



# Isolation and characterization of the hydrophilic BNI compound, 6-methoxy-2(3H)-benzoxazolone (MBOA), from maize roots

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## Abstract

**Background and aims** Biological nitrification inhibition (BNI) is a chemical ecological phenomenon whereby plants specifically suppress nitrification by releasing inhibiting compounds from roots, an effective strategy for improving nitrogen uptake by limiting nitrogen losses from agricultural fields. During this study, we have aimed at characterizing hydrophilic BNI activity released from maize roots to

understand the chemical basis for BNI function in maize.

**Methods** Maize plants were grown hydroponically and root exudates were collected using water-based solutions and hydrophilic BNI activity was extracted. We isolated the target BNI compounds by a combination of chromatographic techniques and bioassays using a recombinant luminescent ammonia-oxidizing bacterium *Nitrosomonas europaea* (pHLUX20).

**Results** We identified 6-methoxy-2(3H)-benzoxazolone (MBOA) as the responsible BNI compound with a median effective dose ( $ED_{50}$ ) = 0.76  $\mu$ M. MBOA inhibited the conversion of  $NH_3$  to  $NH_2OH$  as well as  $NH_2OH$  to  $NO_2^-$  in *N. europaea*, suggesting that MBOA blocks both ammonia monooxygenase and hydroxylamine oxidoreductase enzymatic pathways. Treatment with MBOA significantly suppressed  $NO_2^-$  and  $NO_3^-$  production during soil incubation, but this activity was reduced subsequently due to biodegradation of MBOA by soil microbes. A quantification experiment revealed that MBOA accounted for nearly 50% of the total BNI activity in hydrophilic and hydrophobic exudates from maize roots. A soil incubation test showed that two previously identified benzoxazinoids, HDMBOA and HDMBOA- $\beta$ -glucoside, can be eventually transformed into MBOA.

**Conclusion** We elucidated MBOA as the key component of BNI in maize. Collectively, the present findings will serve as the groundwork for construction of an advanced environment-friendly agricultural system.

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## Introduction

In agriculture, large amounts of nitrogen fertilizers are used for crop production to feed the growing world population. Nearly 50% of the nitrogen fertilizer applied to Poaceae crops (e.g., maize, wheat, and rice) is lost largely because of two soil microbial transformations, nitrification and denitrification, resulting in loss of soil-nitrogen and low nitrogen use efficiency (NUE) (Coskun et al. 2017; Subbarao et al. 2013b; Thakur and Medhi 2019). Nitrification, a stepwise oxidation process from  $\text{NH}_3$  to nitrate ( $\text{NO}_3^-$ ) by soil microbes, plays an important role in the nitrogen cycle. However, excessive production of  $\text{NO}_3^-$  and its high mobility leads to groundwater contamination and generation of harmful greenhouse gas such as  $\text{N}_2\text{O}$  and  $\text{NO}$  (Kuypers et al. 2018; Rivett et al. 2008; Scheer et al. 2020; Stayner et al. 2017; Tian et al. 2020). Thus, overapplication of nitrogen fertilizer (as  $\text{NH}_3$ ) together with generation of excess soil- $\text{NO}_3^-$  provoke serious economic and environmental damage (Subbarao and Searchinger 2021). Therefore, a strategy is needed for suppression of nitrification to increase NUE to correct the imbalances in nitrogen cycle. Biological nitrification inhibition (BNI) provides an innovative way to reduce nitrogen loss from agricultural system (Ghatak et al. 2022; Subbarao and Searchinger 2021). BNI is a chemical ecological phenomenon by which specific natural products (secondary metabolites) secreted from the plant root system, including terpenoids, alkaloids, fatty acids, and phenylpropanoids, that inhibit nitrification and growth of nitrifiers (Subbarao et al. 2009; Subbarao et al. 2013b; Wendeborn 2020). The BNI-possessing crops can retain more  $\text{NH}_4$  in the rhizosphere through circumvention of  $\text{NO}_3^-$  production. Therefore, utilization of BNI has several major advantages. (a) It is eco-friendly: the environmental pollution risk is reduced through using the activity of phytochemicals released from crop root systems. (b) The effect is sustainable: plant crops can continuously biosynthesize and secrete BNI compounds from roots into rhizosphere to keep nitrifier activity under check. (c) Costs are reduced: application of additional agrochemicals is not needed. To date, BNI has been observed in certain plants, including main

staple food crops, namely maize, wheat, rice, and sorghum (Otaka et al. 2022; Subbarao et al. 2021; Subbarao et al. 2013a; Sun et al. 2016; Zakir et al. 2008). Understanding BNI function in maize is important due to: (a) Among staple crops, maize is the most productive crop providing food and feed, (b) It consumes a major portion of nitrogen fertilizer produced globally, and (c) Maize production systems contribute to nitrogen pollution in a major way globally.

To develop a BNI strengthened crop with a higher yield of BNI compounds, understanding the chemical identity of BNI compounds released from roots is essential. For isolation of BNI compounds, a crucial property is whether the root exudates and compounds are water-insoluble (hydrophobic) or water-soluble (hydrophilic) (Subbarao et al. 2013a). Water-insoluble hydrophobic compounds with lower mobility are predominant in the rhizosphere, whereas hydrophilic compounds in water can move more farther from the roots and have wide-reaching influence. Chemical identities of both hydrophobic and hydrophilic BNI compounds in plant crops will lead to a deeper understanding of BNI function in the soil. We have recently reported two major hydrophobic BNI-contributing compounds from the root surface in maize, namely 2,7-dimethoxy-1,4-naphthoquinone (zeanone;  $\text{ED}_{50}=2 \mu\text{M}$ ) and 2-hydroxy-4,7-dimethoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (HDMBOA;  $\text{ED}_{50}=13 \mu\text{M}$ ), together with two analogs of HDMBOA from inside the roots, namely 7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (HMBOA;  $\text{ED}_{50}=91 \mu\text{M}$ ) and HDMBOA- $\beta$ -glucoside ( $\text{ED}_{50}=94 \mu\text{M}$ ) (Fig. S1) (Otaka et al. 2022). This study is aimed at further understanding of the BNI function in maize root systems by focusing on chemical characterization of hydrophilic BNI activity that include (a) Isolation and structural determination of BNI-active components, (b) Determination of mode of inhibitory action of the isolated BNI compounds, and (c) Confirmation of BNI function of isolated compounds using in vitro and in vivo approaches.

## Materials and methods

### Hydroponic cultivation of maize

Ninety seeds of maize ‘Honey Bantam’ (Sakata Seed Corp., Kanagawa, Japan) were wrapped in a

moistened paper towel and incubated in a growth chamber in the dark at 25 °C for 24 h (Fig. S2a). The seeds were then soaked in a plastic bottle containing 200 µM CaSO<sub>4</sub> aqueous solution (aq.) (200 mL) (Fig. S2b). Ten bottles (in total 900 seeds) were prepared and placed in the dark with bubble aeration at 25 °C for 24 h. The seeds were sown in seedling-grow boxes and the seedlings were supplied with 200 µM CaSO<sub>4</sub> aq. (Figure S2c). The seedling-grow boxes were placed in a growth chamber maintained at 29 °C for 13 h/25 °C for 11 h (max/min) with average light intensity of ~360 µmol m<sup>-2</sup> s<sup>-1</sup> (25,000 lx) and relative humidity of 70%.

After 7 days in the seedling-grow boxes, the seedlings were transferred to a hydroponic system with aeration in the growth chamber. The hydroponic system consisted of ten tanks (one tank per 90 L), with each tank having a styrofoam top with eight holes in two rows (Fig. S2d). Six maize plants (one batch) were planted in a hole; in total 480 plants were grown in the ten tanks. The tanks contained nutrient solution with the following composition: KH<sub>2</sub>PO<sub>4</sub> (final concentration: 281 µM), K<sub>2</sub>SO<sub>4</sub> (final concentration: 178 µM), CaCl<sub>2</sub>·2H<sub>2</sub>O (final concentration: 71 µM), MgSO<sub>4</sub>·7H<sub>2</sub>O (final concentration: 150 µM), Fe-EDTA (final concentration: 41 µM), H<sub>3</sub>BO<sub>3</sub> (final concentration: 922 µM), CuSO<sub>4</sub>·5H<sub>2</sub>O (final concentration: 0.3 µM), MnSO<sub>4</sub>·6H<sub>2</sub>O (final concentration: 9.1 µM), Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O (final concentration: 5.2 µM), and ZnSO<sub>4</sub>·7H<sub>2</sub>O (final concentration: 7.7 µM) dissolved in 100 L deionized water. In addition, NH<sub>4</sub>NO<sub>3</sub> was added to the nutrient solution as the nitrogen source at a final concentration of 1.0 mM. During cultivation for the first 21 days, the pH of the nutrient solution was adjusted daily in the range 6.0–6.5 using 1 N HCl or 1 N NaOH. The nutrient solution was replaced with fresh solution every 7 days.

After 21 days, NH<sub>4</sub>NO<sub>3</sub> as the nitrogen source was changed to ammonium sulfate (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> in the nutrient solution at a final concentration of 0.5 mM, and the maize plants were cultivated for an additional 7 days.

#### Collection of root exudates of maize

The 30-day-old maize plants (total 80 batches) (Fig. S2e) were removed from the ten nutrient solution tanks and rinsed with deionized water for

2–3 min. The root exudates of the plants were successively collected with the following four steps (a–d) (Fig. S2f).

- (a). Each batch was transferred to a 2.0 L plastic bottle containing CaSO<sub>4</sub> (final concentration: 200 µM) and NH<sub>4</sub>Cl (final concentration: 500 µM) in aqueous solution (1.8 L), in which only the roots were soaked, for 24 h with aeration (N1 solution).
- (b). The maize plants were removed from the N1 solution and their roots were soaked in 200 µM CaSO<sub>4</sub> aq. (1.8 L) for 24 h with aeration (W1 solution).
- (c). The maize plants were removed from the W1 solution and were moved to aqueous solution (1.8 L) containing CaSO<sub>4</sub> (final concentration: 200 µM) and NH<sub>4</sub>Cl (final concentration: 500 µM) for 24 h with aeration (N2 solution).
- (d). The maize plants were removed from the N2 solution and the roots were soak in 200 µM CaSO<sub>4</sub> aq. (1.8 L) for 24 h with aeration (W2 solution).

In total 320 bottles of the four solutions (N1, W1, N2, and W2) were collected and all solutions were combined (total 480 L).

#### Chemicals and solvents

All chemicals and solvents were purchased from commercial suppliers or prepared as described in this article. Water was purified by a Milli-Q system (Merck Millipore, Billerica, MA, USA) unless indicated otherwise.

#### Instrumentation

Ultraviolet (UV) spectra were measured with a Shimadzu UV-1200 spectrometer. Nuclear magnetic resonance (NMR) spectra were measured in CDCl<sub>3</sub> with a Bruker AVANCE III HD 800 MHz spectrometer. Chemical shifts δ (ppm) were referenced to the residual solvent peaks (δ<sub>H</sub> 7.24 ppm; δ<sub>C</sub> 77.0 ppm), and coupling constants (*J*) were reported in hertz (Hz). Multiplicity was indicated as follows: s=singlet; d=doublet; dd=doublet of doublet; and brs=broad singlet. Liquid chromatography–electrospray ionization–mass spectrometry (LC-ESI-MS) analyses were conducted on a Shimadzu LCMS-2020 instrument (Shimadzu Corp., Kyoto, Japan) equipped with

a TSKgel Super-ODS column (4.6 mm I.D. × 50 mm, 2.3 μm; Tosoh Corp., Tokyo, Japan). The analytical and preparative (prep.) reverse-phase high-performance liquid chromatography (RP-HPLC) was performed on a Shimadzu Prominence LC-20AT instrument (Shimadzu Corp.) with a Shimadzu SPD-M20A photodiode array detector (Shimadzu Corp.), accompanied by a TSKgel Super-ODS column (4.6 mm I.D. × 50 mm, 2.3 μm) or a Cosmosil πNAP column (4.6 mm I.D. × 150 mm, 5.0 μm; Nacalai Tesque Inc., Kyoto, Japan). The optical densities at 660 nm (O.D. 660) was measured on a mini photo 518R photo meter (TAITEC Corp., Nagoya, Japan).

#### Extraction, separation, and purification of BNI compounds from maize root exudates

The solution containing maize root exudates (480 L) was concentrated under reduced pressure at 35 °C to obtain a brown syrup (24 g). A concentrate (24 g) was partitioned between 30% MeOH/70% H<sub>2</sub>O (300 mL) and ethyl acetate (EtOAc) (300 mL), followed by concentration of both layers under vacuum at 35 °C to yield a 30% MeOH aqueous fraction (23.4 g) and an EtOAc fraction (580 mg), respectively. The EtOAc fraction (580 mg) was dissolved in 10% MeOH/90% H<sub>2</sub>O (50 mL), extracted with hexane (50 mL), and both layers were concentrated at 35 °C to obtain a 10% MeOH aqueous fraction (540 mg, fraction M) and a hexane fraction (40 mg), respectively.

Fraction M (540 mg) was separated by RP-HPLC on a TSKgel Super-ODS column using a linear gradient [1% acetonitrile/99% H<sub>2</sub>O (0.1% formic acid) (start) to 30% acetonitrile/70% H<sub>2</sub>O (0.1% formic acid) (6 min) to 100% acetonitrile (9 min), 1.0 mL min<sup>-1</sup>] to give five fractions (M-1–M-5). Fraction M-2 (40 mg, *t<sub>R</sub>* 5.5–8.0 min) was separated by RP-HPLC on a πNAP column [30% acetonitrile/70% H<sub>2</sub>O (0.1% formic acid) (start) to 50% acetonitrile/50% H<sub>2</sub>O (0.1% formic acid) (10 min), 1.0 mL min<sup>-1</sup>], affording five subfractions (M-2-1–M-2-5). Fraction M-2-2 (15 mg, *t<sub>R</sub>* 6.0–8.0 min) was further fractionated by RP-HPLC on a πNAP column [10% acetonitrile/90% H<sub>2</sub>O (0.1% formic acid) (start) to 30% acetonitrile/70% H<sub>2</sub>O (0.1% formic acid) (25 min) to 100% acetonitrile (30 min), 1.0 mL min<sup>-1</sup>] to obtain four fractions (M-2-2-1–M-2-2-4). Fraction M-2-2-1 (2.5 mg, *t<sub>R</sub>* 18.5–22.0 min) was divided into two fractions by

RP-HPLC on a πNAP column [30% acetonitrile/70% H<sub>2</sub>O (0.1% formic acid), 1.0 mL min<sup>-1</sup>], and fractions M-2-2-1-2 (*t<sub>R</sub>* 7.2–7.8 min) and M-2-2-1-1 (*t<sub>R</sub>* 6.8–7.2 min) were dried to isolate compounds **1** (0.5 mg) and **2** (0.7 mg), respectively.

**Compound 1:** colorless crystal (H<sub>2</sub>O); **MS** (pos.) *m/z* 166 [M+H]<sup>+</sup>, (neg.) *m/z* 164 [M-H]<sup>-</sup>; **UV** (H<sub>2</sub>O) λ<sub>max</sub> (log ε) 287 (3.75), 230 (4.01) nm; solubility in water 0.56 mg/mL (25 °C).

**Compound 2:** white powder (MeOH); **MS** (pos.) *m/z* 196 [M+H]<sup>+</sup>; **UV** (acetonitrile:H<sub>2</sub>O 1:1) λ<sub>max</sub> (log ε) 281 (3.58), 231 (4.02), 202 (4.49) nm.

#### Preparation of calibration curves by using HPLC

The purified samples were dissolved in H<sub>2</sub>O or MeOH at concentrations ranging from 0 to 100 ppm. Each 5 μL sample solution was analyzed using a Shimadzu LCMS-2020 instrument on a TSKgel Super-ODS column [1% acetonitrile/99% H<sub>2</sub>O (0.1% formic acid) (start) to 30% acetonitrile/70% H<sub>2</sub>O (0.1% formic acid) (13 min), 0.4 mL min<sup>-1</sup>]. Based on the peak area (mV\*sec) of each compound, a calibration curve was prepared.

#### Bioluminescence assay using *N. europaea* (pHLUX20) [nitrogen source: (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>]

The detailed description of the bioluminescence bioassay was described in our earlier publications (Subbarao et al. 2006). A recombinant *N. europaea* IFO 14298 (pHLUX20) was used (Iizumi et al. 1998). The composition of the culture medium (P medium) for *N. europaea* was as follows: (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (final concentration: 18.9 mM), KH<sub>2</sub>PO<sub>4</sub> (final concentration: 5.1 mM), Na<sub>2</sub>HPO<sub>4</sub> (final concentration: 95.0 mM), NaHCO<sub>3</sub> (final concentration: 6.0 μM), MgSO<sub>4</sub>·7H<sub>2</sub>O (final concentration: 405 μM), CaCl<sub>2</sub>·2H<sub>2</sub>O (final concentration: 340 μM), Fe-EDTA (final concentration: 2.7 μM), and kanamycin-nH<sub>2</sub>SO<sub>4</sub> (0.25 mg) dissolved in 1.0 L water (Lewis and Pramer 1958). The recombinant *N. europaea* (pHLUX20) was grown in a 500 mL baffled Erlenmeyer flask containing 200 mL medium at pH 7.8 on a rotary shaker at 150 rpm at 30 °C for 7 days.

After cultivation, bacterial cells from the three flasks (total 600 mL medium) were concentrated by centrifugation at 9000 rpm at 5 °C for 10 min and

resuspended in 10 mL P medium (O.D. 660=1.0). The bacterial suspension was left in the dark for 10 min at 25 °C. The test sample (extract, fraction, and compound) was dissolved in water or DMSO, from which an aliquot (1.0 µL) was added to 199 µL double-distilled water. To a solution of sample (200 µL) was added 250 µL bacterial culture, then the mixture (total 450 µL) was incubated at 15 °C for 20 min. An aliquot of each resulting mixture (100 µL) was added to 2.5 µL of 10% *n*-decyl aldehyde in ethanol, and the bioluminescence of the sample was measured on a GLOMAX 20/20 Luminometer (Promega Corp., Madison, WI, USA). To test the bacterial condition, fluorescence in *N. europaea* (pHLUX20) was preliminary checked using 0.22 µM allylthiourea as a positive control to show approximately 80% inhibition. Each sample was measured at least three times, and the average value was used to calculate the BNI activity. The BNI activity of each sample is expressed in allylthiourea units (ATU), where 1 ATU means 80% inhibition by allylthiourea on bioluminescence (0.22 µM) (Subbarao et al. 2006).

#### Bioluminescence assay using *N. europaea* (pHLUX20) [nitrogen source: (NH<sub>2</sub>OH)]

A concentrated culture broth of 7-day-old *N. europaea* (pHLUX20) (600 mL) was washed twice with P medium lacking (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and then resuspended with 10 mL P medium containing NH<sub>2</sub>OH (final concentration: 10 µM) as the sole nitrogen source (O.D. 660=1.0). In the same manner as described for the “Bioluminescence assay using *N. europaea* (pHLUX20) [nitrogen source: (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>]”, the BNI activity of the test sample was measured as described earlier (Iizumi et al. 1998; Subbarao et al. 2006).

#### Effect of the BNI compound on NO<sub>2</sub><sup>-</sup> production by *N. europaea* (pHLUX20)

To an aqueous solution of the BNI compound (1.0 mL, final concentration: 2.0–50 µM) was added P medium (100 mL) containing (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (final concentration: 750 µM) or NH<sub>2</sub>OH (final concentration: 20 µM) in a 500 mL baffled Erlenmeyer flask. Instead of the BNI compound, water (1.0 mL) was added to medium as a control. A 250 µL aliquot of concentrated 7-day-old *N. europaea* (pHLUX20) suspension (O.D. 660=1.0), which was washed twice

by P medium lacking a nitrogen source [(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and NH<sub>2</sub>OH], was inoculated into each medium and incubated on a rotary shaker at 150 rpm at 30 °C. An aliquot of the reaction mixture at 0 min (inoculated time), 1 h, and 3 h after inoculation was collected from each flask. The concentration of NO<sub>2</sub><sup>-</sup> was measured using the NO<sub>2</sub>/NO<sub>3</sub> Assay Kit-C II (Colorimetric) Griess Reagent Kit (NO<sub>2</sub><sup>-</sup> concentration range: 10–100 µM) (Dojindo Co., Kyoto, Japan) or the NO<sub>2</sub>/NO<sub>3</sub> Assay Kit-FX (Fluorometric) 2,3-Diaminonaphthalene (DAN) Kit (NO<sub>2</sub><sup>-</sup> concentration range: 1.0–10 µM) (Dojindo Co.) in accordance with the manufacturer’s protocol. The absorbance of the Griess products in the test samples were measured at 540 nm, and the fluorescence of the DAN products were measured at λ<sub>ex</sub>=360 nm/λ<sub>em</sub>=450 nm, both of which were conducted on an EnSpire multimode plate reader (PerkinElmer, Waltham, MA, USA). The concentration of NH<sub>4</sub><sup>+</sup> was measured using the LabAssay™ Ammonia Kit (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan), for which the absorbance of the indophenol blue products was measured at 630 nm. Each experiment (Griess reaction, DAN reaction, and indophenol blue reaction) was replicated three times.

#### Effect of the BNI compound on growth of *N. europaea* (pHLUX20)

A 7-day-old *N. europaea* (pHLUX20) suspension (250 µL, O.D. 660=1.0) was inoculated into 100 mL P medium containing (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (final concentration: 18.9 mM) as the nitrogen source and the BNI compound dissolved in H<sub>2</sub>O (1.0 mL, final concentration: 10 µM) in a 500 mL baffled Erlenmeyer flask. Instead of the BNI compound, water (1.0 mL) was used as a blank control. Each medium was incubated on a rotary shaker at 150 rpm at 30 °C. An aliquot of culture medium (3.0 mL) was subsequently collected from each flask at 0 min (inoculated time), day 1, day 3, and day 5 after inoculation. The value of O.D. 660 for each sample was corrected by that of the blank control containing only P medium.

To analyze the stability of the BNI compound in the bacterial culture, collected samples were centrifuged at 7000 rpm for 10 s before HPLC. Each 5 µL of supernatant was analyzed by HPLC on a TSKgel Super-ODS column [1% acetonitrile/99% H<sub>2</sub>O (0.1% formic acid) (start) to 30% acetonitrile/70% H<sub>2</sub>O



(0.1% formic acid) (13 min) to 100% acetonitrile (18 min), 0.4 mL min<sup>-1</sup>].

#### Soil incubation test for determining potential nitrification

Potential soil nitrification was determined using a modified soil slurry protocol (Vazquez et al. 2020). The brief protocol is as follows: Soil samples collected from the JIRCAS experimental farm in Tsukuba City were used (Typic Hapludands; pH 6.0 in water; clay 54.8%, silt 26.3%, sand 18.9%; C/N ratio=29.2/2.5 mg g<sup>-1</sup> soil, CEC=119.1 mEq/100 g). The soil was air-dried at room temperature and sifted through a 2 mm sieve. To 100 mL nitrogen fertilizer solution [KH<sub>2</sub>PO<sub>4</sub> (final concentration: 300 μM), K<sub>2</sub>HPO<sub>4</sub> (final concentration: 700 μM), (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (final concentration: 750 μM), water (98 mL)] was added air-dried soil (10 g) in a 500 mL baffled Erlenmeyer flask. After the addition of BNI compounds [0.8 mg (final concentration: 50 μM) and 1.6 mg (final concentration: 100 μM)], each soil solution was sealed with a silico plug and incubated on a rotary shaker at 100 rpm at 20 °C. An aqueous solution of the synthetic nitrification inhibitor dicyandiamide (DCD, final concentration=327 μM) and water was used as a positive control and blank control, respectively. The sterilized soil was used as a negative control. An aliquot of soil mixture (soil + buffer) at 1 h, day 1, day 2, day 3, day 4, day 5, and day 7 after incubation was collected into a 1.5 mL tube from each flask. After centrifugation of the assay solution at 7000 rpm for 10 s, each supernatant was immediately transferred to a new 1.5 mL tube. All collected samples were stored in a deep-freezer at -80 °C until use. The concentration of NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> in each sample was measured using the NO<sub>2</sub>/NO<sub>3</sub> Assay Kit-C II (Colorimetric) Griess Reagent Kit (Dojindo Co.) or the NO<sub>2</sub>/NO<sub>3</sub> Assay Kit-FX (Fluorometric) 2,3-Diaminonaphthalene Kit (NO<sub>2</sub><sup>-</sup> concentration range: 1.0–10 μM) (Dojindo Co.). To measure the concentration of the BNI compound in the sample, a 5 μL aliquot of the supernatant was analyzed by LC-ESI-MS on a TSKgel Super-ODS column [1% acetonitrile/99% H<sub>2</sub>O (0.1% formic acid) (start) to 30% acetonitrile/70% H<sub>2</sub>O (0.1% formic acid) (13 min) to 100% acetonitrile (18 min), 0.4 mL min<sup>-1</sup>].

#### Analysis of stability of BNI compounds in soil

Air-dried soil (100 mg) was moistened with 500 μL water in a glass vial. To 5 μL solution of the BNI compound (350 μM), moistened soil was added and the soil mixture was incubated at 25 °C. An aliquot of reactant (20 μL) was collected at 0 min (inoculated time), 1 h, 3 h, 6 h, and 24 h after inoculation. Each collected sample was centrifuged, and the 5 μL supernatant was analyzed by LC-ESI-MS with monitoring at 210 nm [1% acetonitrile/99% H<sub>2</sub>O (0.1% formic acid) (start) to 30% acetonitrile/70% H<sub>2</sub>O (0.1% formic acid) (13 min), 0.4 mL min<sup>-1</sup>]. The sterilized soil was used as a negative control.

## Results

#### Isolation and identification of the hydrophilic BNI compound from root exudates of maize

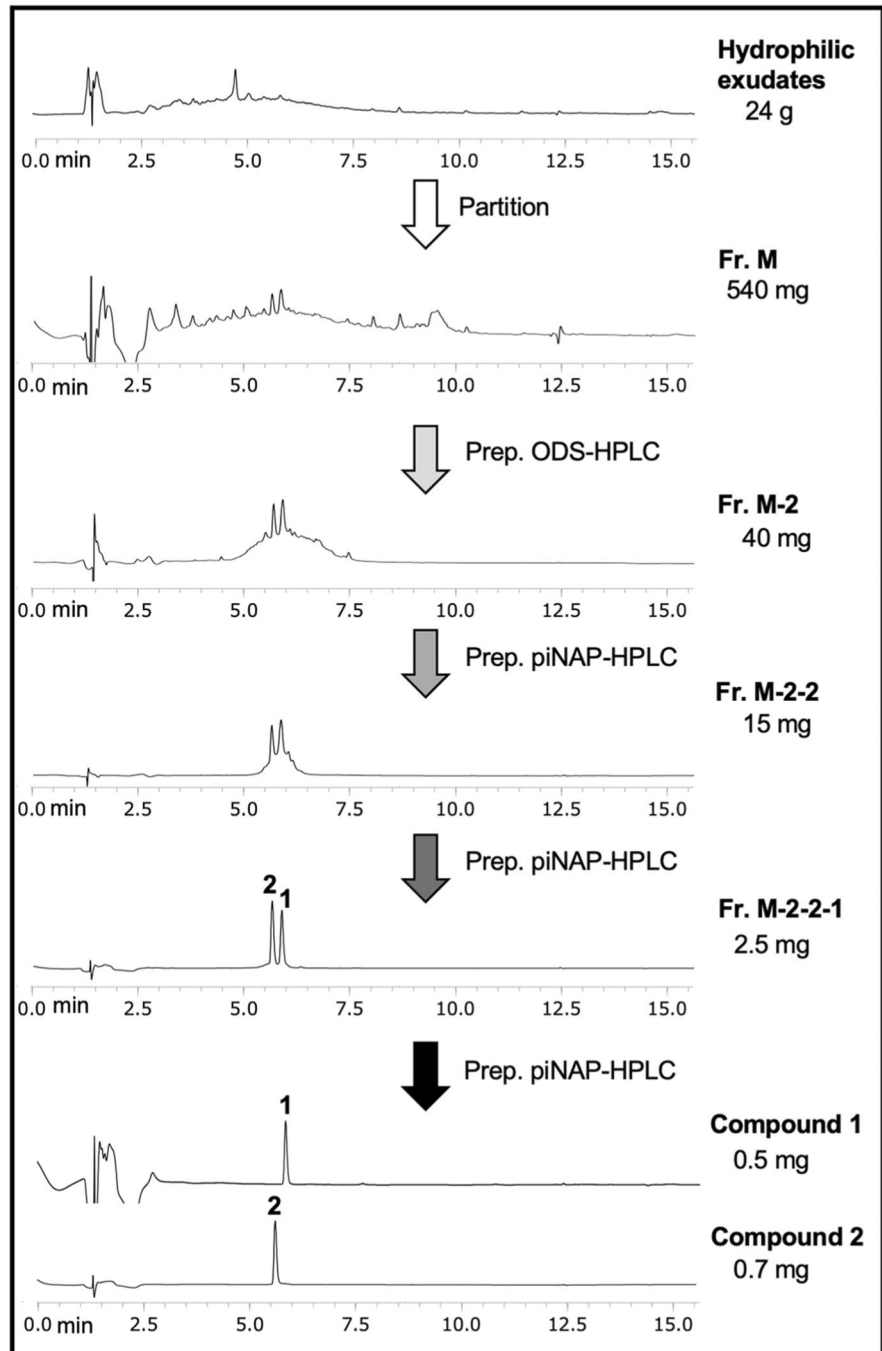
To explore hydrophilic BNI compounds in exudates from maize roots, we began our study on the basis of the following planned steps (I–IV): I. Hydroponic cultivation of maize; II. Soaking of intact maize roots into aerated CaCl<sub>2</sub> aqueous solutions under induction of BNI by NH<sub>4</sub>Cl; III. Concentration of the combined soaked solutions to obtain hydrophilic root exudates; and IV. Isolation of hydrophilic BNI compounds in the root exudates based on the results of a bioluminescent assay using *N. europaea* (pHLUX20).

For collection of hydrophilic BNI compounds from maize roots, we concentrated 480 L extraction solution from 30-day-old maize roots to collect root exudates (24 g). Because the hydrophilic root exudates (24 g) contained not only maize metabolites but also other compounds, such as inorganic salts from fertilizer, we partitioned the exudates between EtOAc and 30% MeOH aqueous solutions. In the first bioassay, the EtOAc fraction (580 mg) showed nitrification inhibitory activity against *N. europaea*, whereas the 30% MeOH layer (19.4 g) containing inorganic salts had no such activity. Hence, we further partitioned the EtOAc fraction between hexane and 10% MeOH aqueous solutions. Because BNI activity was concentrated in the 10% MeOH aqueous fraction (fraction M; 540 mg) (18,256 ATU), we then aimed to purify the BNI compound.

We narrowed down the target BNI fraction, composed of two peaks **1** and **2**, through successive purification of fraction M (540 mg) based on a bioassay-guided fractionation method (Fig. 1). Then, we separated and isolated two BNI candidates, **1** (0.5 mg) and **2** (0.7 mg), that showed similar UV

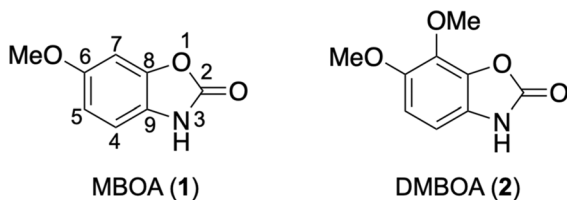
spectral patterns (Fig. S3). The structures of both compounds were elucidated as 6-methoxy-2(3*H*)-benzoxazolone (MBOA, **1**) and 6,7-dimethoxy-2(3*H*)-benzoxazolone (DMBOA, **2**) from the NMR ( $^1\text{H}$  and  $^{13}\text{C}$ ) and MS data, together with comparison of their spectroscopic data with previously

**Fig. 1** Stepwise purification and isolation of the BNI compound. Two candidates **1** and **2** were isolated based on the bioassay-guided fractionation method. Reverse-phase-HPLC analyses of the obtained fractions and compounds were performed on a TSKgel Super-ODS column [injected volume: 5  $\mu\text{L}$ , 1% acetonitrile/99%  $\text{H}_2\text{O}$  (0.1% formic acid) (start) to 30% acetonitrile/70%  $\text{H}_2\text{O}$  (0.1% formic acid) (13 min) to 100% acetonitrile (18 min), 0.4  $\text{mL min}^{-1}$ , at 210 nm]



reported data in the literature (Figs. 2, S4, and S5, Tables 1 and S1) (Hasegawa et al. 1992; Kosemura et al. 1995; Nagao et al. 1985). The difference in chemical structure between MBOA (1) and DMBOA (2) is the presence of a methoxy group at C-7 (Fig. 2).

MBOA (1), which belongs to the benzoxazolone group, was first identified in 1955 from the root of two *Coix* species, including adlay (*Coix lacrymajobi* L.) (Koyama 1955; Koyama and Yamato 1955). In 1957, two groups individually reported the isolation, identification, and biological activities of MBOA (plant-resistance factor and antimicrobial activity) from maize and wheat seedlings (Smisman et al. 1957; Virtanen et al. 1957). DMBOA (2) was first identified from dried maize tissue that appeared to be a degradation product of benzoxazinoids (BXs) (Klun et al. 1970). Several studies on the anti-auxin activity of DMBOA have been reported (Anai et al. 1996; Kosemura et al. 1995; Kosemura et al. 1994; Venis and Watson 1978).



**Fig. 2** Chemical structures of 6-methoxy-2(3H)-benzoxazolone (MBOA, 1) and 6,7-dimethoxy-2(3H)-benzoxazolone (DMBOA, 2). The numbering on the benzoxazolone skeleton for 1 is also shown

**Table 1**  $^{13}\text{C}$ - (201 MHz) and  $^1\text{H}$ -NMR (800 MHz) data of MBOA (1) in  $\text{CDCl}_3$ . \*Data from literature ( $^1\text{H}$ -NMR in  $\text{CDCl}_3$  and  $^{13}\text{C}$ -NMR in  $\text{DMSO}-d_6$ ) (Hasegawa et al. 1992; Nagao et al. 1985). These data of isolated MBOA (1) were identical to an authentic standard

position	$\delta_{\text{C}}$		$\delta_{\text{C}}^*$	$\delta_{\text{H}}$ , mult. (J in Hz)	$\delta_{\text{H}}^*$	HMBC
1 (O)						
2	155.9	C	155.1			
3 (N)						
4	109.9	CH	109.9	6.95, d (8.6)	6.96, d (8.3)	5, 6, 8, 9
5	109.7	CH	109.1	6.70, dd (8.6, 2.4)	6.72, d (8.3, 2.4)	4, 6, 7, 9
6	156.2	C	155.4			
7	97.5	CH	97.1	6.82, d (2.4)	6.84, d (2.4)	5, 6, 8, 9
8	144.6	C	144.3			
9	122.6	C	123.9			
6-OCH <sub>3</sub>	56.0	CH <sub>3</sub>	55.8	3.78, s	3.81, s	6
NH[b]				7.68 brs	8.55 brs	

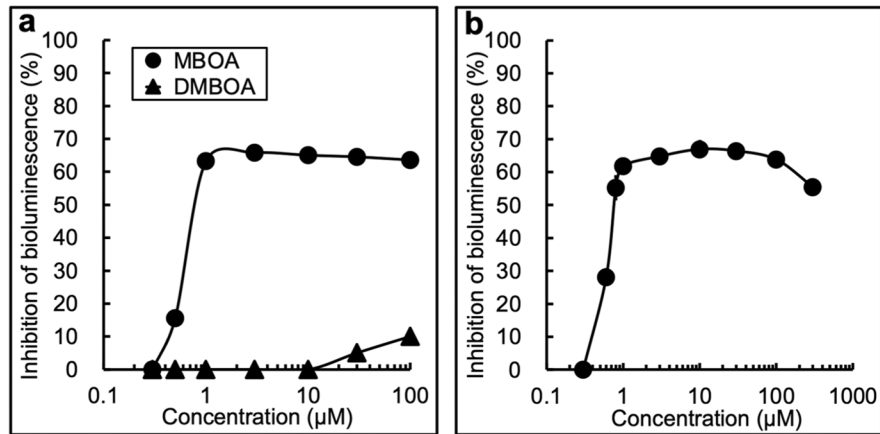
## Bioactivity of BNI compound in hydrophilic root exudates of maize

MBOA (1) showed strong BNI activity, whereas the activity of DMBOA (2) was markedly weaker (Fig. 3a). Moreover, a dose–response curve for standard MBOA (FUJIFILM Wako Pure Chemical Corp.) reproduced that of isolated 1, having an  $\text{ED}_{50}$  value of 0.76  $\mu\text{M}$  (Fig. 3b). Thus, we elucidated MBOA to be the strongest BNI compound in hydrophilic root exudates of maize. Remarkably, in contrast to other reported BNI compounds, the shape of the dose–response curve for MBOA to *N. europaea* (pHLUX20) was unprecedented. Previously reported BNI compounds, including zeanone ( $\text{ED}_{80}=8 \mu\text{M}$ ), HDMBOA ( $\text{ED}_{80}=70 \mu\text{M}$ ), and HDMBOA- $\beta$ -glucoside ( $\text{ED}_{80}=300 \mu\text{M}$ ), displayed a linear correlation between their bioluminescence inhibition and concentration (Fig. S6), from which each  $\text{ED}_{80}$  was calculated (Otaka et al. 2022; Subbarao et al. 2009; Subbarao et al. 2013a). In contrast, MBOA displayed more than 50% inhibition in a dosage range of 0.76–300  $\mu\text{M}$  but did not attain 80% inhibition (Figs. 3b and S6). These results indicated that MBOA had a different function in nitrification from those of known nitrification inhibitors.

## Mode of action of MBOA on *N. europaea*

To validate the influence of MBOA in nitrification, we then tested whether MBOA inhibited the oxidation pathway from  $\text{NH}_3$  (ammonium ion form,  $\text{NH}_4^+$ ) to  $\text{NO}_2^-$  in *N. europaea*. Two concentrations of MBOA (2.0 and 10  $\mu\text{M}$ ) were added to *N. europaea*





**Fig. 3** Dose–response curves of isolated MBOA (1) and DMBOA (2) (a) and that of standard MBOA (b). A 7-day-old pure *Nitrosomonas europaea* (pHLUX20) culture was treated with the indicated concentrations of MBOA for 20 min. The bioluminescence of the control reaction after the addition of water instead of BNI compounds is defined as 100% biolumi-

nescence. The dose–response curve is a plot of the treated dose (μM) on the X-axis versus the response (percentage inhibition of bioluminescence) on the Y-axis. (b) Error bars indicate the standard deviation of the mean ( $n=3$ ). Error bars are not visible as they are smaller than symbols

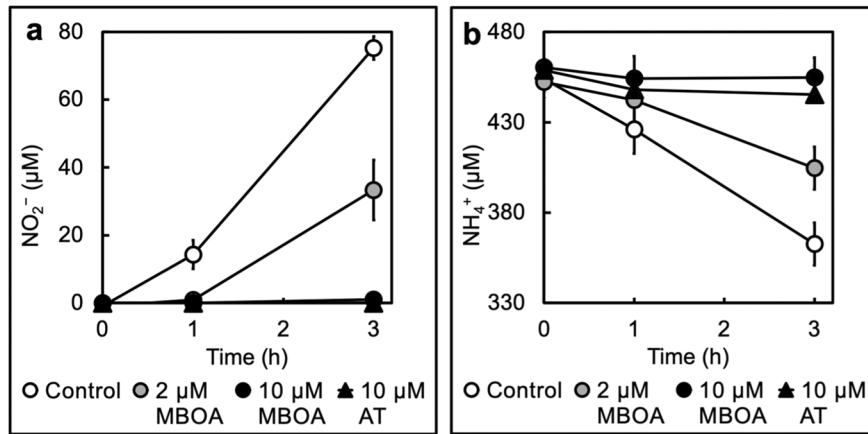
(pHLUX20) broth containing  $(\text{NH}_4)_2\text{SO}_4$  as the sole nitrogen source, which was incubated on a rotary shaker. An aliquot of each bacterial broth was collected at 0 min, 1 h, and 3 h after inoculation. The concentrations of both  $\text{NO}_2^-$  and  $\text{NH}_4^+$  in the sample were measured based on the Griess reaction for 10–100 μM  $\text{NO}_2^-$ , the DAN reaction for 0.1–10 μM  $\text{NO}_2^-$ , and the indophenol blue reaction for  $\text{NH}_4^+$  (Ivancic and Degobbi 1984; Nussler et al. 2006; Tsikas 2007).

MBOA inhibited the production of  $\text{NO}_2^-$  from *N. europaea*, with the inhibition rate ranging from approximately 50% at 2 μM and >99% at 10 μM at 3 h after inoculation (Fig. 4a). The concentration of  $\text{NH}_4^+$  was maintained by MBOA, indicating that  $\text{NH}_4^+$  consumption was suppressed (Fig. 4b). Similarly, allylthiourea, a commercial synthetic nitrification inhibitor, suppressed  $\text{NO}_2^-$  production together with  $\text{NH}_4^+$  consumption (Fig. 4a and b). Hence, these results proved that MBOA could inhibit the AMO pathway in *N. europaea*.

As *N. europaea* derives vital free energy from AMO-catalyzing oxidation of  $\text{NH}_4^+$  (Prosser 1990), we proposed that MBOA might inhibit the growth of *N. europaea*. To test this hypothesis, we assessed the effect of MBOA on the growth of *N. europaea* (pHLUX20) by measuring the O.D. at 660 nm to estimate the concentration of bacteria.

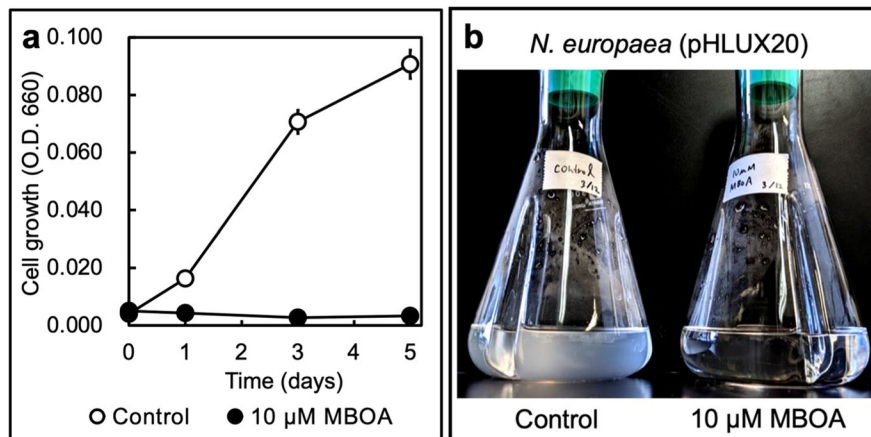
As expected, treatment with MBOA distinctly inhibited the cell growth of *N. europaea* (pHLUX20) for 5 days after inoculation (Fig. 5a and b). It has been reported that AOB can oxidize a variety of aromatic compounds (Chang et al. 2002; Keener and Arp 1994). In addition, AMO are potent oxidizers of not only  $\text{NH}_4^+$  but also various small molecules (e.g., allylsulfide and  $\text{C}_2\text{H}_4$ ) as substrates that show competitive nitrification inhibition (Juliette et al. 1993; Keener and Arp 1994). To test the stability and potential as a substrate of MBOA against *N. europaea*, we analyzed a culture of MBOA-treated *N. europaea* (pHLUX20) for 5 days by RP-HPLC monitored at 287 nm. Consequently, MBOA was not metabolized by *N. europaea* because the peak area of MBOA did not vary (Fig. S7). These results demonstrated that MBOA alone, but no other metabolized compounds, could suppress AMO activity in *N. europaea* in addition to cell growth.

Next, we refocused on the finding that MBOA incompletely inhibited the bioluminescence of *N. europaea* (pHLUX20) (Figs. 3 and S6); nevertheless, it strongly suppressed  $\text{NO}_2^-$  production from *N. europaea* without oxidation of  $\text{NH}_4^+$  (Fig. 4). We used recombinant *N. europaea* (pHLUX20) harboring an expression plasmid vector for bacterial luciferase genes (*luxA* and *luxB*) under the transcriptional control of the HAO gene (*hao*) promoter



**Fig. 4** Time-course analysis of  $\text{NO}_2^-$  production (a) and  $\text{NH}_4^+$  consumption (b) from *Nitrosomonas europaea* (pHLUX20) in the presence of MBOA. A 7-day-old *N. europaea* (pHLUX20) solution (250  $\mu\text{L}$ , O.D. 660=1.0) was inoculated into 100 mL P medium [nitrogen source:  $(\text{NH}_4)_2\text{SO}_4$ ] upon addition of MBOA (2 and 10  $\mu\text{M}$ ) and incubated at 150 rpm and 30 °C.

Allylthiourea (AT, 10  $\mu\text{M}$ ) and water was used as a positive control and control, respectively. After cultivation for 0 min (start: inoculated time), 1 h, and 3 h, an aliquot of the medium was collected to measure the  $\text{NO}_2^-$  and  $\text{NH}_4^+$  concentrations. Error bars indicate the standard deviation of the mean ( $n=3$ ). Invisible error bars were smaller than symbols



**Fig. 5** Time-course analysis of cell growth (a) and appearance (b) of *Nitrosomonas europaea* (pHLUX20) in  $(\text{NH}_4)_2\text{SO}_4$  medium with or without MBOA. A 7-day-old *N. europaea* suspension (250  $\mu\text{L}$ , O.D. 660=1.0) was inoculated to 100 mL P medium containing  $(\text{NH}_4)_2\text{SO}_4$  (final concentration: 18.9 mM) upon treatment with MBOA (10  $\mu\text{M}$ ) and grown on a rotary shaker at 150 rpm at 30 °C for 5 days. Water (1.0 mL) was used instead of MBOA as the control. (a) Cell growth (O.D. 660) was monitored on a mini photo 518R photometer at 0 min

(start: inoculated time), day 1, day 3, and day 5 after inoculation. The experiment was replicated three times. Error bars indicate the standard deviation of the mean ( $n=3$ ). Invisible error bars were smaller than symbols. (b) A representative image of *N. europaea* grown for 5 days under treatment with MBOA (right flask) and the control (left flask). A turbid culture in the right flask contains grown *N. europaea*, which was suppressed by MBOA

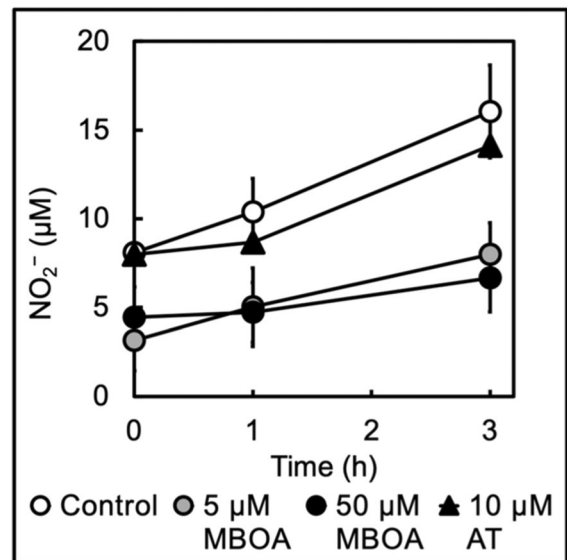
(Iizumi et al. 1998; Meighen 1993; Sayavedra-Soto et al. 1994). The biochemical basis of the bacterial luciferase, a heterodimeric ( $\alpha\beta$ ) flavin monooxygenase, reaction involves oxidation of a reduced

flavin mononucleotide (FMNH<sub>2</sub>) and a long-chain aliphatic aldehyde (decyl aldehyde in this study) by molecular oxygen to generate oxidized FMN (Campbell et al. 2009; Gibson and Hastings 1962;

Meighen and Hastings 1971; Ulitzur and Hastings 1979). Thus, loss of light emission means a decrease of the reducing power in the bacterial cell or destruction of cellular metabolic pathways by nonspecific inhibitors, such as acetone, respiratory inhibitors ( $\text{NaN}_3$  and  $\text{Na}_2\text{S}$ ), and heavy metals ( $\text{HgCl}_2$ ) (Backhaus and Grimme 1999; Hooper and Terry 1973; Parvez et al. 2006). In addition, inhibition of AMO decreases the reducing power in the cell of *N. europaea* (pHLUX20), causing the loss of light emission, which is recovered upon addition of hydroxylamine ( $\text{NH}_2\text{OH}$ ) as a substrate for HAO (Iizumi et al. 1998). Because synthetic allylthiourea targets primarily the AMO pathway and does not affect the luminescence of  $\text{NH}_2\text{OH}$ -treated *N. europaea* (pHLUX20) cells, the electron supply to luciferase via activation of HAO is suggested not to be linked to the presence of an AMO inhibitor that causes a decrease in light emission by blocking the electron flow within the cell (Iizumi et al. 1998). In an additional experiment, we unexpectedly observed that AMO-inhibiting MBOA remarkably increased the intensity of bioluminescence of *N. europaea* incubated in P medium containing  $\text{NH}_2\text{OH}$  as the sole nitrogen source (Fig. S8). Based on these results, we hypothesized that the incomplete inhibition of bioluminescence of *N. europaea* (pHLUX20) in  $(\text{NH}_4)_2\text{SO}_4$  medium by MBOA was correlated with HAO activity in the cell, which catalyzed the oxidative conversion of  $\text{NH}_2\text{OH}$  to  $\text{NO}_2^-$ .

To examine the effect of MBOA on HAO, we incubated *N. europaea* (pHLUX20) in culture medium containing  $20 \mu\text{M}$   $\text{NH}_2\text{OH}$  as the sole nitrogen source under treatment with MBOA in aerobic conditions. MBOA ( $5 \mu\text{M}$  and  $50 \mu\text{M}$ ) suppressed  $\text{NO}_2^-$  production by more than 80% relative to that of the control after inoculation, whereas  $\text{NO}_2^-$  production was not affected by  $10 \mu\text{M}$  allylthiourea (Fig. 6).

In contrast to the inhibitory activity of MBOA on both AMO and HAO, the bioluminescence of *N. europaea* (pHLUX20) in  $\text{NH}_2\text{OH}$  medium (Figs. 6 and S8) was enhanced. Given that nonspecific inhibitors (e.g.,  $\text{HgCl}_2$  and  $\text{Na}_2\text{S}$ ) decrease light emission in *N. europaea* (pHLUX20) (Iizumi et al. 1998), one possible explanation of this phenomenon is that the reducing power for luciferase was supplied via activation of other metabolic pathways. Further studies on protein–compound interactions using HAO and MBOA are required.



**Fig. 6** Time-course analysis of  $\text{NO}_2^-$  production converted from  $\text{NH}_2\text{OH}$  by *N. europaea* (pHLUX20) in the presence of MBOA. A culture broth of 7-day-old *N. europaea* (pHLUX20) ( $250 \mu\text{L}$ , O.D.  $660=1.0$ ) was inoculated into  $100 \text{ mL}$  P medium containing  $20 \mu\text{M}$   $\text{NH}_2\text{OH}$  as the sole nitrogen source and MBOA ( $5 \mu\text{M}$  and  $50 \mu\text{M}$ ) and incubated at  $150 \text{ rpm}$  and  $30 \text{ }^\circ\text{C}$ . Allylthiourea (AT,  $10 \mu\text{M}$ ) and water was used instead of MBOA as the positive control and control, respectively. After the incubation for  $0 \text{ min}$  (start: inoculated time),  $1 \text{ h}$ , and  $3 \text{ h}$ , an aliquot of the broth was collected to measure the  $\text{NO}_2^-$  concentration. Error bars indicate the standard deviation of the mean ( $n=3$ ). Invisible error bars were smaller than symbols

Collectively, these results showed that MBOA inhibited the AMO and HAO pathways, together with cell growth of *N. europaea* (pHLUX20).

#### BNI ability of MBOA in soil incubation test

To assess the potency of MBOA against nitrification in the soil, we performed a time-course analysis of  $\text{NO}_2^-$  production in a soil incubation test. We incubated soil ( $10 \text{ g}$ , dried weight) in  $(\text{NH}_4)_2\text{SO}_4$  buffer solution ( $100 \text{ mL}$ ) under treatment with MBOA ( $0.8 \text{ mg}$  in  $100 \text{ mL}$  solution:  $50 \mu\text{M}$ ;  $1.6 \text{ mg}$  in  $100 \text{ mL}$  solution:  $100 \mu\text{M}$ ) and sampled a soil slurry for 7 days after inoculation. We measured the concentrations of  $\text{NO}_2^-$  and  $\text{NO}_3^-$  in each sample based on the DAN reaction method.

MBOA dose-dependently suppressed  $\text{NO}_2^-$  and  $\text{NO}_3^-$  production for 4 days of incubation (Fig. 7a and b). The  $50 \mu\text{M}$  and  $100 \mu\text{M}$  MBOA-treated soil

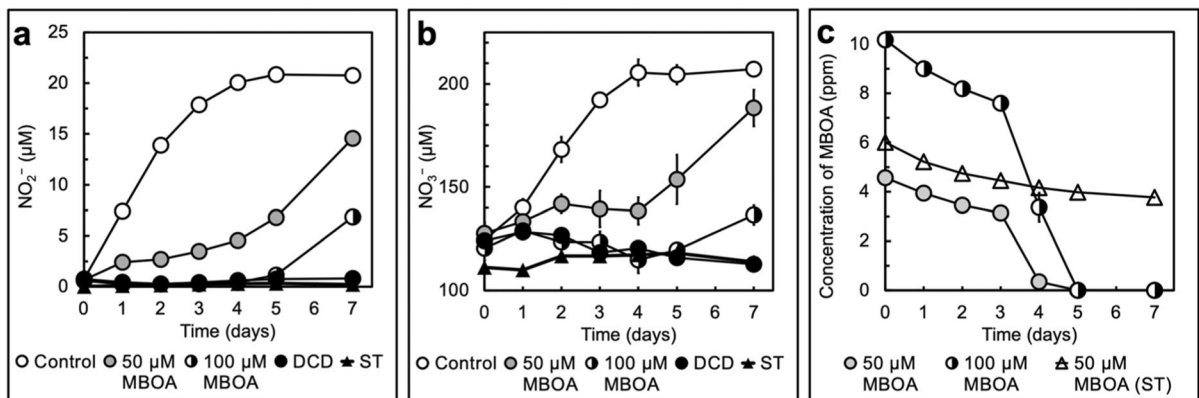
incubated for 4 days suppressed  $\text{NO}_2^-$  production by 80% and >99%, respectively (Fig. 7a). In addition, we have confirmed the inhibitory effect of DCD (a commercial nitrification inhibitor) which suppressed the production of  $\text{NO}_2^-$  and  $\text{NO}_3^-$  in these soil incubation studies (Fig. 7a and b). In contrast, the production of  $\text{NO}_2^-$  and  $\text{NO}_3^-$  was notably enhanced in MBOA-treated soil after incubation for 5 days (Fig. 7a and b).

In parallel with the soil incubation assay, we examined the stability of MBOA in the soil for 7 days. We quantified the peak area of MBOA in MBOA-treated soil by RP-HPLC monitored at 287 nm (Fig. S9). The concentration in the soil of MBOA (50  $\mu\text{M}$  and 100  $\mu\text{M}$  treatments) decreased during incubation for 4 days and was not detected after incubation for 5 days (Figs. 7c and S10). In contrast, MBOA remained detectable in sterilized soil for 7 days (Figs. 7c and S10). Hence, it was strongly suggested that MBOA was biodegraded by soil microorganisms during incubation. In addition, the concentrations of  $\text{NO}_2^-$  and  $\text{NO}_3^-$  in MBOA-treated soil increased concomitant with MBOA degradation (Fig. 7a–c).

## Discussion

Total BNI activity of MBOA in exudates from maize roots

A combination of chromatography guided by a bioluminescence assay enabled us to identify MBOA as the major BNI-active compound in hydrophilic exudates of maize roots (Figs. 1, 2 and 3). To clarify the contribution of MBOA to total BNI activity, we quantified MBOA in fraction M, based on its peak area at 287 nm by HPLC, which was fractionated from hydrophilic exudates (Figs. 1, S9, and S11). The weight of MBOA in fraction M (540 mg) was calculated to be 5.0 mg (Fig. S11). Given that the total BNI activity of hydrophilic exudates (24 g, 18,256 ATU) was concentrated in fraction M containing the BNI-active MBOA (5.0 mg, BNI activity per amount: 0.396  $\mu\text{g}/\text{ATU}$ ), the total BNI activity of MBOA in hydrophilic exudates was calculated as 12,605 ATU (Fig. 8a). As other BNI compounds have not been identified owing to their weak activities, we elucidated MBOA as the predominant BNI-contributing compound in hydrophilic exudates of maize roots (contributing 69%



**Fig. 7** Effect of MBOA on nitrification (a and b) and degradation of MBOA in soil (c). MBOA (0.8 mg for 50  $\mu\text{M}$  and 1.6 mg for 100  $\mu\text{M}$ ) was added to air-dried soil (10 g) in 750  $\mu\text{M}$   $(\text{NH}_4)_2\text{SO}_4$  buffer solution (100 mL). Water was added to the soil solution, instead of MBOA, as the control. An aqueous solution of dicyandiamide (DCD, final concentration: 327  $\mu\text{M}$ ) was used as a positive control. Sterilized soil (ST) was used as a negative control. Each soil solution was incubated on a rotary shaker at 100 rpm at 20 °C. An aliquot of the soil solution was collected at 1 h, days 1, 2, 3, 4, 5, and 7 after the start of incubation. All collected solutions were centrifuged, and the supernatant was used for analysis of  $\text{NO}_2^-$

and  $\text{NO}_3^-$  production and MBOA stability. Error bars indicate the standard deviation of the mean ( $n=3$ ). Invisible error bars were smaller than symbols. (c) Stability of MBOA in soil was estimated based on the peak area of MBOA monitored at 287 nm by RP-HPLC. MBOA (50  $\mu\text{M}$  and 100  $\mu\text{M}$ ) was maintained in non-sterilized soil for 4 days, but was subsequently not detected owing to microbial biodegradation. MBOA was not degraded in sterilized soil (ST) for 7 days. The difference in initial concentration after addition of MBOA (0.8 mg) between non-sterilized and sterilized soil is attributed to the absorptivity of MBOA by each soil

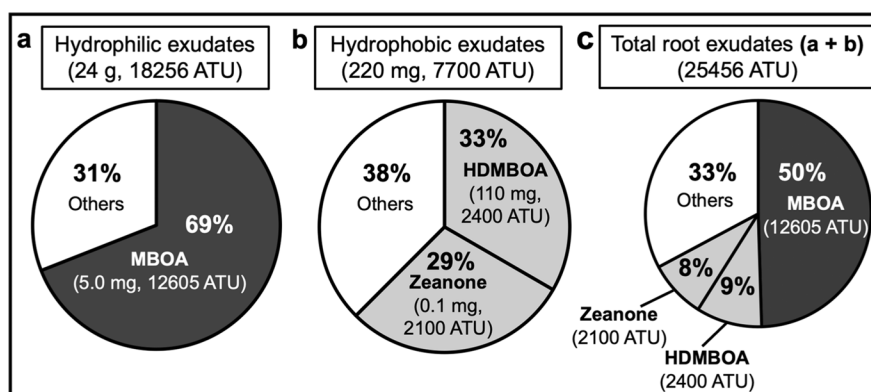
of the total BNI activity). In our previous study, we have identified HDMBOA (110 mg, 2400 ATU) and zeanone (0.1 mg, 2100 ATU) as the major hydrophobic BNI-active compounds released from maize roots (220 mg, 7700 ATU), together accounting for 62% of the total BNI activity (Fig. 8b) (Otaka et al. 2022). By combining the hydrophilic and hydrophobic exudates, it was revealed that MBOA accounts for nearly 50% of the total BNI activity released from maize roots (Fig. 8c). In turn, HDMBOA and zeanone respectively contributed 9% and 8% to the BNI activities of maize. Hence, we determined that MBOA is the predominant BNI-contributing compound in all exudates of maize roots.

Although a diverse range of bioactivities among secondary metabolites have been discovered, unlike primary metabolites (i.e., protein, starch, and sugar), secondary metabolites are not considered essential for the growth and development of plants. In addition, the total productivity of our identified BNI secondary metabolites is much smaller than that of root tissue in maize; therefore, the metabolic cost for biosynthesis of BNI compounds from the carbon source in roots may have a negligible impact on the growth of maize.

Collectively, the present results suggested that three BNI-active compounds (MBOA, zeanone, and HDMBOA) are largely responsible for BNI activity released from maize roots, which can be the targets for genetic improvement efforts to improve BNI-capacity in maize.

#### Occurrence mechanism of MBOA from maize

Although the benzoxazolone MBOA was the most abundant BNI-contributing compound in maize root exudates, the biosynthetic pathway to form the 1,3-benzoxazol-2-one skeleton has not been elucidated. To date, many researchers have investigated benzoxazolones generated by non-enzymatic reactions from more chemically reactive biosynthesized benzoxazinoids (BXs) with a 1,4-benzoxazin-3-one skeleton (Cotton et al. 2019; Klun et al. 1970; Kosemura et al. 1994; Virtanen et al. 1960; Woodward et al. 1978). In particular, MBOA has been reported to be the primary product from three BXs: HDMBOA, 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA), and HMBOA (Fig. S1), which have N-methoxy, N-OH, and N-H groups, respectively (Atkinson et al. 1991; Grambow et al. 1986). HDMBOA has been reported to be the dominant BX in organic exudates of maize seedlings (Otaka et al. 2022; Zhang et al. 2000). The half-life of HDMBOA in aqueous solution is much shorter than that of the other two BXs because the N-methoxy group is less stable than the N-OH and N-H groups, which we also confirmed by auto-conversion of HDMBOA into MBOA in water (Fig. S12) (Grambow et al. 1986; Maresh et al. 2006). One intermediate in the conversion of HDMBOA to MBOA, *o*-imidoquinone linked to formic acid and MeOH, has been established (Fig. S13) (Grambow et al. 1986; Maresh et al.



**Fig. 8** Contributions of identified compounds to BNI activities in root exudates of maize (a–c). (a) MBOA was identified as the most abundant BNI-active compound in hydrophilic exudates. (b) Two major BNI-contributing compounds, HDMBOA and zeanone, were identified in hydrophobic exudates

(Otaka et al. 2022). (c) Sum of total BNI activities for hydrophilic and hydrophobic exudates (a + b) indicating that MBOA contributed approximately half of the total BNI activity in root exudates



2006). Importantly, we also observed that HDMBOA was converted to MBOA whether in sterilized or non-sterilized soil within 24 h (Fig. 9a), strongly suggesting that this phenomenon does not involve biological conversion in soil.

As an internal compound in maize roots, HDMBOA is stored not only as a stand-alone compound (agricone form) but also in a glycosylated form (HDMBOA- $\beta$ -glucoside), which can be enzymatically hydrolyzed into agricone (HDMBOA) and glucose (Ahmad et al. 2011; Niemeyer 2009; Otaka et al. 2022). We have previously observed that both  $\beta$ -amylase and  $\beta$ -glucosidase, which are generally present in higher plants and microbes, catalyze the hydrolysis of HDMBOA- $\beta$ -glucoside to give HDMBOA and MBOA (Gu et al. 2006; Lauriere et al. 1992; Otaka et al. 2022). Based on these results, we hypothesize that HDMBOA- $\beta$ -glucoside can be hydrolyzed by maize roots and/or soil microbes. In maize roots, hydrolysis of HDMBOA- $\beta$ -glucoside is performed by a metabolic process in the root tissue, such as turnover of cytoplasmic material, detachment of the root cap, and root elongation (Brzobohaty et al. 1993; McLoughlin et al. 2018). However, soil microbes can also hydrolyze HDMBOA- $\beta$ -glucoside. Indeed, we observed that HDMBOA- $\beta$ -glucoside was irreversibly converted into MBOA in non-sterilized soil, but this biological reaction was not observed in sterilized soil (Fig. 9b). HDMBOA generated via biological hydrolysis is then non-biologically converted into MBOA, as shown in Fig. 9c.

Taken together, we suggest that part of the released MBOA from maize roots can also come from transformations of BXs accumulated both on the surface and within roots. In particular, HDMBOA and HDMBOA- $\beta$ -glucoside are suggested to be the crucial precursors of MBOA.

#### Plausible BNI mechanism in maize

On the basis of the collective results, we propose a comprehensive BNI mechanism for maize (Fig. 10) (Otaka et al. 2022). Maize releases three compounds responsible for BNI activity from the roots: MBOA ( $ED_{50}=0.76 \mu\text{M}$ ) in the hydrophilic portion, and zeaxone ( $ED_{50}=2 \mu\text{M}$ ) and HDMBOA ( $ED_{50}=13 \mu\text{M}$ ) in the hydrophobic portion (Figs. 2 and S1). MBOA has the ability to suppress nitrification in the soil, in which it can block at least the AMO and HAO pathways in

*N. europaea* (Figs. 4, 5, 6 and 7). BNI activity attributable to MBOA can be diffused from the rhizosphere because of its water-solubility ( $0.56 \text{ mg/mL}$  at  $25 \text{ }^\circ\text{C}$ ), whereas the BNI-effective area of naturally occurring zeaxone and HDMBOA may be limited in the rhizosphere owing to their low affinity with water.

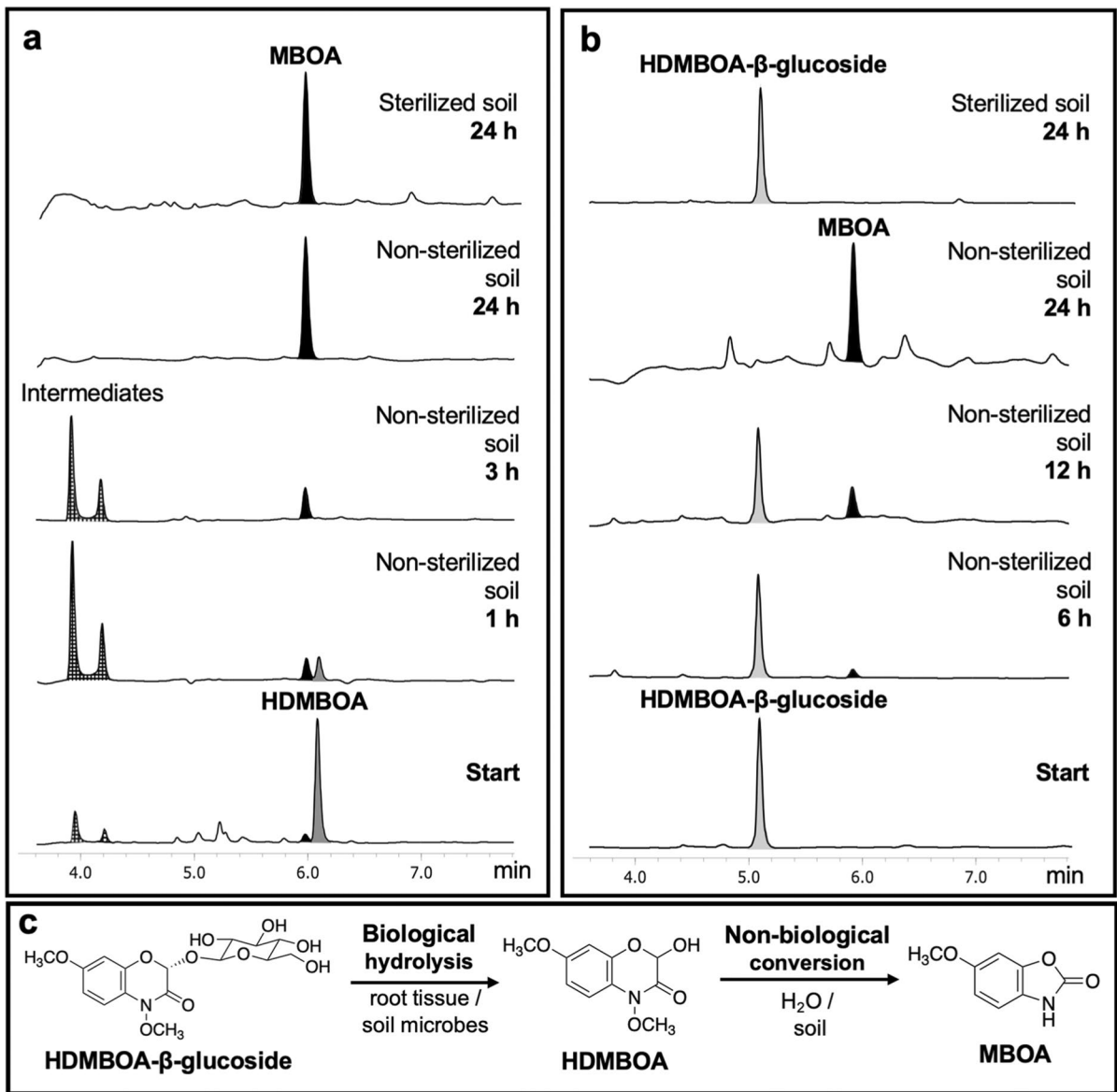
Released HDMBOA is eventually converted into the more stable MBOA via a non-biological process (Fig. 9a and c). When conversion of  $110 \text{ mg}$  HDMBOA ( $0.488 \text{ mmol}$ ) in hydrophobic exudates ( $220 \text{ mg}$ ) gives rise to MBOA ( $80.6 \text{ mg}$ ,  $0.488 \text{ mmol}$ ), the total BNI activity caused by MBOA ( $12,605 \text{ ATU}$ ) is potentially increased to  $351,539 \text{ ATU}$  (Hiradate 2006). It has been reported that the basic condition ( $>\text{pH } 7.0$ , in phosphate buffer) favors the rapid conversion from HDMBOA to MBOA (Maresh et al. 2006). In addition, acidification of the maize rhizosphere has been reported when maize takes up  $\text{NH}_3$  by the release of  $\text{H}^+$  from root cells (Gollany and Schumacher 1993; Jing et al. 2010; Zhou et al. 2009). Therefore, it is crucial to consider that the yield of MBOA from HDMBOA is regulated by the physicochemical conditions in the rhizosphere (e.g., pH, water content, temperature, and electric charge). In addition to HDMBOA, HDMBOA- $\beta$ -glucoside released from within the roots is also eventually transformed to MBOA via biological hydrolysis and following non-biological conversion of HDMBOA, which enters the rhizosphere together with water to exhibit BNI activity (Fig. 9b and c).

Although MBOA is much more stable than BXs (HDMBOA and HDMBOA- $\beta$ -glucoside) in a soil environment, it can be biochemically degraded to downstream products by soil microorganisms that weaken the BNI activity (Figs. 7c and S10) (Friebe et al. 1998; Macias et al. 2009). However, in parallel with the degradation of MBOA, MBOA may be continuously supplied as long as maize can release BXs into the rhizosphere. Hence, the longevity of BNI activity derived from MBOA is suggested to be sustained in the soil, which is an advantage for maize. Therefore, we attributed MBOA to be the key component of BNI in maize (Fig. 10).

#### Conclusion

We have established that MBOA as the major hydrophilic BNI-active compound released from maize



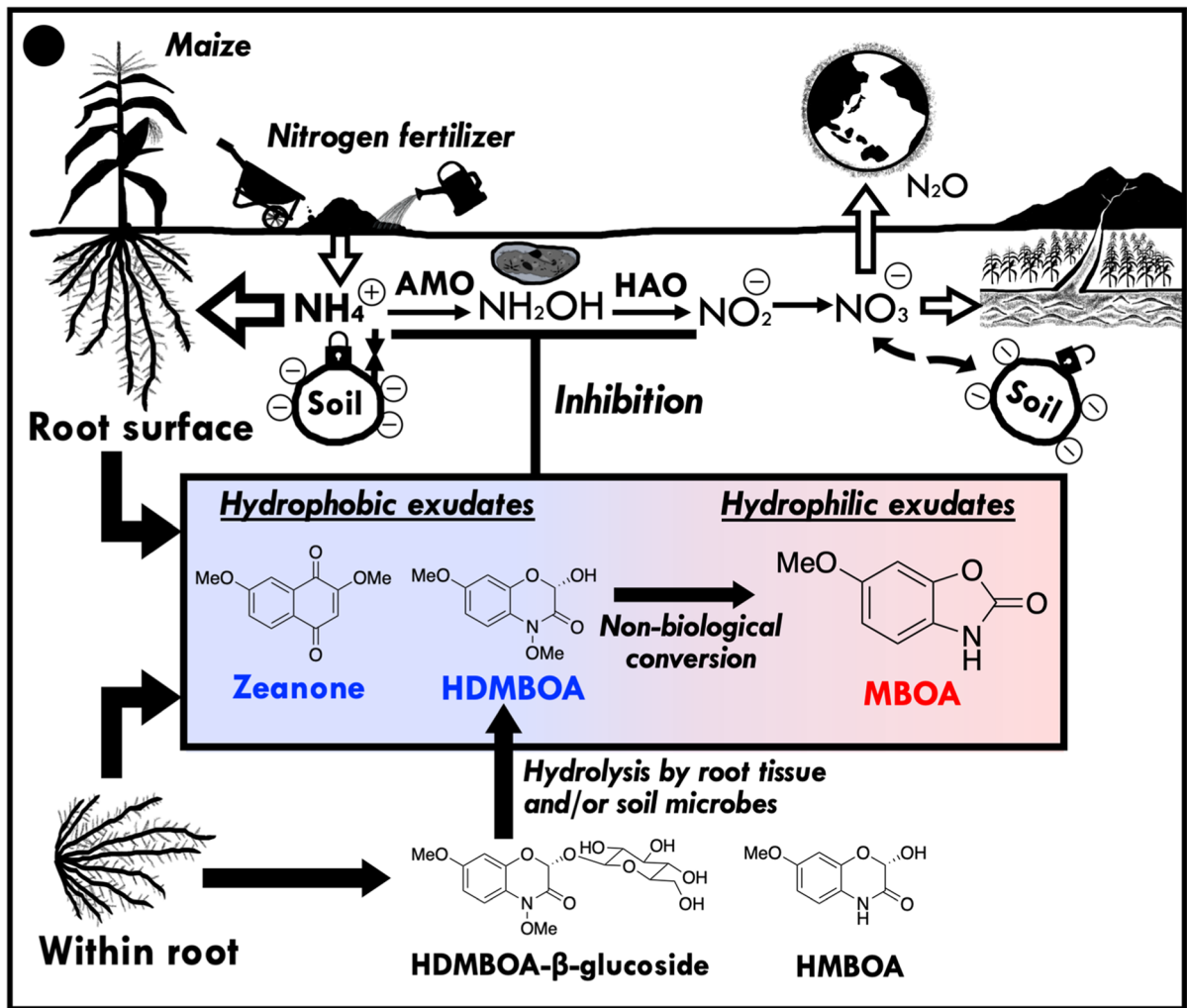


**Fig. 9** Time-course analyses of conversion of HDMBOA (a) and HDMBOA-β-glucoside (b) to MBOA in soil and the generation mechanism of MBOA from HDMBOA-β-glucoside (c). (a and b) To 5 μL solution of HDMBOA (350 μM) in acetone or HDMBOA-β-glucoside (350 μM) in MeOH was added wet soil (100 mg in 500 μL water). The mixture in a glass vial was incubated at 25 °C for 24 h. Sterilized soil was used as a negative control. After each sample was centrifuged, the superna-

tant of each collected sample (5 μL) was analyzed using HPLC with monitoring at 210 nm [1% acetonitrile/99% H<sub>2</sub>O (0.1% formic acid) (start) to 30% acetonitrile/70% H<sub>2</sub>O (0.1% formic acid) (13 min), 0.4 mL min<sup>-1</sup>]. (a and c) HDMBOA was non-biologically converted into MBOA in soil after 24 h. (b and c) HDMBOA-β-glucoside was irreversibly hydrolyzed to MBOA in soil. In sterilized soil, the peak of HDMBOA-β-glucoside was retained

roots. MBOA suppressed the conversion not only of NH<sub>3</sub> to NO<sub>2</sub><sup>-</sup> (AMO pathway) but also NH<sub>2</sub>OH to NO<sub>2</sub><sup>-</sup> (HAO pathway) in *N. europaea*. In a soil incubation test, over 80% of NO<sub>2</sub><sup>-</sup> production was

suppressed by 50 μM MBOA; however, its soil-residence time is only 5 days and degraded subsequently from soil microbial activity. Quantification experiments revealed that MBOA contributed nearly to 50%



**Fig. 10** Proposed BNI mechanism in maize. Maize can secrete three BNI-active compounds from the roots: zeanone and HDMBOA in hydrophobic exudates, and MBOA in hydrophilic exudates. Abundant HDMBOA is non-biologically

converted into MBOA after secretion into the rhizosphere. HDMBOA- $\beta$ -glucoside (within the roots), when released, is transformed into MBOA via biological hydrolysis and following auto-conversion of HDMBOA in the soil

of the BNI activity released from maize roots. Two BXs, HDMBOA and HDMBOA- $\beta$ -glucoside (which are chemically and biologically unstable in soil), are converted to a more stable BNI-active MBOA in the soil (through a soil-chemical process). Overall, we have shown that MBOA is a key component in the BNI activity of maize.

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**Author contributions** Junnosuke Otaka, Guntur Venkata Subbarao, and Tadashi Yoshihashi designed the experiments. Junnosuke Otaka performed chemical experiments, including structural elucidation of compounds. Junnosuke Otaka, Guntur Venkata Subbarao and Jiang MingLi performed bioassays using *N. europaea*. Junnosuke Otaka and Jiang MingLi performed the soil incubation experiment. All authors contributed to the writing of the manuscript.

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**Data availability** The datasets generated during the current study are available from JIRCAS on reasonable request.

## Declarations

**Competing interests** The authors have no relevant financial or non-financial interests to disclose.

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