO_3 [1200], KNO_3 [400], $Ca(NO_3)_2$ [400], HNO_3 [600], K_2HPO_4 [200], $MgSO_4$ [200], $MgCl_2$ [100], Na_2SiO_3 [200], FeNA-EDTA [50], H_3BO_3 [10.0], $MnSO_4$ [4.0], $ZnSO_4$ [4.0], $CuSO_4$ [1.0], Na_2MoO_4 [1.0]. The nutrient solution was exchanged every second day and pH of fresh nutrient solution was adjusted at 4.8. It is important to emphasize the NH_4^+:NO_3^- ratio (1:2.5) of this nutrient solution. Although it is acknowledged that *B. humidicola* feeds dominantly on NO_3^- (>90%, as evidenced by N uptake analysis, Figure S1), the small portion of NH_4^+ turned out to essentially support vigorous plant growth. In contrast, pure NO_3^-

nutrition resulted in development of leaf chlorosis, so that the "*Preculture* treatment" (pure NH_4^+ vs. pure NO_3^-) had to be restricted to a period of 10 days (Experiment 2). The nutrient solution used for plant propagation was also the basis for all trap solutions used in the experiments.

In Experiment 1, for factor "*pH*," the pH of the trap solution was adjusted to the target values of 4.2 and 6.8 by addition of HCl or Na_2CO_3, respectively. The pH was not further corrected in the course of exudate collection since the collection time was restricted to 4 h, during which no major pH alterations occurred. Factor "*Trap solution*" consisted of a NH_4^+ and NO_3^- treatment. The NH_4^+ treatment was achieved by replacing all NO_3^- containing salts of the above-described nutrient solution by the respective chlorides and adjusting total N to the same final concentration of 4200 μM by addition of NH_4Cl. The NO_3^- treatment was obtained by replacing NH_4NO_3 of the above-described nutrient solution by 800 μM of both KNO_3 and $Ca(NO_3)_2$, yielding the same final N content of 4200 μM. Exudate collection was started at 9:00 h, 3 h after the onset of irradiation.

In Experiment 2, the pH was adjusted to 4.8 (corresponding to pH of propagation medium). Experimental factors "*Preculture*" consisted of NH_4^+ and NO_3^- treatments, while "*Trap solution*" consisted of NH_4^+, NO_3^-, and K^+ treatments. The NH_4^+ and NO_3^- treatments were prepared as in Experiment 1. The K^+ treatment was equal to the sole NH_4^+ treatment, but NH_4Cl was replaced completely by KCl (no N source). Exudates were collected over a period of 6 h, starting exudate collection at 9:00 h (3 h after onset of irradiation).

2.3 | Root exudate collection and sample processing

After root exudate collection, the trap solution was filtered to remove root debris (Whatman Grade 2). A 10% aliquot was frozen (−20°C) until analysis of NH_4^+ and NO_3^- (for N uptake calculations), total sugars (TS), and amino acids (AA). The remaining 90% were subjected to secondary metabolite extraction by liquid-phase extraction: NaCl was added to 1 L of the nutrient solution until saturation. The solution was extracted with 300 ml of ethyl acetate in a separating funnel. The ethyl acetate phase was filtered through a layer of 2 cm of anhydrous Na_2SO_4 to remove the remaining water. The extraction of the nutrient solution was repeated. Both ethyl acetate extracts were pooled and concentrated in vacuo to a final volume of 1.5 ml and stored at 4°C until 3-*epi*-brachialactone analysis. Prior to LC–MS measurements, ethyl acetate extracts were dried under N_2 and resuspended in acetonitrile/H_2O (80/20, v/v).

2.4 | Quantification of NH_4^+ and NO_3^- in trap solution

Both N forms were quantified colorimetrically, NH_4^+ as green ammonium salicylate complex at 667 nm, NO_3^- as yellow ionized form derived from alkalinization with sodium salicylate at 410 nm, according to Borrero Tamayo et al. (2017).

2.5 | Quantification of total sugars (TS) and amino acids (AA) in root exudates

Total sugars were quantified colorimetrically based on furfural formation upon dehydration of carbohydrates by concentrated H_2SO_4, which can be stained via condensation with anthrone (Turula et al., 2010). To 100 μl of root exudate samples, 900 μl of anthrone reagent (0.1% anthrone in 13.8 M H_2SO_4) was added. Staining was achieved by incubation at 40°C for 20 min. Concentration was subsequently determined spectrophotometrically at 620 nm, using a standard curve based on glucose in the range from 0 to 500 μM.

Amino acids were quantified via LC–MS (Accela HPLC/LTQ Velos MS, Thermo Scientific). Prior to AA quantification, root exudates were derivatized using the AccQ Tag Ultra Derivatization Kit (Waters). The pH was adjusted between 7 and 8, thereafter, 10 μl of sample was mixed with 70 μl of borate buffer and the respective volume of the AccQ-Tag-reagent was added. Samples were thoroughly mixed and incubated at 55°C for 15 min. After brief chilling, samples were diluted with 400 μl acetonitrile/H_2O (1/4, v/v) and analyzed via LC–MS using a commercial, mixed standard. LC–MS details are provided in Table S1.

2.6 | Acid hydrolysis of root tissue extracts and digestion with α- and β-glucosidase

Root tissue extracts were obtained by cutting 1 g of frozen root (fresh material, FM) tissue into small pieces, transferring the material into a falcon-tube, adding 4 ml of MeOH/H_2O (70/30, v/v) and blending with an Ultraturrax. Extracts were then filtered (Whatman Grade 2 followed by 0.2 μm syringe filter) and stored cool (4°C) until 3-*epi*-brachialactone quantification via LC–MS.

Acid hydrolysis was performed on an aliquot of the extract by adding 2 M TFA in ratio 1 to 1 and heating the sample to 80°C for 30 min. After cooling, organic compounds were extracted by liquid phase extraction and samples were prepared for LC–MS analysis as described before.

Digestion with α- and β-glucosidase was performed on EtOH extracts obtained from lyophilized, ball-milled root tissue. Accordingly, 3 g of the dry root material (DM) was extracted in 30 ml EtOH by incubation in ultrasonic bath for 30 min. The obtained extract was filtered (Whatman Grade 2), dried by rotary evaporation and resuspended in acetate-buffer (0.1 M, pH 5). The sample was divided into three equal aliquots, the first was not further treated, whereas the second and third aliquots were spiked with five units of α- and β-glucosidase, respectively. All aliquots were incubated at 37°C for 24 h, extracted by liquid-phase extraction and prepared for LC–MS analysis as described before.

2.7 | Quantification of 3-*epi*-brachialactone

Quantity of 3-*epi*-brachialactone was determined via LC–MS, using a QExactive Plus Electrospray Mass Spectrometer (Thermo Fisher Scientific), coupled to an Agilent 1290 Ultra Performance Liquid Chromatography System equipped with a Waters ACQUITY UPLC HSS 1.8 μm 2.1 × 150 mm column. Measurement parameters were: ESI positive, HESI Source, Capillary Temp 360°C, Sheath gas 60, Auxin gas 20, Probe Heater 380°C, Targeted-SIM scan: 335.221 m/z, resolution 70.000. The respective eluents and eluent gradient are provided in Table S2. Quantification was performed using a 3-*epi*-brachialactone standard ranging from 0.25 to 2.0 g L^{-1}. The standard had been isolated and purified as described in Egenolf et al. (2020).

2.8 | Calculation of proton motive force ΔP

The proton motive force ΔP is composed of ΔΨ (membrane potential) und ΔpH (H^+ gradient) and can be quantified as an electrochemical gradient, according to the following adaptation of the Nernst equation (Kotyk, 1983; West, 1980):

$$\Delta P = -\Delta\Psi - (2.3 * R * T/F) * \Delta pH \quad (1)$$

with R = gas constant, T = temperature in °K, F = Faraday constant.

At 25°C, this leads approximately to the following relation, which was used for the calculations:

$$\Delta P = -\Delta\Psi - 60\,mV * \Delta pH \quad (2)$$

The membrane potential ΔΨ of root cells has been reported to range from 120 to 180 mV and cytosolic pH from 7.3 to 7.6 (Marschner, 1995). Whereas NH_4^+/NO_3^- feeding has been reported to, respectively, depolarize/hyperpolarize root hair cell membranes (Ayling, 1993; McClure et al., 1990; Ullrich & Novacky, 1990), the membrane potential of root hairs has been reported to remain relatively unaffected by trap solution pH alterations (15–30 mV variability for pH changes between 4 and 7) (Dunlop & Bowling, 1978; McClure et al., 1990). Accordingly, for comparison of different pH treatments, the proton motive force was approximated based on an assumed default ΔΨ of 150 mV and ΔpH as the difference between the trap solution pH and an assumed cytosolic pH of 7.5.

2.9 | Statistics

Statistics were performed and plots were created with R version 3.5.3 (R Core Team, 2018) using the packages "lme4," "lsmeans," "multcompView," and "ggplot2." The "lme4" package was used to fit linear models with 3-*epi*-brachialactone, TS or AA release as response variables and Block, pH and Trap solution (Experiment 1) or Block, Preculture and Trap solution (Experiment 2) as effects. Studentized residuals were inspected graphically for normality and homogeneity. Package "lsmeans2" was used to perform ANOVA and "multcompView" to prove statistical significance of treatment effects using Tukey Test. Package "ggplot2" was used to create the figures.

3 | RESULTS AND DISCUSSION

3.1 | Rhizosphere pH and nutritional N form determine exudation rates of 3-*epi*-brachialactone

In Experiment 1, both experimental factors had a significant effect on exudation rates of 3-*epi*-brachialactone (N form: P = 0.0001; pH: P = 0.0087; N form*pH: P = 0.0424). Especially the combination of NH_4^+ nutrition and low pH (4.2) prompted 3-*epi*-brachialactone exudation (Figure 1). These results are in line with previous reports revealing that, in the case of *B. humidicola*, exudation of nitrification inhibitors in general is dependent on both nutritional N form and pH (Subbarao, et al., 2007) and that exudation of brachialactone is increased under NH_4^+ rather than NO_3^- nutrition (Subbarao et al., 2009). This confirms that the release of both brachialactone C3-epimers is triggered under the same ecophysiological conditions.

3.2 | Uniform primary metabolite exudation rates indicate that 3-*epi*-brachialactone is released by a regulated transport mechanism

Previous studies on root exudation indicated increased leaching of low molecular weight (LMW) compounds (amino acids, phenolics) as a consequence of cell membrane damage induced by Ca limitation associated with low external pH (Cakmak & Marschner, 1988; Koyama et al., 2001). To evaluate whether the observed NH_4^+-induced

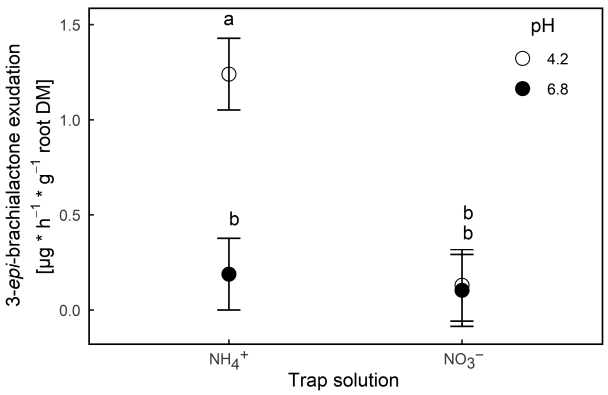

FIGURE 1 Exudation of 3-*epi*-brachialactone by roots of *B. humidicola* into trap solutions differing in nitrogen source (x-axis) and pH (legend). Trap solutions are complete nutrient solutions with nitrogen as ammonium (NH_4^+ treatment) or nitrate (NO_3^- treatment). Results represent the least-square means of four biological replications, error bars indicate standard error (SE) of the mean. Values with same letters do not differ significantly for least-square means (α = 0.05). Presented data are from Experiment 1

stimulation of 3-*epi*-brachialactone release may similarly reflect passive leaching of LMW compounds, because of membrane damage induced by low pH of the growth medium (Souri & Neumann, 2018), we profiled the pattern of sugar (TS) and amino acid (AA) exudation. Neither trap solution pH nor nutritional N form had a significant influence on the release of TS (N form: $P = 0.09$; pH: $P = 0.17$) or AA (N form: $P = 0.83$; pH: $P = 0.79$) (Figure 2). This indicated that the described stimulatory effects are specific to 3-*epi*-brachialactone and, hence, are not founded on a treatment-induced loss of plasma membrane selectivity/integrity that may have led to increased metabolite leaching. The specificity of the observed effects on 3-*epi*-brachialactone exudation suggested that it is rather released via a regulated exudation mechanism, i.e. by primary or secondary transporters or through channel proteins-facilitated diffusion.

3.3 | NH_4^+ does not constitute an essential prerequisite for the release of 3-*epi*-brachialactone

Experiment 2 corroborated the outcomes of Experiment 1, with a trend for higher 3-*epi*-brachialactone exudation rates under NH_4^+ compared to NO_3^- nutrition (Figure 3, x-axis). Pre-culture had a significant influence ($P = 0.0073$) on 3-*epi*-brachialactone exudation, confirming earlier reports on higher NI exudation rates of plants precultured with NH_4^+ as N source (Subbarao et al., 2009; Subbarao, Wang, et al., 2007). Nevertheless, the results do not confirm NH_4^+ to constitute an essential prerequisite for 3-*epi*-brachialactone release since complete replacement of NH_4^+ by K^+ (no N source) resulted in equally high 3-*epi*-brachialactone exudation rates (Figure 3). Plant roots generally accumulate both NH_4^+ and K^+ at similar rates (Macduff et al., 1997; Scherer et al., 1984) and, in both cases, positive charge influx during ion uptake has to be counterbalanced by the secretion of H^+ ions (Alvarez-Pizarro et al., 2014; Schubert & Yan, 1997). This indicated that the stimulating effect of NH_4^+ compared to NO_3^- nutrition on 3-*epi*-brachialactone exudation resulted from the associated cation surplus during nutrient uptake and not from a physiological response associated to NH_4^+ as nutritional N form.

To address the question whether the nutritional N form influences 3-*epi*-brachialactone accumulation in planta, the contents of 3-*epi*-brachialactone in root tissue were quantified in dependence of pre-culture (NH_4^+ or NO_3^-). Root tissue extracts were analyzed for 3-*epi*-brachialactone prior and after acid hydrolysis and enzymatic digestion with α- and β-glucosidase to detect potentially glycosylated forms, since many plant terpenoids in plant tissues are known to exist as glycosides (Rivas et al., 2013). However, an accurate quantification of 3-*epi*-brachialactone in root extracts was impaired by a high background noise and very low quantities ranging around 2–8 μg g^{-1} root DM (data not shown). Thus, a clear conclusion on the influence of different nutritional N forms on 3-*epi*-brachialactone levels in planta was not possible. Moreover, neither acid hydrolysis nor the digestion with α- and β glucosidase increased 3-*epi*-brachialactone contents in root extracts. The detected tissue concentrations corresponded to the amount secreted in only 2 h (at exudation rates of 1–4 μg h^{-1} g^{-1} root DM), suggesting that internal 3-*epi*-brachialactone pools were readily re-synthesized upon cytosolic depletion without pre-accumulation of storage pools. In this regard, the existence of other yet unidentified precursor pools is currently not completely excluded.

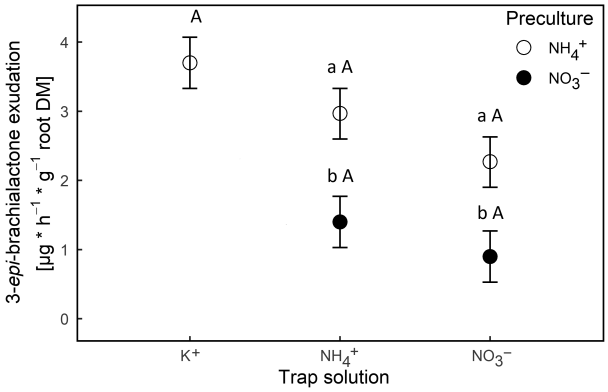

FIGURE 3 Exudation of 3-*epi*-brachialactone by roots of *B. humidicola* into different trap solutions (x-axis) after being raised on different nitrogen sources (legend). Trap solutions are complete nutrient solutions with nitrogen as ammonium (NH_4^+ treatment), nitrate (NO_3^- treatment) or completely replaced by potassium (K^+ treatment), pH of trap solution was adjusted at 4.8. Results represent least-square means of three biological replications, error bars indicate standard error (SE) of the mean. Different uppercase letter indicates statistical differences for least-square means ($\alpha = 0.05$) between treatments, different lowercase letters indicate statistical differences between pretreatments ($\alpha = 0.05$). Presented data are from Experiment 2

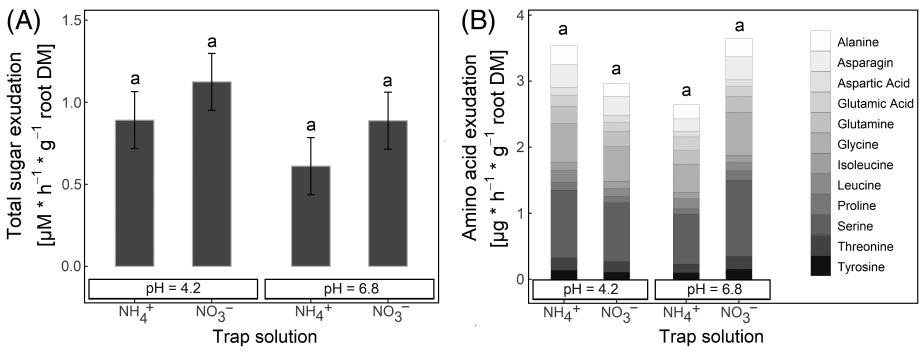

FIGURE 2 Total sugar (A) and amino acid (B) exudation by roots of *B. humidicola* into trap solutions differing in nutritional N form and pH (x-axis)

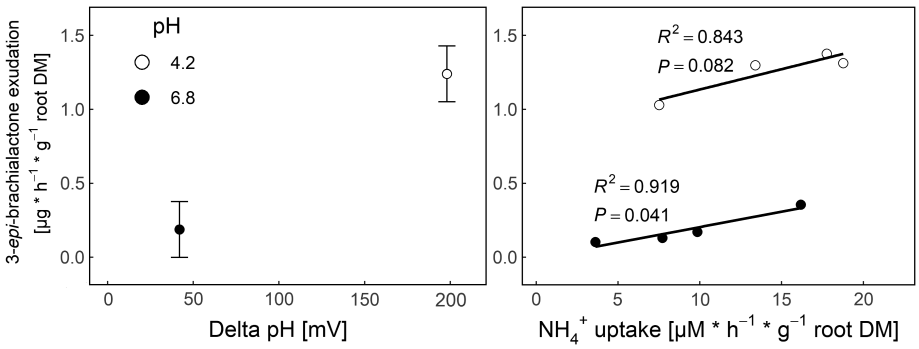

FIGURE 4 Correlations between transmembrane proton gradient (ΔpH) and 3-*epi*-brachialactone release (left) and NH_4^+ uptake and 3-*epi*-brachialactone release (right). The transmembrane proton gradient was calculated based on the difference between trap solution pH and an assumed cytosolic pH of 7.5. Presented data are from the NH_4^+ treatment of Experiment 1

3.4 | Exudation of 3-*epi*-brachialactone is driven by the proton motive force along the plasma membrane

The presented data indicated that the exudation of 3-*epi*-brachialactone depends on external pH and the cation-anion balance during nutrient uptake. Due to the high share of N among all essential nutrients, the cation-anion balance depends ultimately on the N form taken up by the plant (NH_4^+ vs. NO_3^-). A surplus in cation uptake (NH_4^+ feeding) is counterbalanced by the secretion of H^+ out of the root, contributing to the acidification of the apoplast (Hoffmann et al., 1992; Kosegarten et al., 1999). Therefore, both low external pH and a surplus of cations during nutrient uptake (NH_4^+/K^+ treatments) are translated into a proton gradient along the plasma membrane. It seems plausible that this might be the driving force for 3-*epi*-brachialactone exudation via secondary transport.

Certainly, the transmembrane proton gradient is established and maintained by the PM H^+ATPase. Its activity is regulated through phosphorylation/dephosphorylation in the short term and at the transcriptional level in the long term (Zhang et al., 2017). In this regard, an increased expression of root cell PM H^+ATPase under NH_4^+ nutrition has been shown to sustainably increase root cell PM H^+ATPase concentration and activity (Alvarez-Pizarro et al., 2011; Zhu et al., 2012). Considering that, at current state of knowledge, differences in internal 3-*epi*-brachialactone concentrations seem to be ruled out as an explanation for the pretreatment effect observed in Experiment 2, differences in PM H^+ATPase concentration and activity would possibly offer a valid explanation.

The fact that, under NO_3^- nutrition, a decrease of the trap solution pH from 6.8 to 4.2 (Experiment 1) did not increase 3-*epi*-brachialactone exudation rates may be explained by the dissipation of the proton gradient along the plasma membrane during anion (NO_3^-) uptake. The initially proposed theory that NH_4^+ nutrition (∼ cation surplus) stimulates NI release (Subbarao, Wang, et al., 2007) would then have to be revised: 3-*epi*-brachialactone (and probably also other NIs) release is driven by the proton gradient along the membrane of root cells (low rhizosphere pH), as long as this gradient is not dissipated by H^+-driven anion uptake (NO_3^-).

To corroborate this argumentation, the proton motive force expected for the two pH treatments of Experiment 1 was calculated. Assuming a constant membrane potential of 150 mV and a cytosolic pH of 7.5, the calculation gives a ΔpH of 0.7 (∼ 40 mV) for the high pH treatment (6.8) versus 3.5 (∼ 200 mV) for the low pH treatment (4.2) or a ΔP (proton motive force) of 190 mV versus 350 mV, respectively. Hence, a pH change from 6.8 to 4.2 corresponds to a fivefold increase of ΔpH (from 40 to 200 mV), leading to an approximately twofold increase of ΔP (from 190 to 350 mV). This goes along with an approximately fivefold increase of the 3-*epi*-brachialactone exudation rate (from 0.25 to 1.25 µg h^{-1} g^{-1} root DM, Figure 1). Secondary transport rates should correlate with ΔP rather than with ΔpH. Nevertheless, a linear response of secondary transport rates to ΔpH has been reported, e.g. for secondary active H^+/glucose co-transporters in Chlorella algae (Schwab & Komor, 1978) or secondary active phosphate uptake in white clover (Dunlop & Bowling, 1978). As a supporting argument, we plotted 3-*epi*-brachialactone release rates against the dominant factors determining the proton motive force (ΔP), i.e. the pH gradient along the plasmamembrane (ΔpH) and cation uptake rates (in this case NH_4^+ uptake). The linear response suggested a mechanistic relationship between pH, cation uptake and 3-*epi*-brachialactone exudation (Figure 4). Although membrane transport of many LMW terpenoids, representing lipophilic and frequently volatile compounds with high membrane permeability, seems to occur mainly by diffusion (Weston et al., 2012; Yazaki et al., 2008), selective transport mechanisms have also been described for various secondary metabolites, including terpenoids (Shoji, 2014). The currently known transport proteins are ATP-binding cassette (ABC) and multidrug and toxic compound extrusion family (MATE) transporters, mediating the transport of organic compounds in plants (Shoji, 2014). For terpenoids, ABC transporters have been described, serving as ATP-driven primary transporters that mediate the movement of a range of molecules such as strigolactones and diterpenes across cellular membranes (Shoji, 2014). In contrast to directly ATP-energized ABCs, MATEs are secondary transporters that achieve the transport of primary and secondary metabolites (such as flavonoids, alkaloids, xenobiotics, citrate) using the proton motive force. Transport can occur through several membrane systems of the plant cell by an ATPase-mediated preformation of an electrochemical H^+-gradient, associated with a proton antiport mechanism (Santos et al., 2017; Shoji, 2014). Although no terpenoid transport processes via MATE proteins have been described yet (Shoji, 2014), the presented data obviously match the transport dynamics described for MATE transporters. These may thus represent a promising target for prospective gene expression analyzes related to the release of brachialactones in roots of *B. humidicola*.

4 | CONCLUSIONS AND OUTLOOK

Rhizosphere pH and nutritional N form have been demonstrated to influence the exudation of NI, e.g. brachialactone in the case of *B. humidicola*. Here, we confirmed this relation for 3-*epi*-brachialactone, the so far most bioactive known brachialactone of *B. humidicola* (Egenolf et al., 2020). We propose that 3-*epi*-brachialactone is excreted via secondary transport mechanisms with the proton gradient along the plasma membrane determining exudation rates. As experimentally verified, rhizosphere conditions favoring a stronger proton gradient, e.g. low rhizosphere pH or excess cation (NH_4^+ or K^+) nutrition, increased the exudation rate, whereas conditions lowering the gradient, e.g. excess anion (NO_3^-) nutrition, resulted in a decrease.

Interestingly, similar NI release patterns were reported for other crops (e.g. *S. bicolor*, *L. racemosus*, *O. sativa*). Especially for the polar NI fraction in *S. bicolor*, pH and N form determined NI release (Di et al., 2018). Moreover, the NI release correlated strongly with PM-H^+ATPase (Zhu et al., 2012), a scenario taken as evidence for exudation via MATE transporters in various plant species (Kochian et al., 2015; Magalhaes et al., 2018; Zhang et al., 2017). The similarities of the exudation dynamics of the NIs from *S. bicolor* and *B. humidicola* are obvious, raising the question if similar transport mechanisms are involved in both cases.

As exudation rates of the mentioned NIs can be explained mechanistically (based on the physiology of secondary transport), BNI does not—as commonly proposed—constitute a regulated response to pH and NH_4^+ availability in the rhizosphere. However, observed effects were recorded in hydroponic culture with defined pH as well as amounts and forms of nutrient supply (e.g. pure NH_4^+ nutrition vs. NO_3^- supply). To which extent this concept applies to the highly variable rhizosphere properties in terms of pH and cation-anion ratios under natural growth conditions remains to be verified.

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AUTHOR CONTRIBUTIONS

Konrad Egenolf drafted the presented study, wrote the manuscript and modified it according to suggestions and corrections of the co-authors. Supriya Verma developed Experiment 1 as part of her M.Sc. thesis and supported the conductance of Experiment 2. Jochen Schöne and Iris Klaiber developed the methodology for 3-*epi*-brachialactone quantification and performed respective LC–MS measurements. Jacobo Arango was the leading scientist at CIAT. Günter Neumann and Georg Cadisch co-supervised the study. Frank Rasche was leading senior scientist of the study. All authors contributed to manuscript revision, read, and approved the submitted version.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as all new created data is already contained within this article.

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SUPPORTING INFORMATION

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