

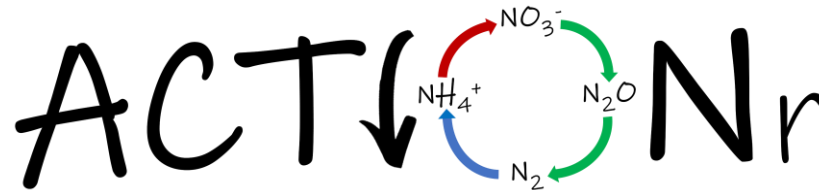


PhD Summer School

“Biological Nitrification Inhibition: Integrating Microbial Functions, Plant Traits, and Technological Innovations for Sustainable Nitrogen Cycling”

Practical Training

Fast-track, high-throughput screening for BNIs in ammonia-oxidizing bacteria



Research Action Network for Reducing Reactive Nitrogen Losses from Agricultural Ecosystems

Project No. 101079299

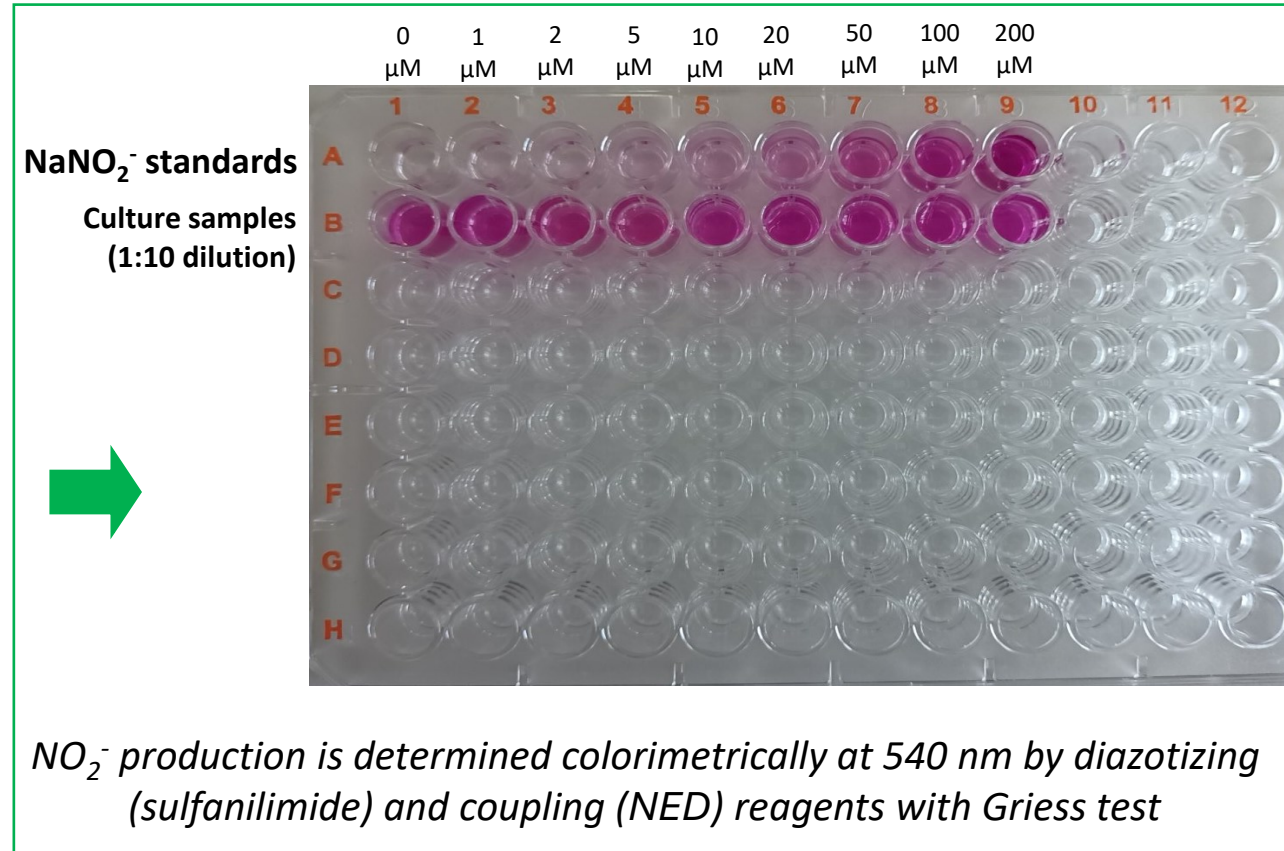


University of Thessaly
12-16 May 2025

Selected AOB strain for the fast-track assay

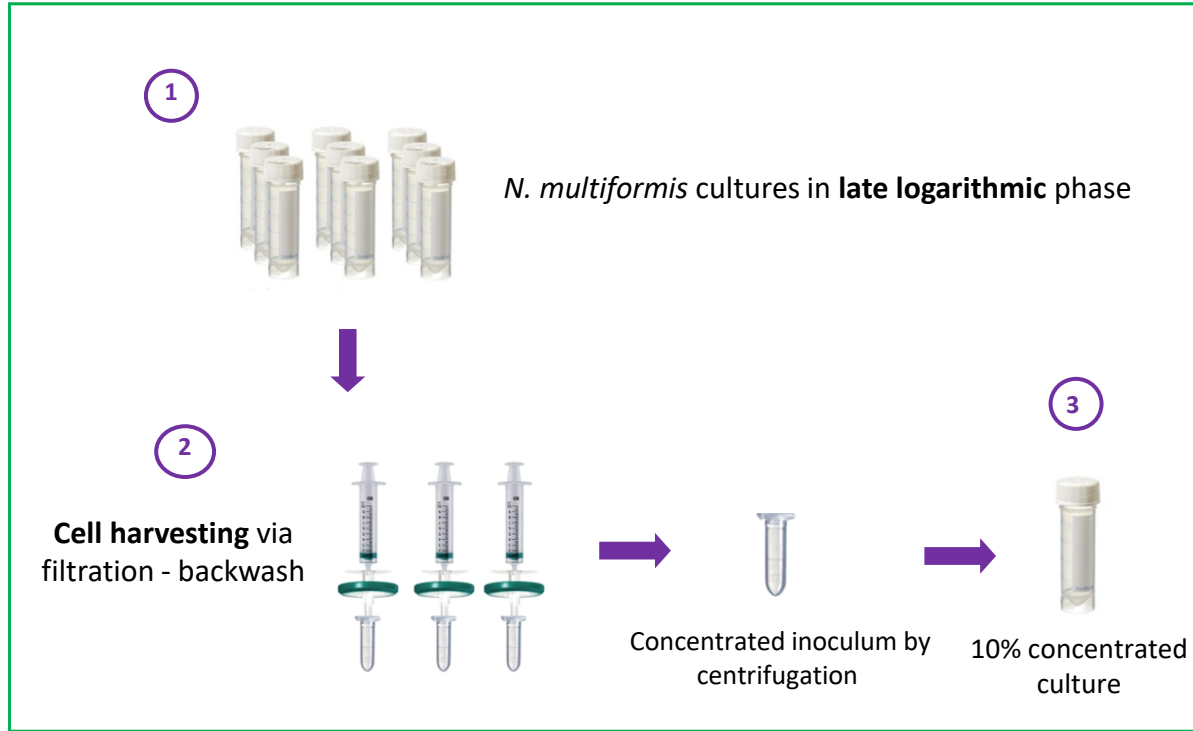
Nitrosospira multififormis (ATCC 25196)

- Screening up to six different compounds—each at four concentrations and in triplicate—can be accommodated within a single 96-well plate, allowing for effective estimation of EC₅₀ values.
- *N. multififormis* cultures in **late logarithmic phase**
→ **800-1000 μM NO_2^-**
- Skinner and Walker (SW) medium: **1mM NH_4^+ substrate**
- Grown statically at 28°C



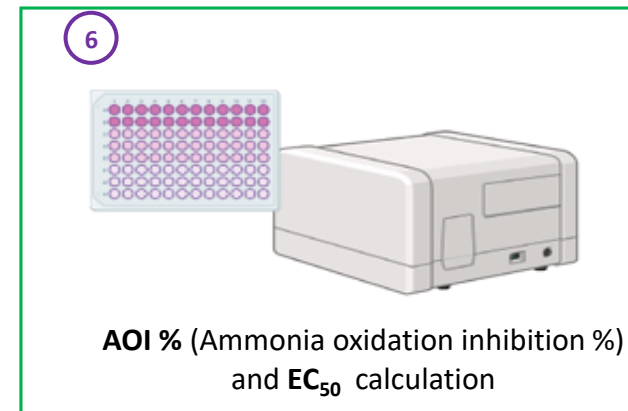
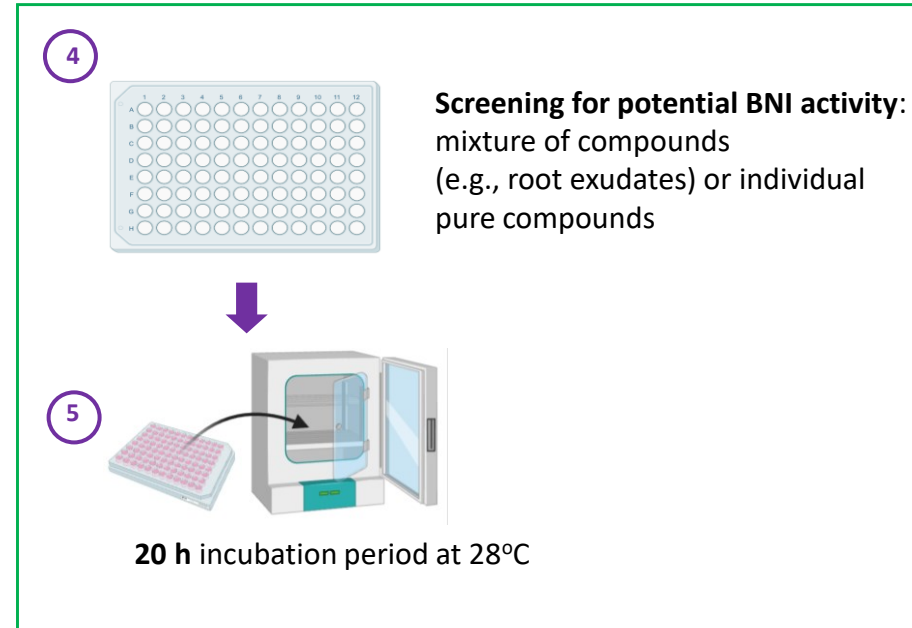
Fast-track pipeline

Stage 1: Cell harvesting & inoculation



Ghatak et al. Plant Biotechnology (submitted)

Stage 2: Cell plating & sample application



Stage 3: Colorimetric NO_2^- quantification

Fast-track pipeline – Stage #2: Plate setup for BNI application

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	ddH ₂ O	ddH ₂ O	ddH ₂ O	DMSO	DMSO	DMSO						
C	50	50	50	100	100	100	250	250	250	500	500	500
D	0.02	0.02	0.02	0.05	0.05	0.05	0.10	0.10	0.10	0.15	0.15	0.15
E												
F												
G												
H												

Negative
controls

MHPP

(positive control)

Root exudates
(samples)

1) **200 µL concentrated culture (10%)**
in each well (rows B-D)

2) **20 µL negative controls:**
0.09% (v/v) ddH₂O and DMSO

3) **20 µL positive control:**
Methyl 3-(4-Hydroxyphenyl) propionate
(**MHPP**) at final concentrations: 50, 100,
250 and 500 µM

4) **20 µL root exudates:**
(hydrophobic)
at final concentrations 0.02, 0.05, 0.1
and 0.15 µg µL⁻¹

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Fast-track pipeline – Stage #3: Plate setup for NO_2^- quantification

➤ Transfer of samples from the **incubated** plate to a **new** 96-well plate

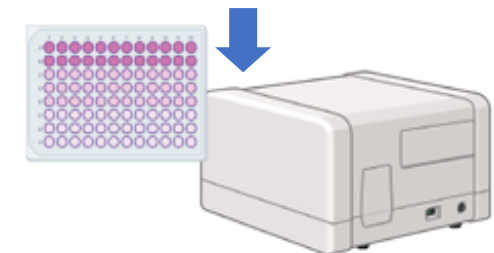
	1	2	3	4	5	6	7	8	9	10	11	12
NaNO₂ standard solutions	A 0	1	2	5	10	20	50	100	200			
Negative controls	B ddH ₂ O	ddH ₂ O	ddH ₂ O	DMSO	DMSO	DMSO						
MHPP (positive control)	C 50	50	50	100	100	100	250	250	250	500	500	500
Root exudates (samples)	D 0.02	0.02	0.02	0.05	0.05	0.05	0.10	0.10	0.10	0.15	0.15	0.15
Dilution 1:10												
	F											
	G											
	H											

1) **100 μL of standard solutions:**
0, 1, 2, 5, 10, 20, 50, 100, 200 μM NaNO₂

2) **90 μL SW + 10 μL of samples**
(from each well of the incubated plate) – **Sample dilution 1:10**

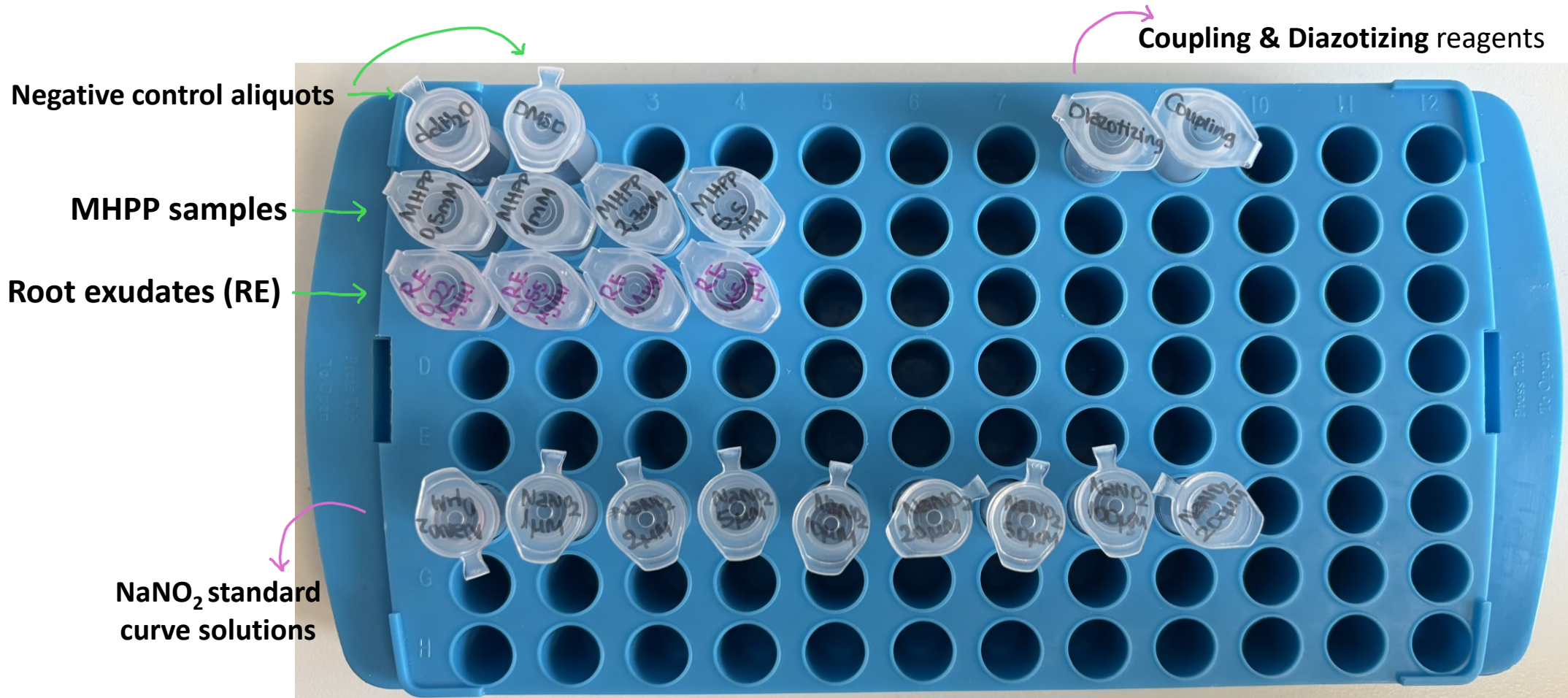
3) **20 μL Diazotizing reagent**

4) **20 μL Coupling reagent**



Nitrite determination at 540 nm

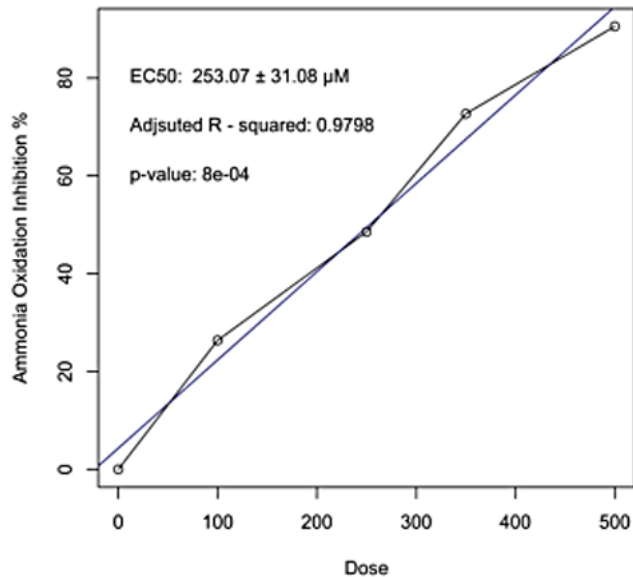
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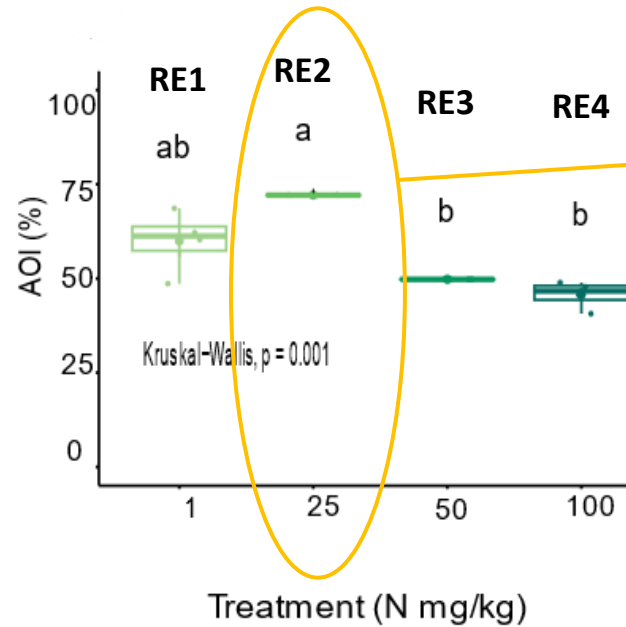
Result Analysis

- Negative Controls: 800 -1000 μM
- $\text{AOI}\% = - (\text{Normalized } \text{NO}_2_{\text{sample}} - \text{Normalized } \text{NO}_2_{\text{control}})$
- EC_{50} calculation for MHPP and root exudate (RE)

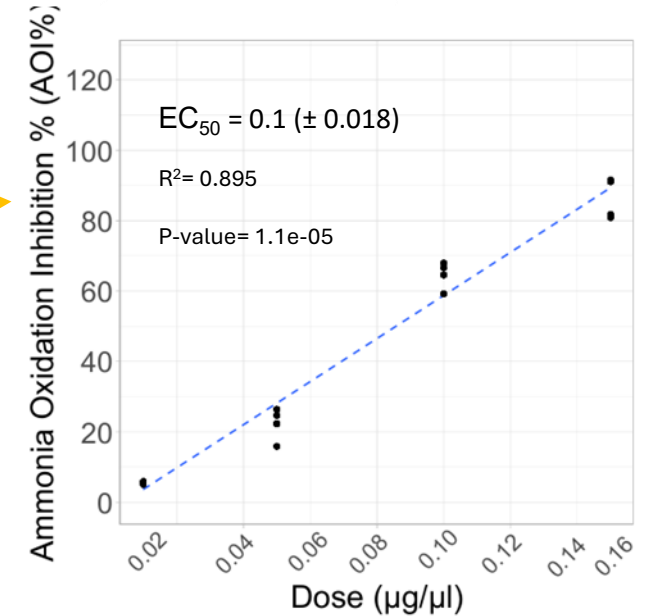
MHPP results



REs inhibition profiles (preliminary screening)



RE2 EC₅₀



THANK YOU



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wien



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