James White

Bionutrient Food Association
Soil and Nutrition Conference (Nov. 5, 2020)

Soil Microbes and Seed-Transmitted Endophytes Determine Health of Crops

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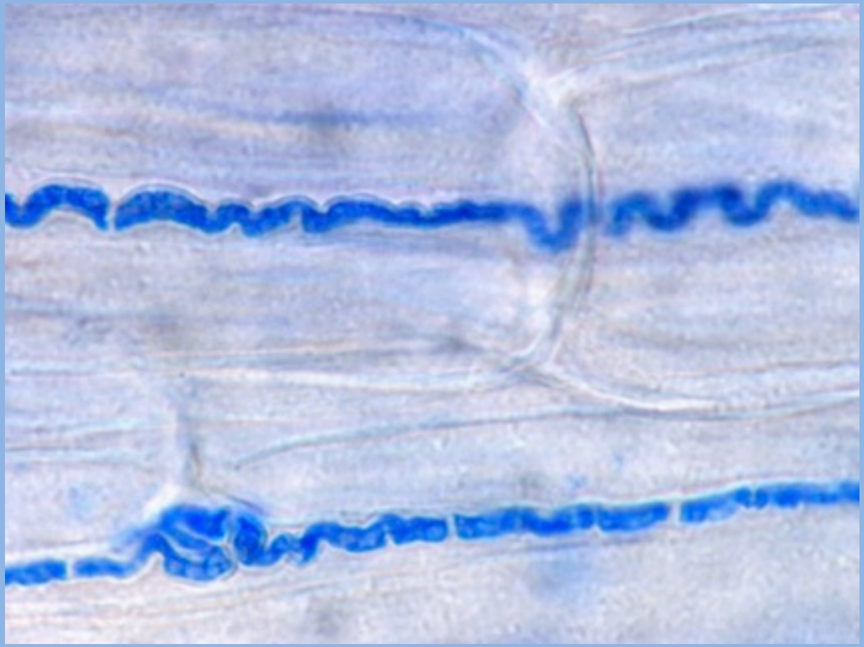
Nov. 5, 2020

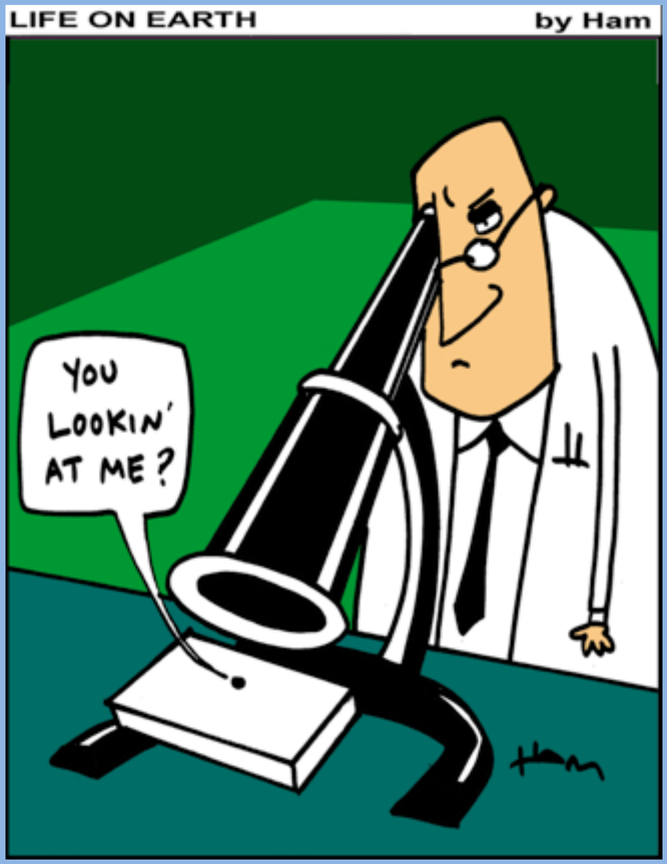
What are endophytes?

(Botany): Endophytic/endosymbiotic non-pathogenic microbes (fungi, bacteria or algae) present asymptomatically for all or part of their life cycles in tissues of plants.



Fungal hyphae of endophyte in stem tissue of tall fescue grass.

All plants naturally have endophytes!



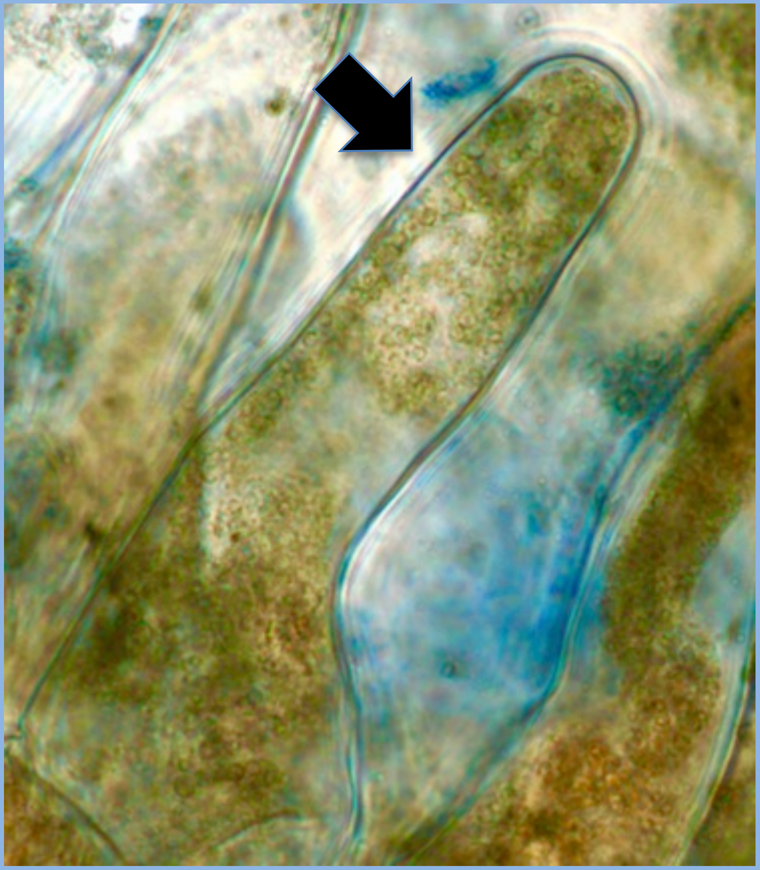
Endophytes are everywhere!

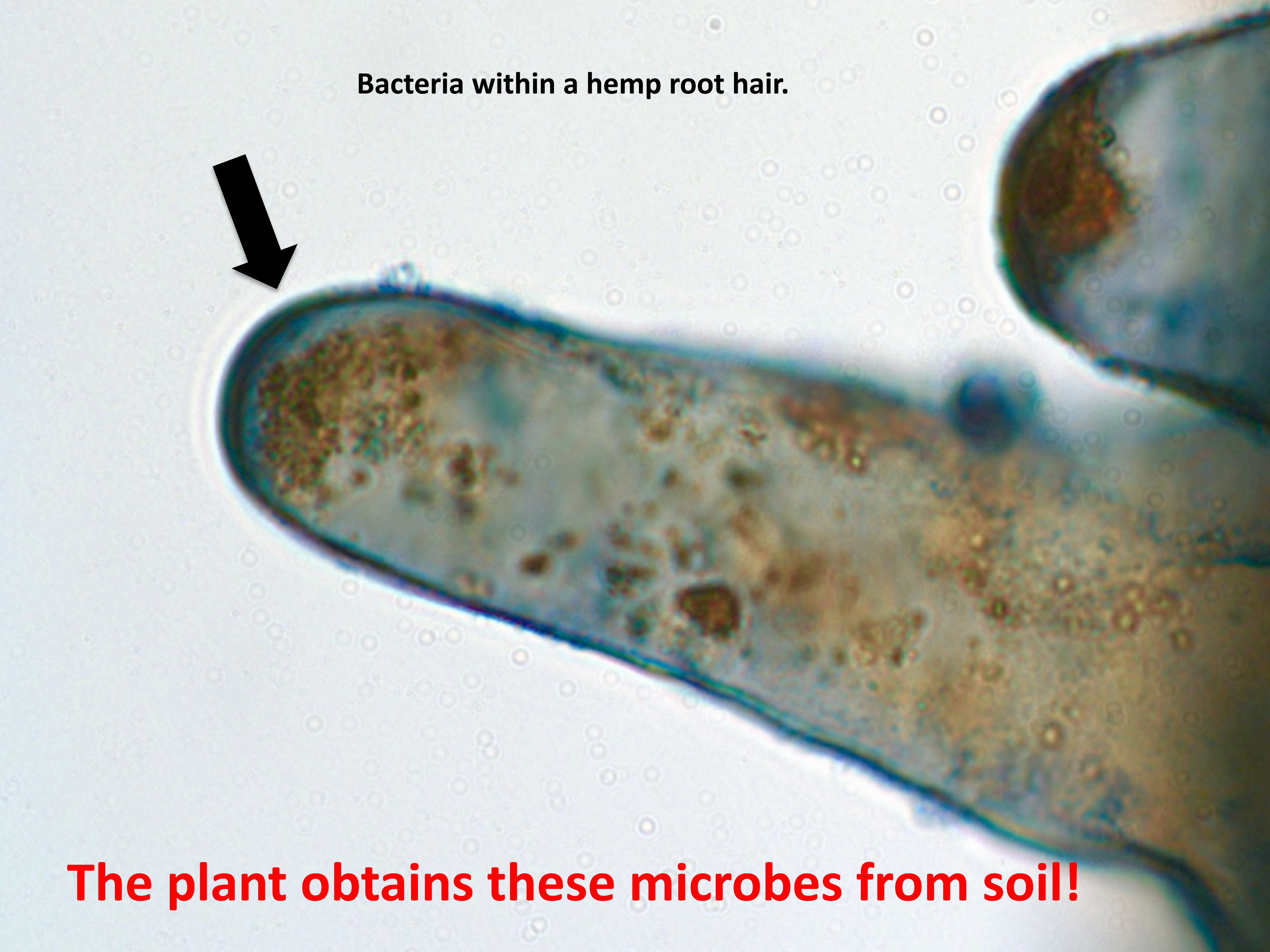
Every plant contains multiple endophytes!

Hemp seeds carry endophytes!

Hemp seedling root hairs

Root hairs showing bacteria (arrows) within hairs.



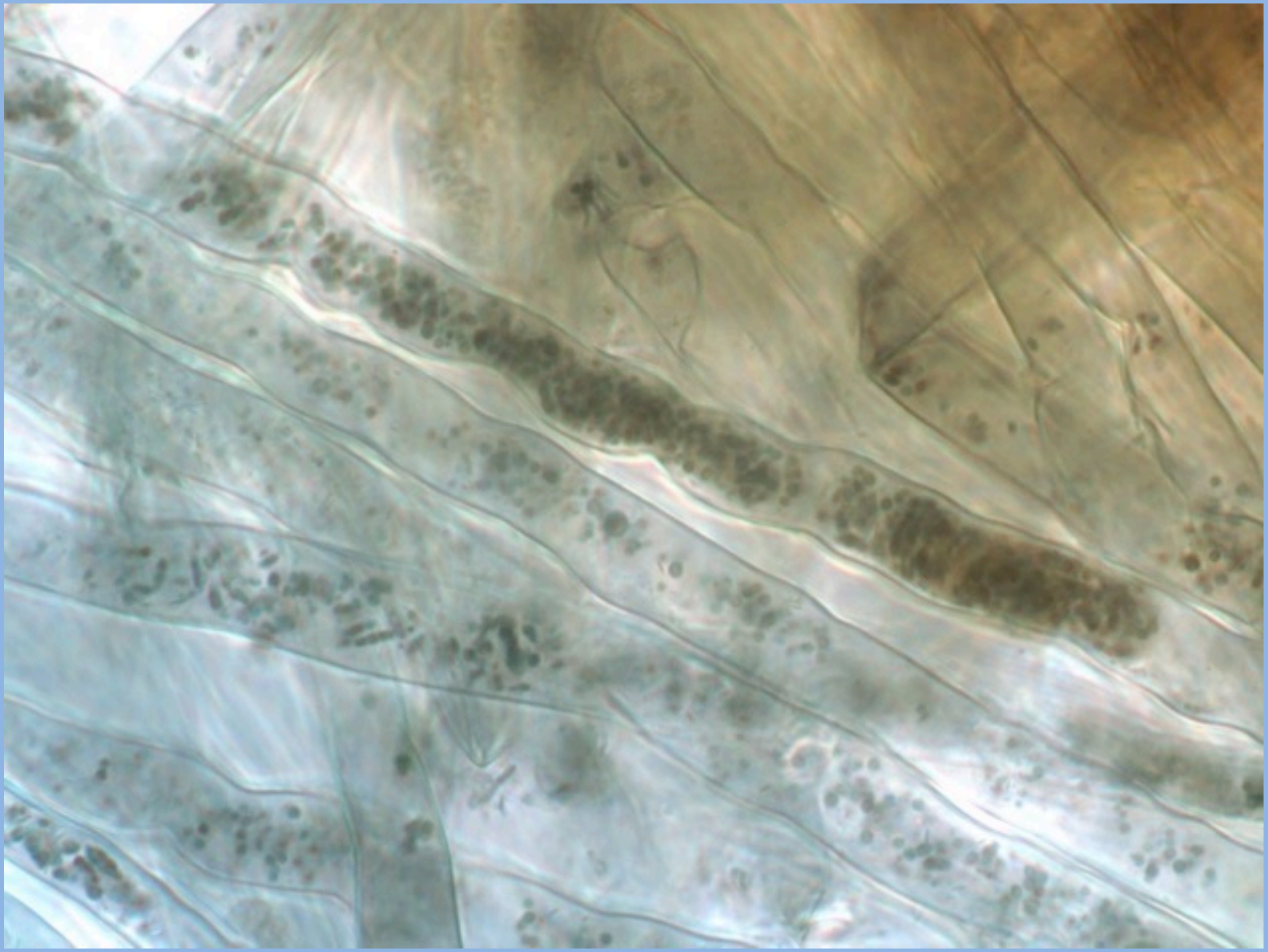

Bacteria within a hemp root hair.
The plant obtains these microbes from soil!

'Cadushy' cactus: *Subpilocereus repandus* in Bonaire

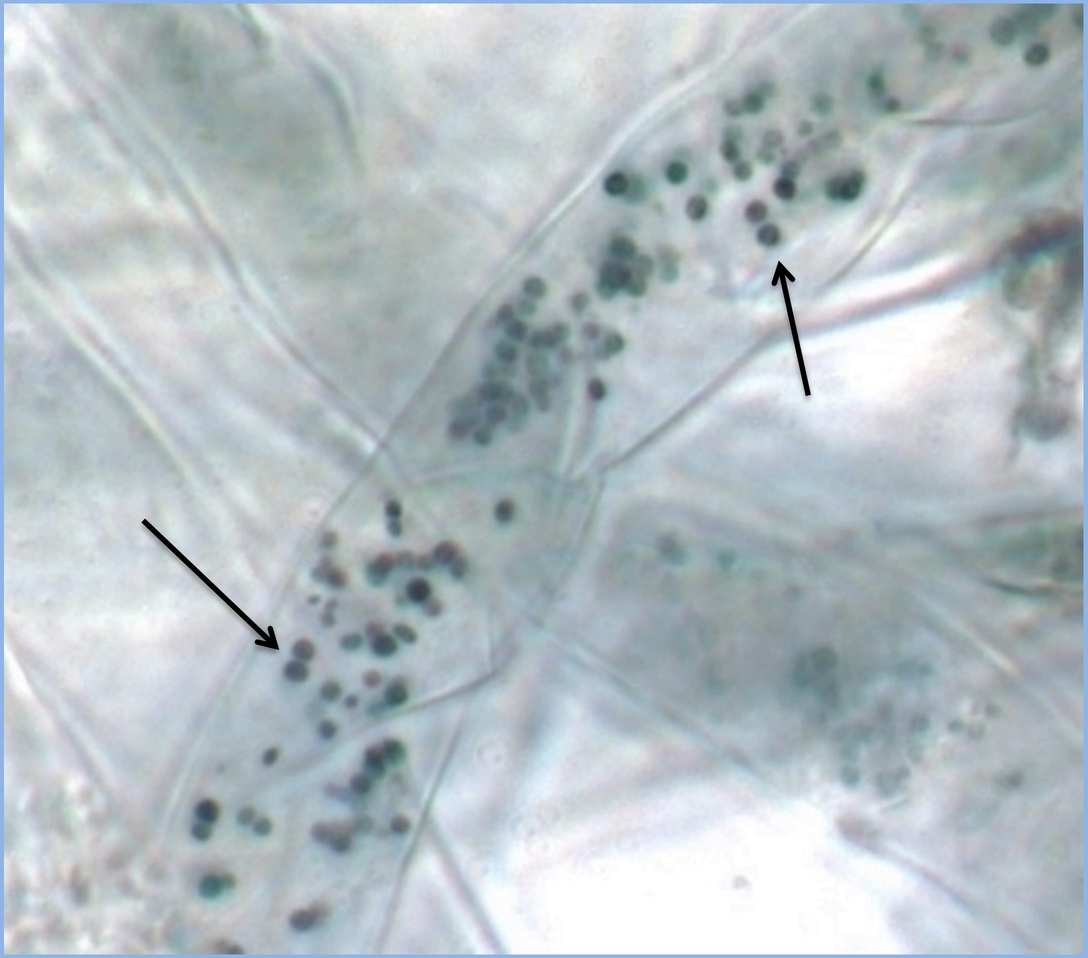
Seeds

Cadushy seedling

Bacteria in root hairs (Stained in DAB followed by aniline blue).



Bacteria in root hairs showing recently divided pairs



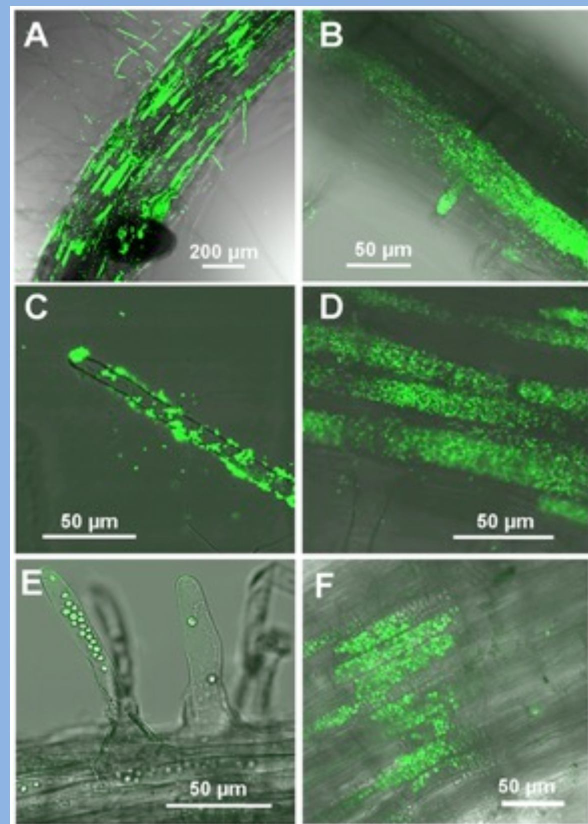
Microbial Endophytes

- Improve plant stress tolerance
- Suppress plant pathogenic fungi
- Modulate root development
- Improve nutrient absorption
- Alter chemical constituents of plants

Figure 1. Roots of axenically grown Arabidopsis and tomato were incubated with E coli or yeast expressing green fluorescent protein (GFPE. coli or GFPyeast).

“Rhizophagy”

Do plant roots consume bacteria to obtain nutrients?



‘Turning the Table:
Plants Consume Microbes as a Source of Nutrients’

Chany Paungfoo-Lonhienne

Paungfoo-Lonhienne C et al. 2010.
Turning theTable: Plants Consume Microbes as a Source of Nutrients.
PLoS ONE 5(7): e11915, doi:10.1371/journal.pone.0011915

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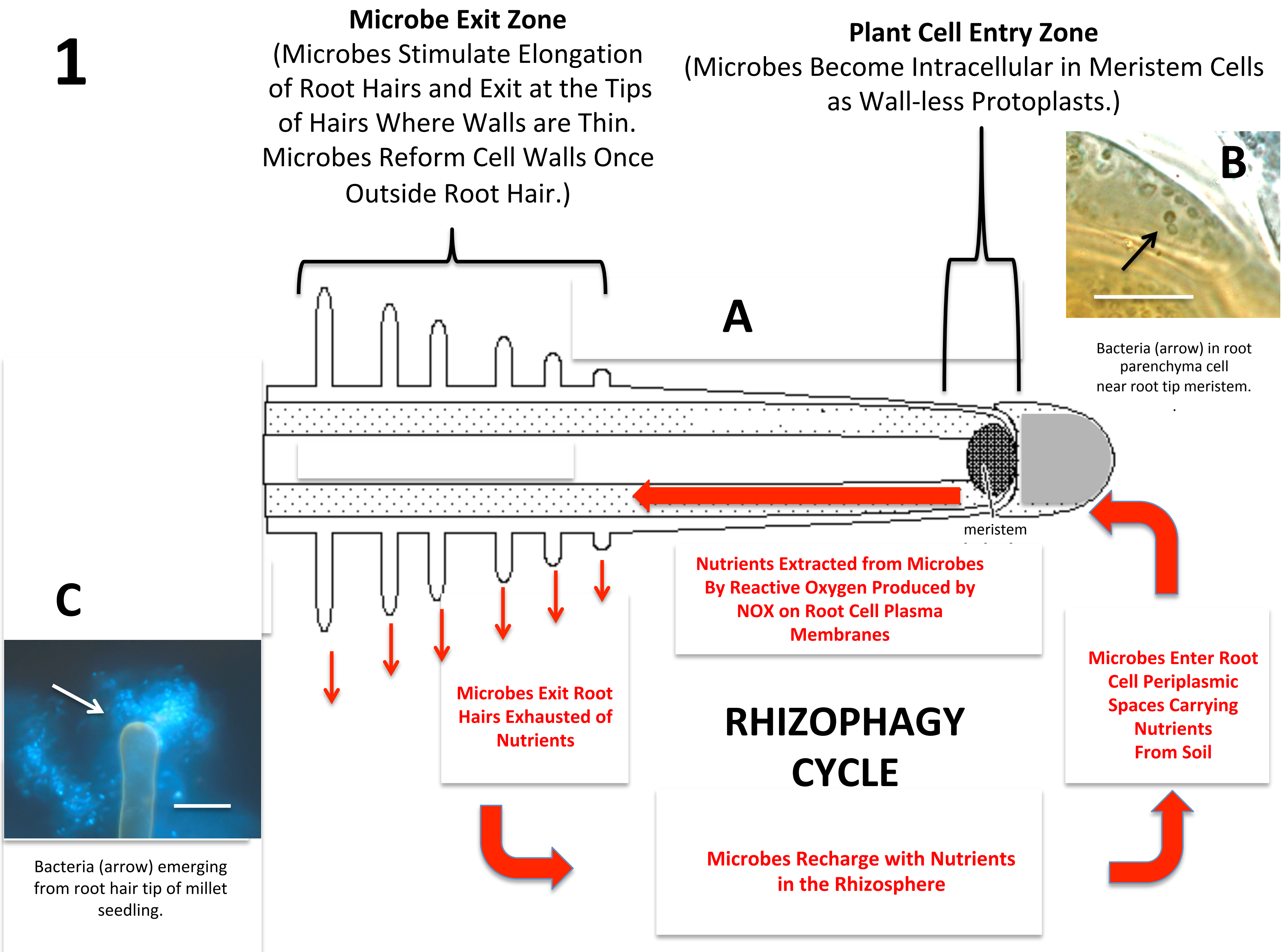
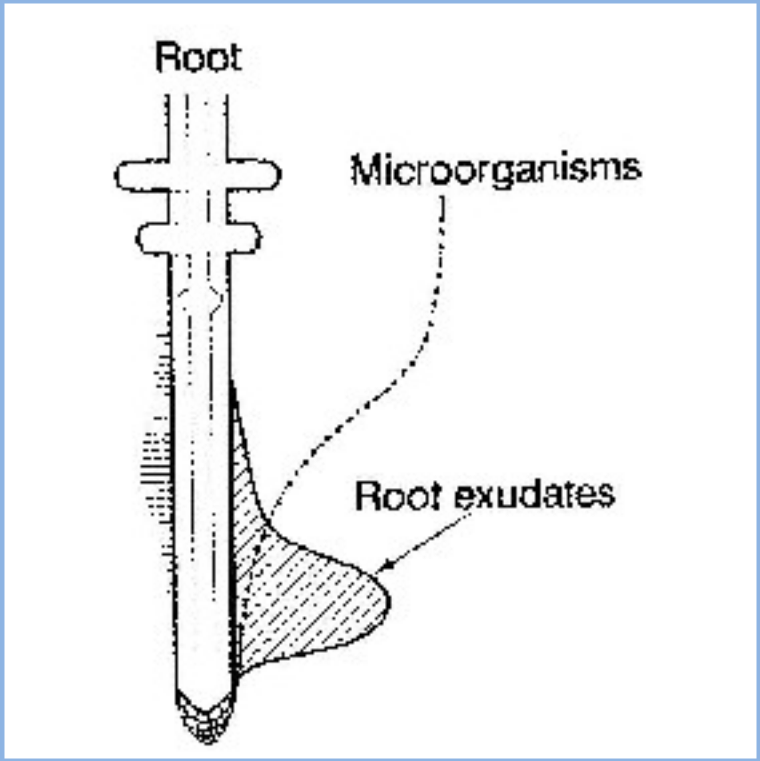

Microbe Exit Zone
(Microbes Stimulate Elongation of Root Hairs and Exit at the Tips of Hairs Where Walls are Thin. Microbes Reform Cell Walls Once Outside Root Hair.)
Plant Cell Entry Zone
(Microbes Become Intracellular in Meristem Cells as Wall-less Protoplasts.)
A
B
Bacteria (arrow) in root parenchyma cell near root tip meristem.
meristem
Nutrients Extracted from Microbes By Reactive Oxygen Produced by NOX on Root Cell Plasma Membranes
C
Microbes Exit Root Hairs Exhausted of Nutrients
RHIZOPHAGY CYCLE
Microbes Enter Root Cell Periplasmic Spaces Carrying Nutrients From Soil
Microbes Recharge with Nutrients in the Rhizosphere
Bacteria (arrow) emerging from root hair tip of millet seedling.

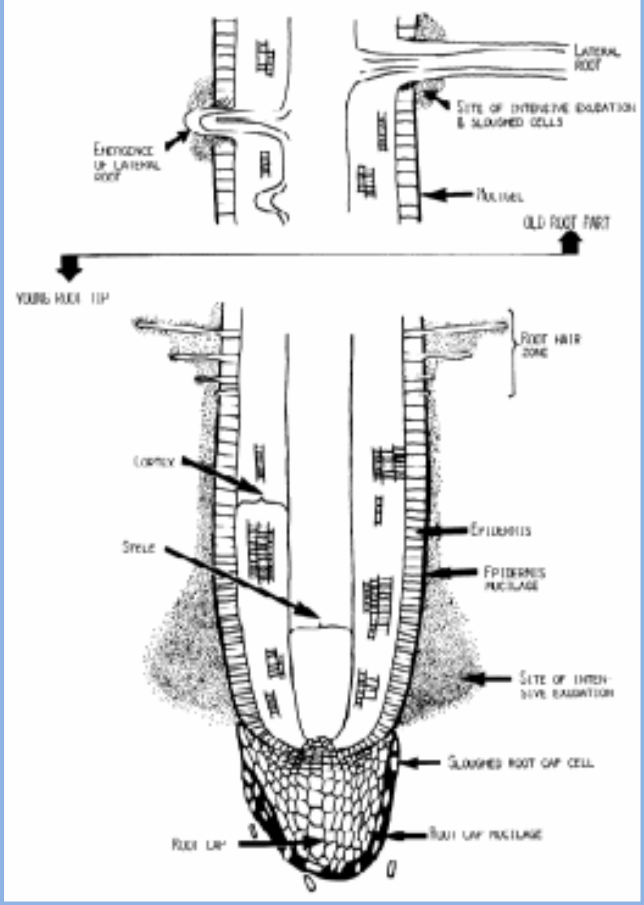
Bacterial symbiosis: germinating tall fescue seed showing seed-transmitted bacteria (*Bacillus* spp., *Pseudomonas* sp., etc..)


Richard Chen

Root exudation zones determined by 14C experiments.

Plants manipulate bacteria by cultivating microbes in
The root exudate zone near tip of root.



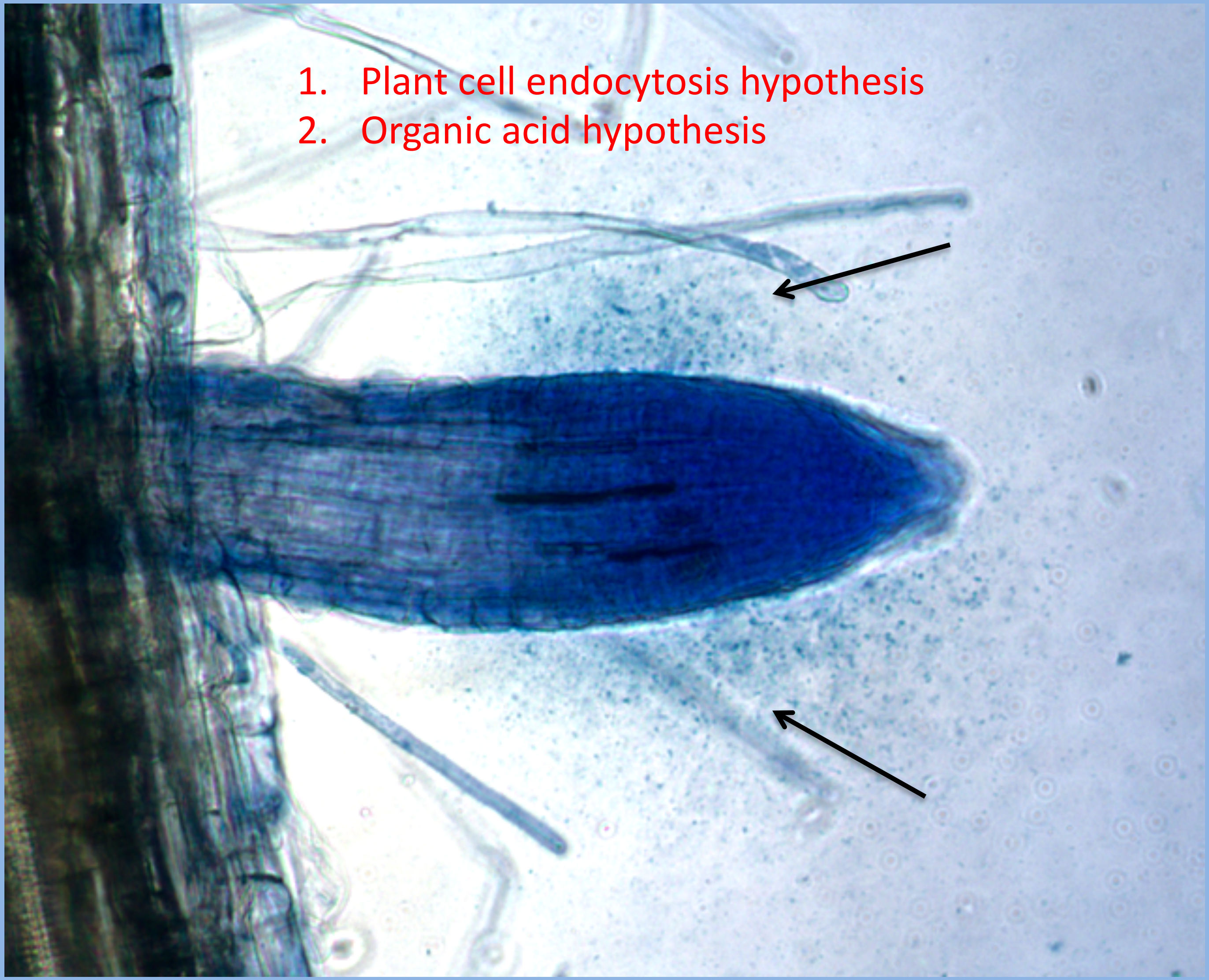


Secretion of exudates in a zone proximal to root tip meristems facilitates microbe entry into cells of the plant meristem.

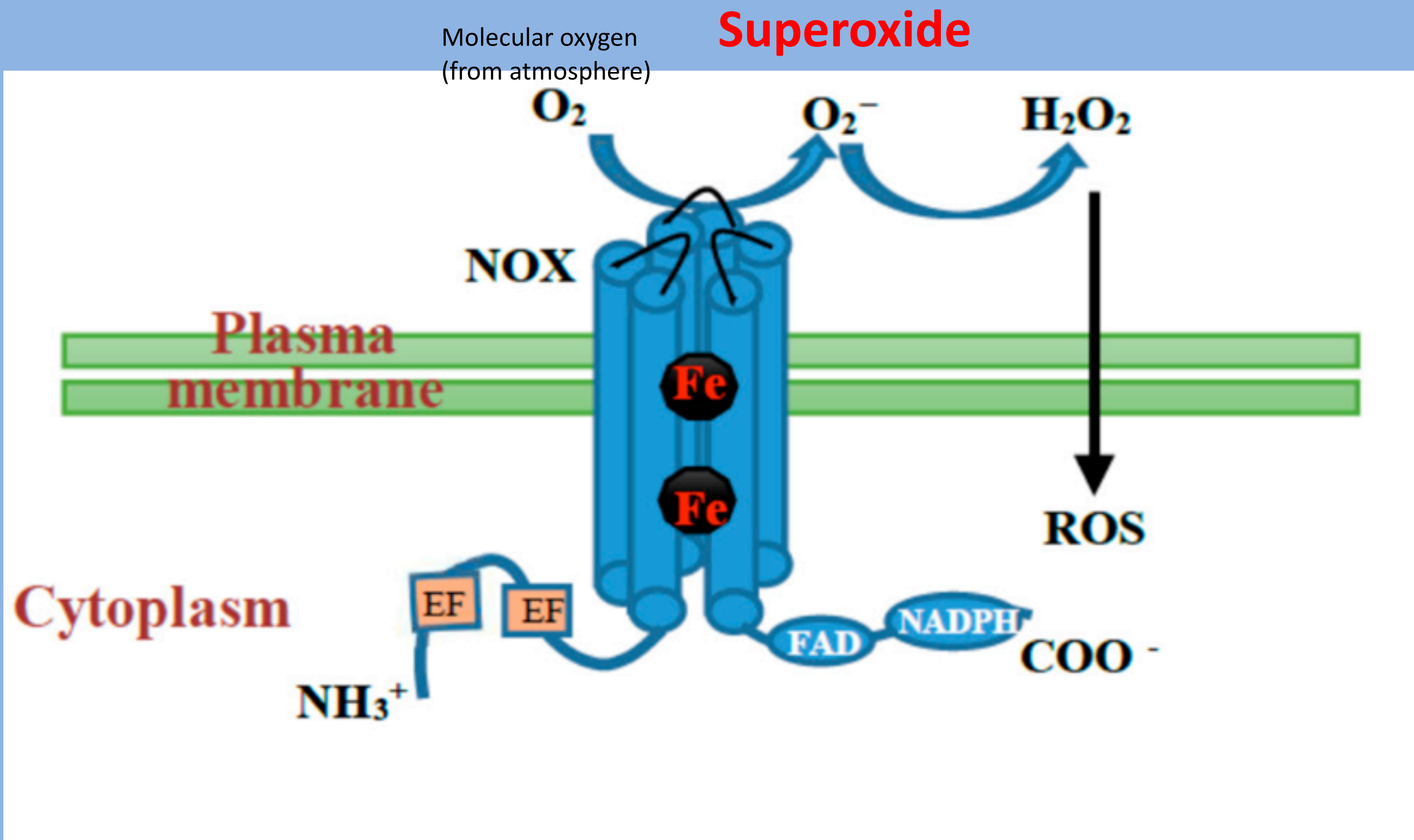
Marschner, H., 1995. Mineral Nutrition of Higher Plants, 2nd edn., Academic Press, London.

FUNCK-JENSEN, D. & HOCKENHULL, J. 1984. Root exudation, rhizosphere microorganisms and disease control. Växtskyddsnotiser 48: 3-4, 49-54.

Bacteria entering root epidermal cells in the 'zone on intracellular colonization' at the root tip meristem. A cloud of bacteria (arrows) is seen around the root tip meristem where intracellular colonization is occurring. The blue stain is aniline blue.



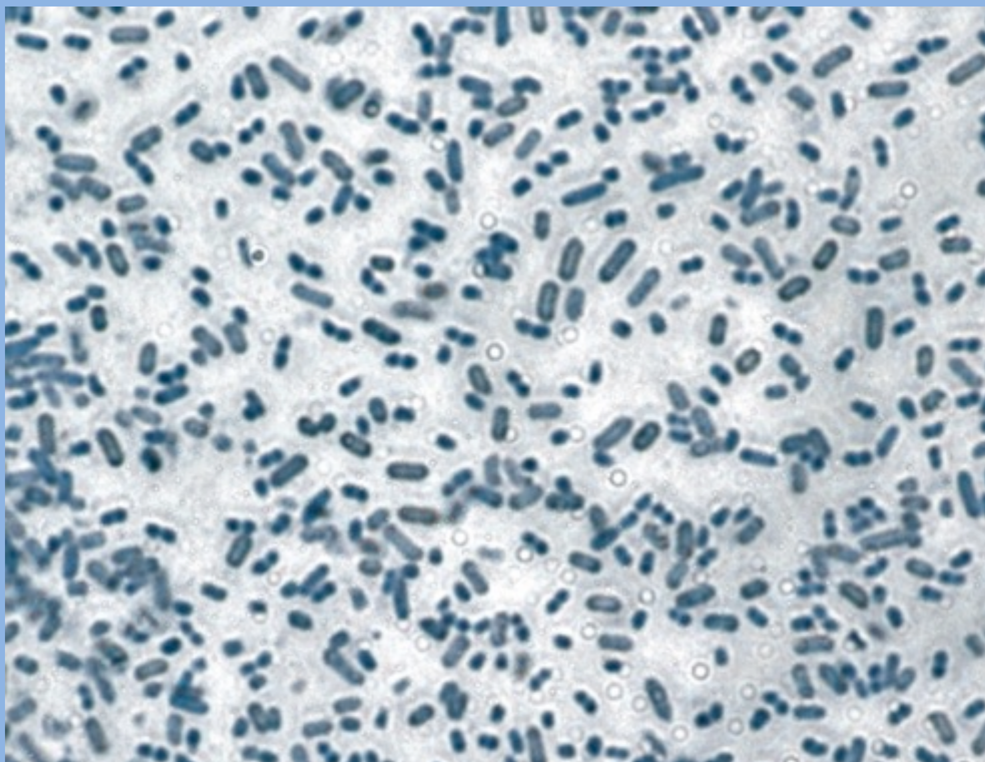
REACTIVE OXYGEN DEFENSE RESPONSE OF THE ROOT CELL INVOLVES MEMBRANE-BOUND NADPH OXIDASES (NOX)



Chemical equation: $NADPH + 2O_2 \xrightarrow{NOX} NADP^+ + 2O_2^- + 2H^+$

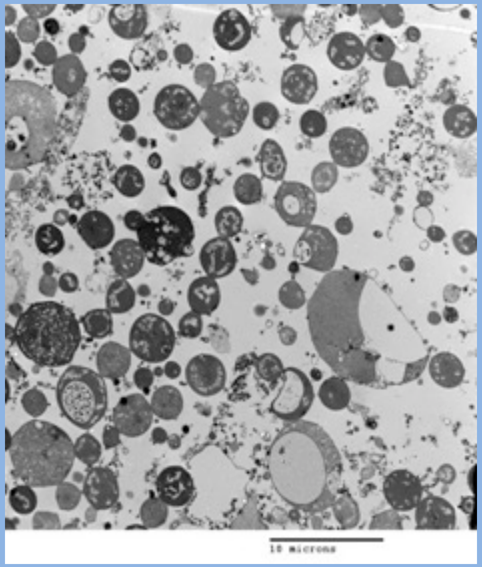
Bacterium *Bacillus subtilis*

Bacteria with cell walls (rods)



Reactive oxygen (superoxide)

Spherical bacterial protoplasts (no cell walls)

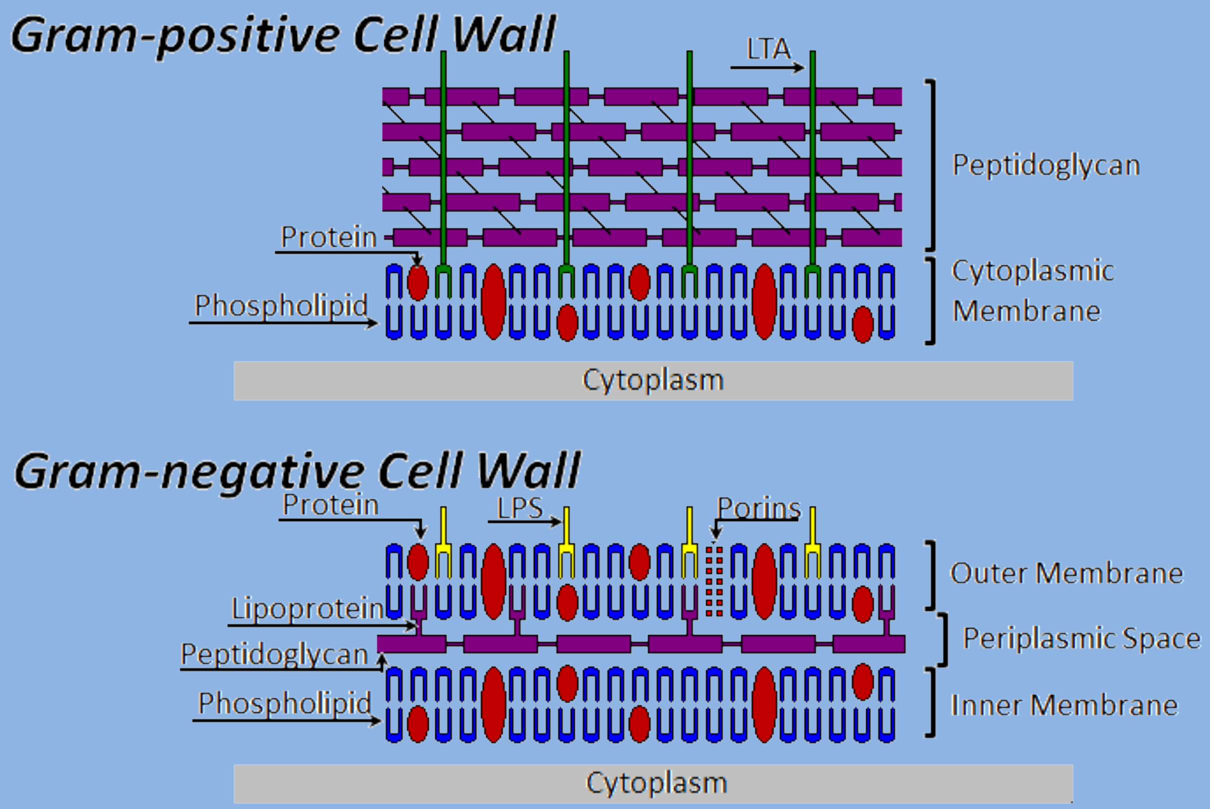


Bacterial protoplasts are called L-forms.

Inside root cells superoxide strips cell walls off of the microbes!

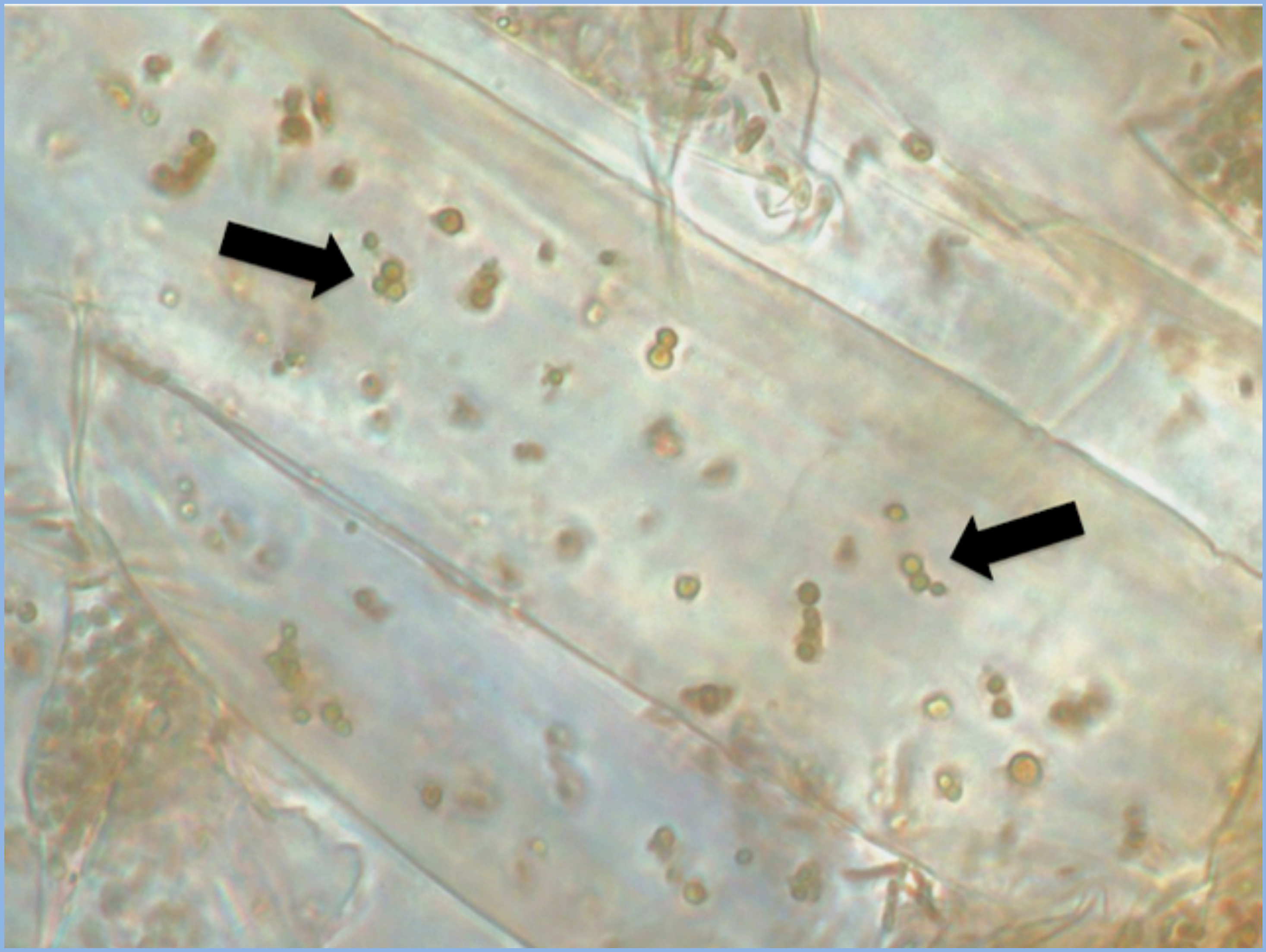
What is contained in bacterial cell walls?

Nitrogen!!!

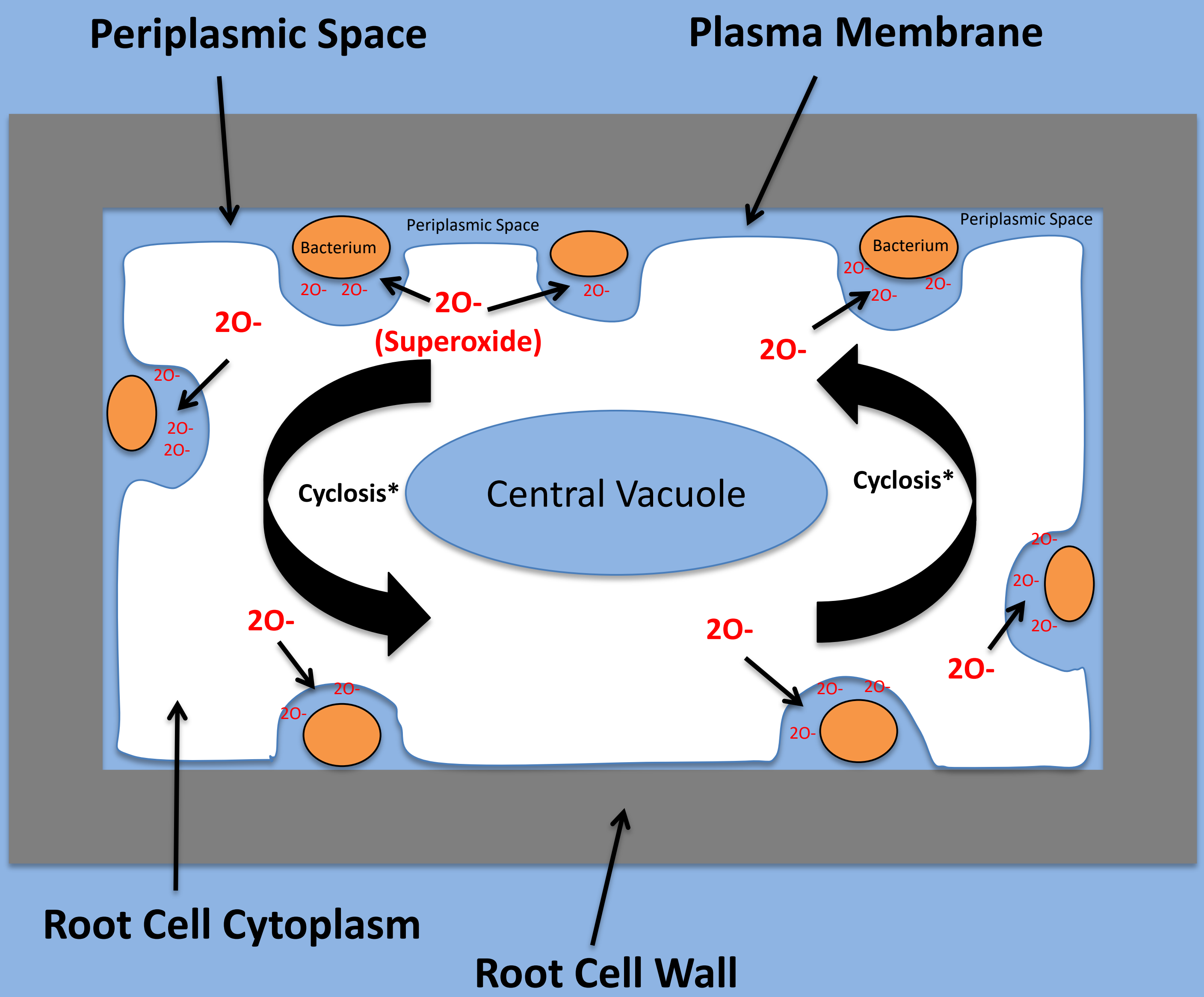


- Gram - bacteria = amino acids = alanine, glutamic acid, diaminopimelic acid
- Gram + bacteria = amino acids = alanine, glutamine, lysine, glycine

Phragmites root stained with diaminobenzidine DAB to visualize reactive oxygen around bacterial protoplasts (arrows). Reactive oxygen is visualizable as brown or red coloration around bacteria. The reactive oxygen is the result of superoxide produced by NADPH oxidases on the root cell plasma membranes. The reactive oxygen extracts nutrients from the bacteria (mostly pseudomonads) that are symbiotic with *Phragmites*.

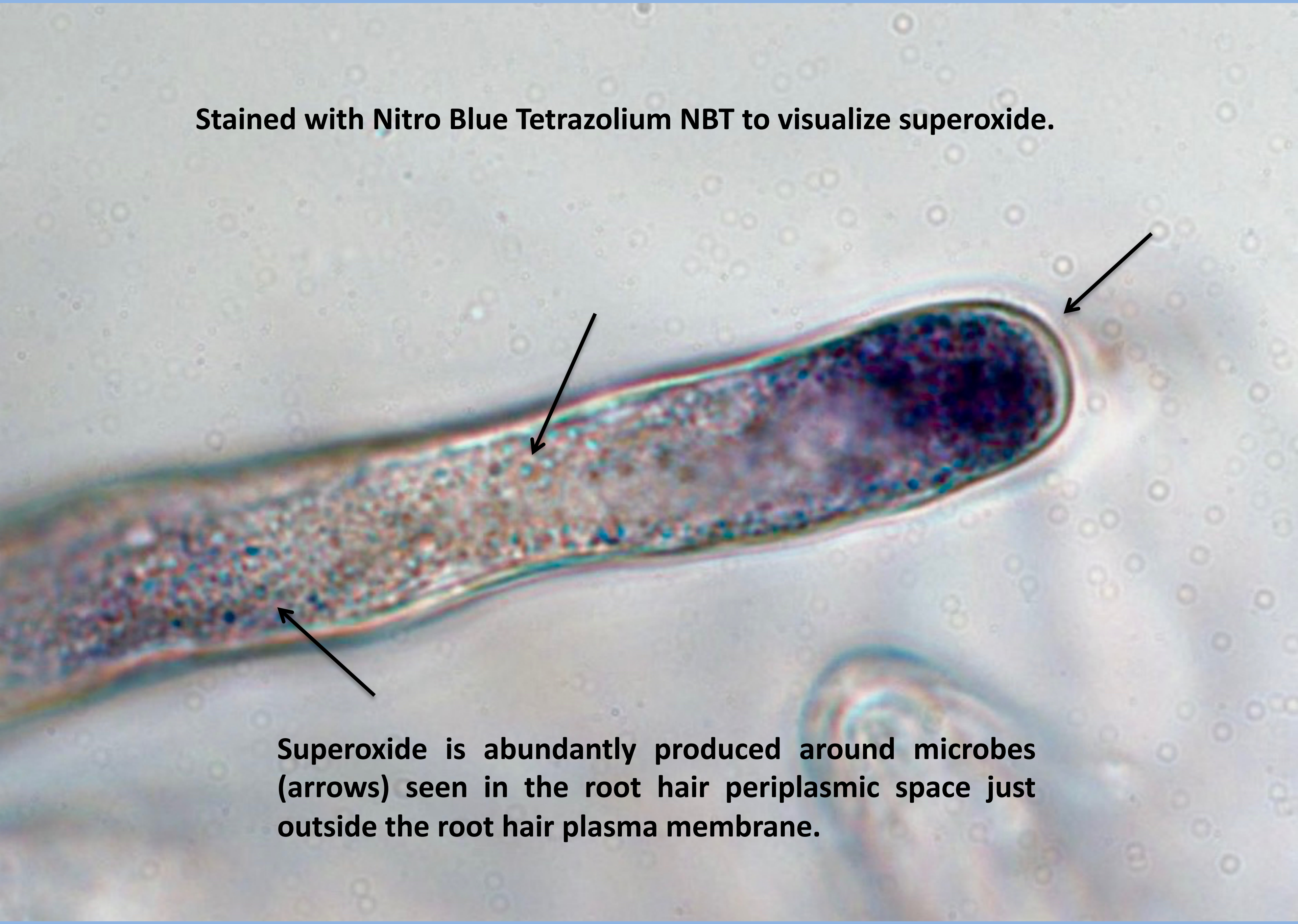


Bacterial Protoplasts in Periplasmic Space are Subjected to Host-Produced Superoxide.



*Cyclosis = Cytoplasmic Movement

Stained with Nitro Blue Tetrazolium NBT to visualize superoxide.

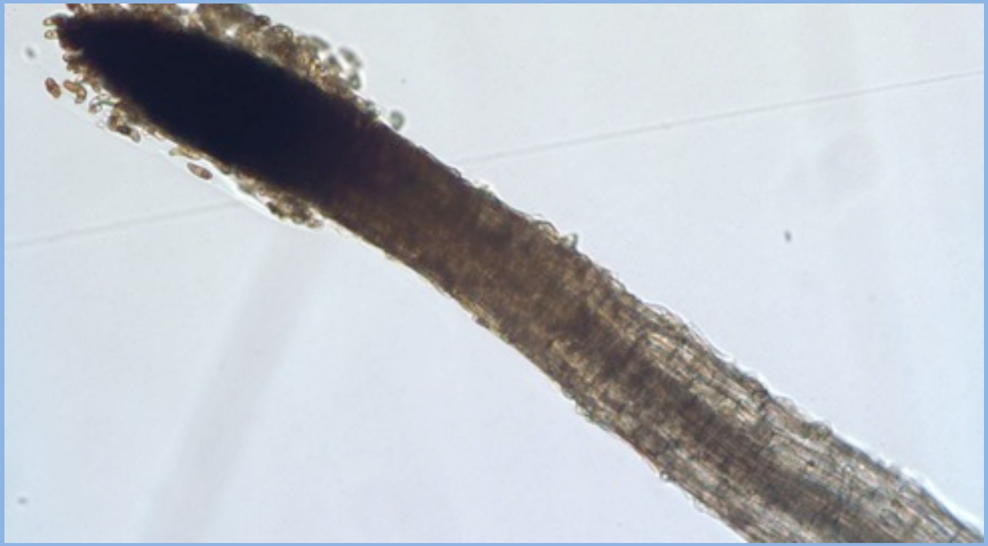


Superoxide is abundantly produced around microbes (arrows) seen in the root hair periplasmic space just outside the root hair plasma membrane.

Rhizophagy cycle microbes modulate development of seedlings

- Microbes trigger the gravitropic response in roots
- Microbes trigger root hair elongation
- Microbes increase root branching
- Microbes increase root and shoot elongation

Bermuda grass seedling root in agarose without microbes showing absence of root hairs



Root tip

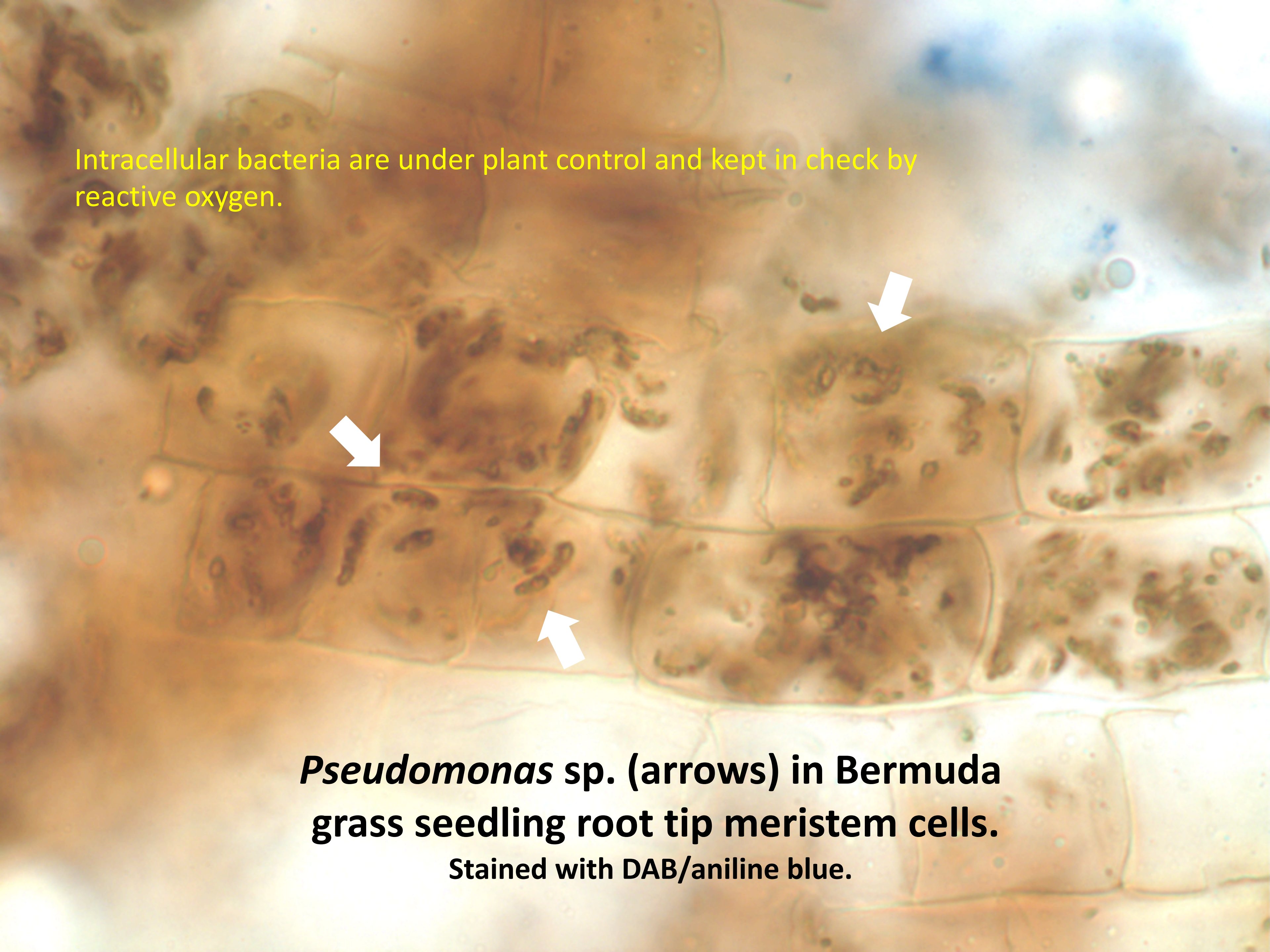
More developed region of seedling root

Bermuda grass root containing *Pseudomonas* (bacterium)

Proposed route of endophyte colonization of root and reentry to rhizosphere from root hairs

RHIZOPHAGY CYCLE

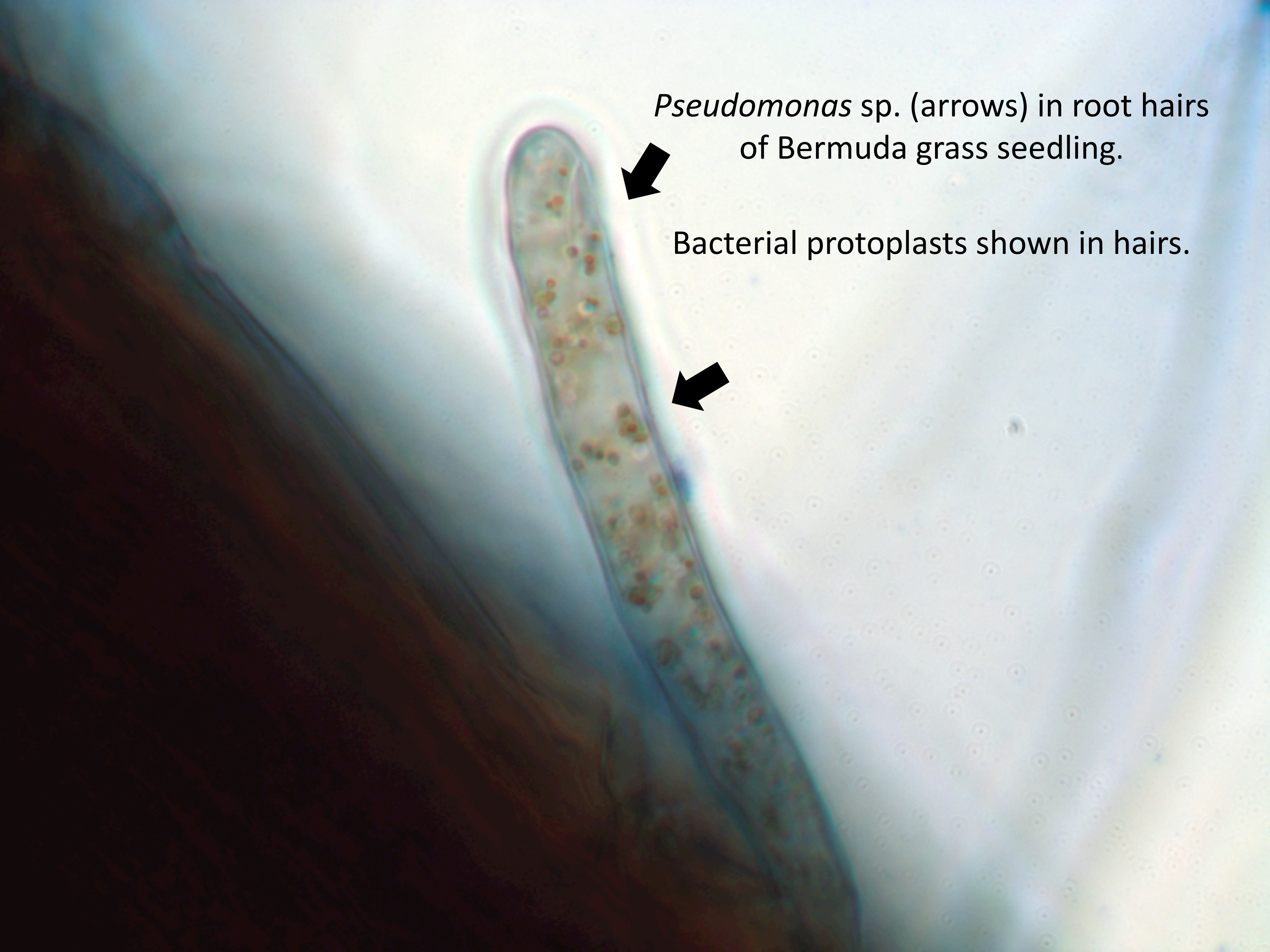
Bacteria (from seed coat) → Colonize root tip meristem (enter cells) → Intracellular in root parenchyma → Bacteria stimulate root hair formation In root epidermis → Bacteria emerge to surface of hair as the hair elongates → Bacteria colonize soil rhizosphere → Bacteria acquire nutrients in rhizosphere → Colonize root tip meristem (enter cells)



***Pseudomonas* sp. (arrows) in Bermuda grass seedling root tip meristem cells.**

Stained with DAB/aniline blue.


Bermuda grass seedling root containing *Pseudomonas* endophyte.
All brown spots in roots are intracellular bacteria.

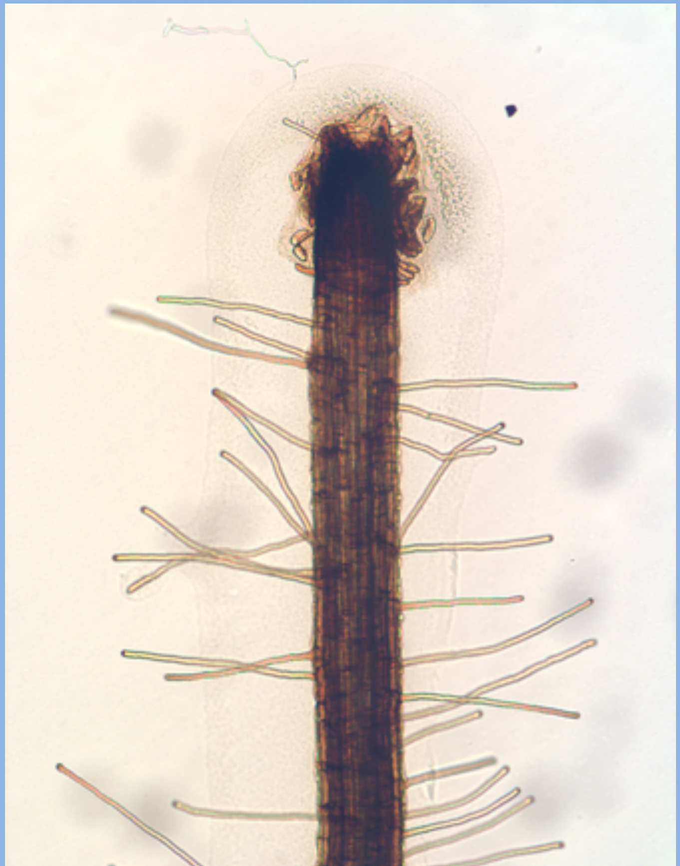

Pseudomonas sp. (arrows) in root hairs of Bermuda grass seedling.
Bacterial protoplasts shown in hairs.

What is the function of root hairs?

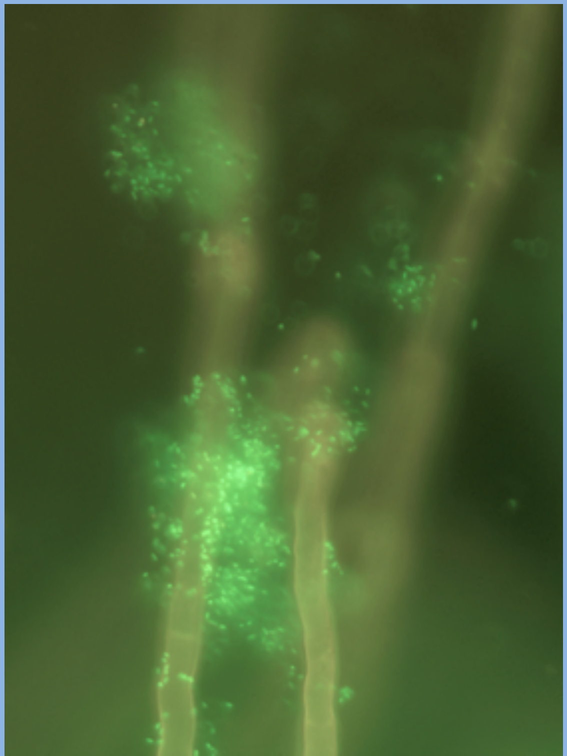
James White 11/5/2017
Rutgers University

Root hairs function to eject rhizophagy microbes out into the soil where they may acquire nutrients.

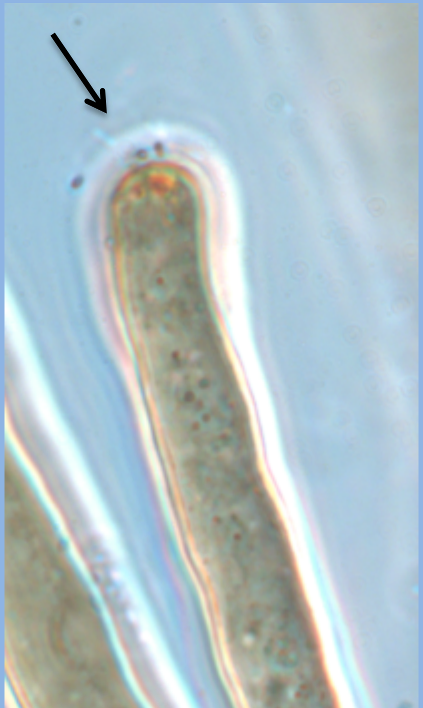
Root growing in agarose showing extension of root hairs beyond the rhizoplane and the bacterial biofilm on the rhizoplane.



Bacteria emerging from tips of elongating root hairs. Stained with nuclear stain Syto 13.

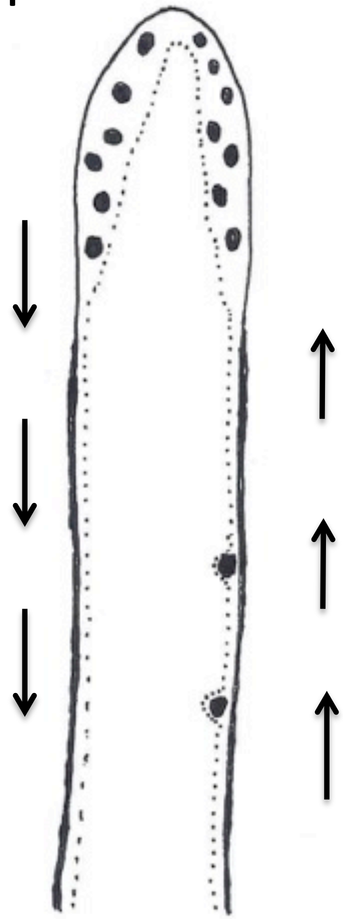


Bacteria emerging from root hair tip. Bacteria in hairs are present as wall-less L-forms. Bacteria reform their walls after exiting from the tip of the hair.

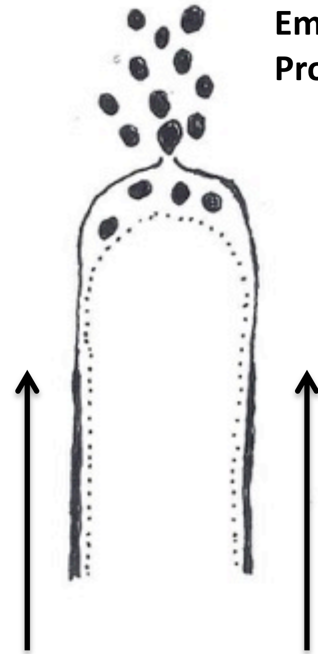


Cyclosis/Expansion Wave Mechanism for Microbe Expulsion from Root Hairs in Rhizophagy Cycle

1. Cyclosis moves microbes to tip and facilitates replication of microbe protoplasts.



Emerging Microbe Protoplasts



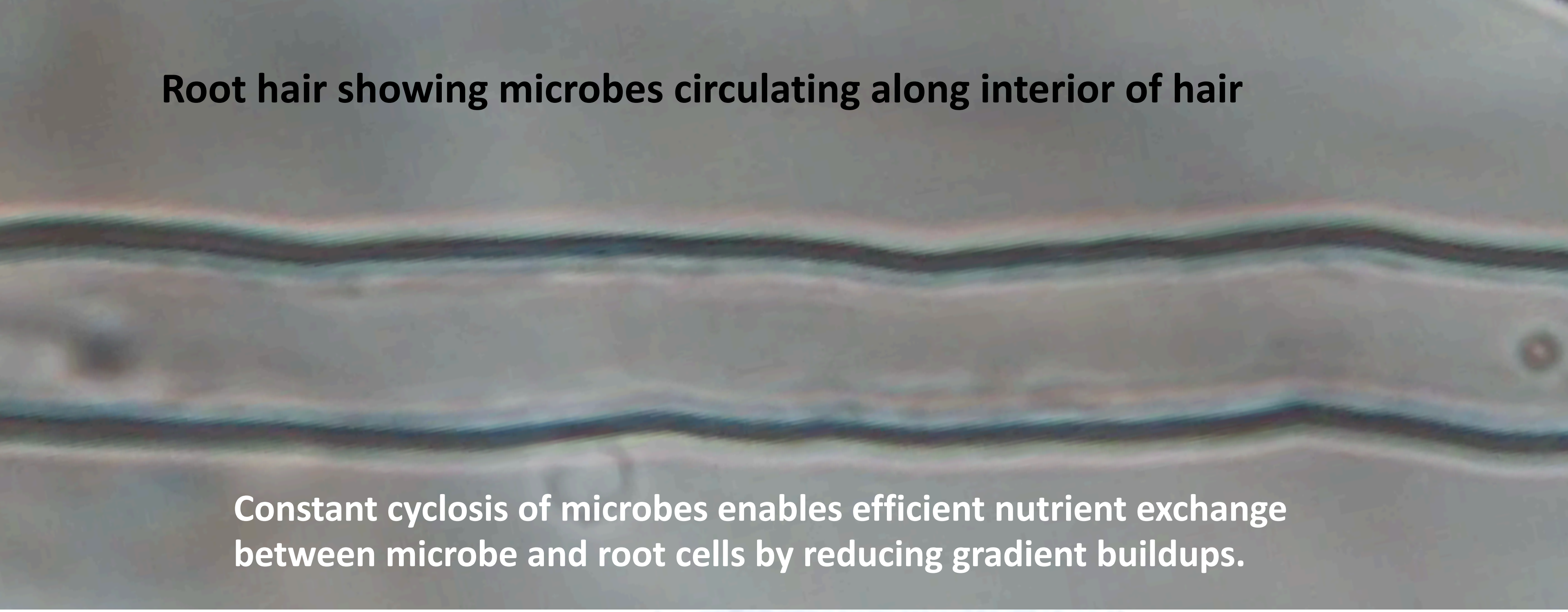
Expansion Wave

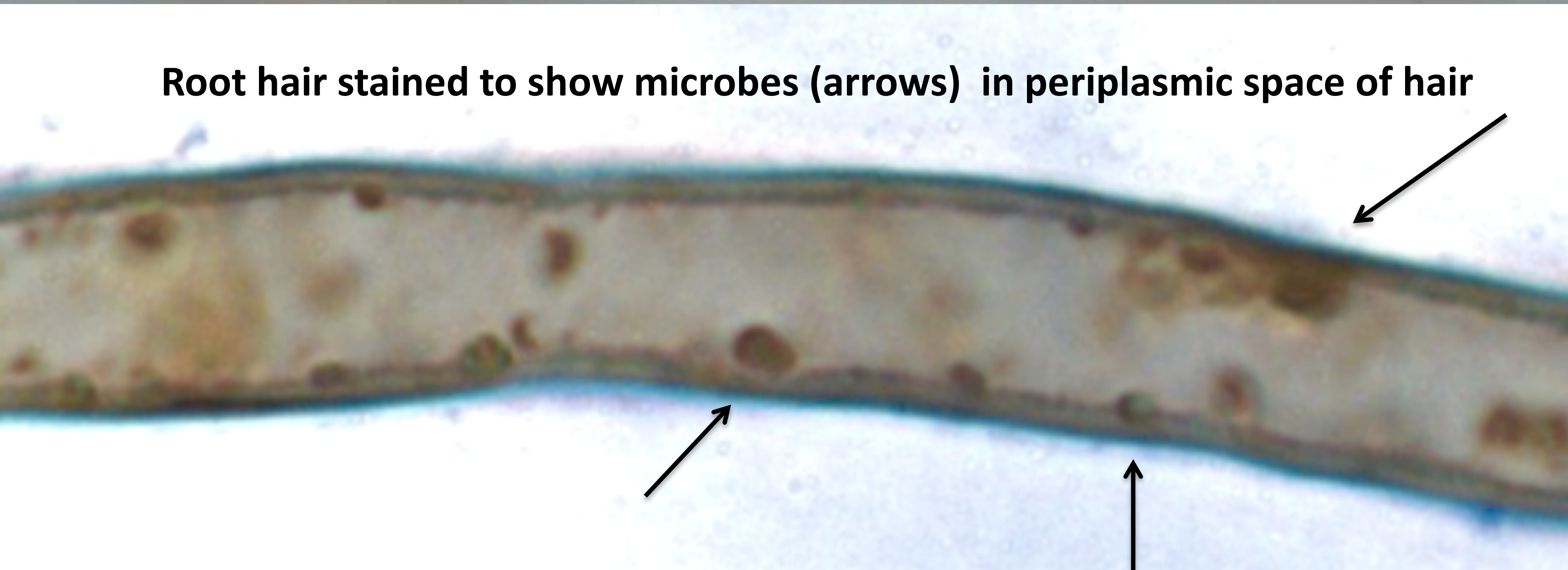
2. A wave of expansion in the hair protoplast begins in the base of the hair and progresses to the tip of the hair. This expansion wave forces microbe protoplasts through pores that form in the thin wall at the hair tip.

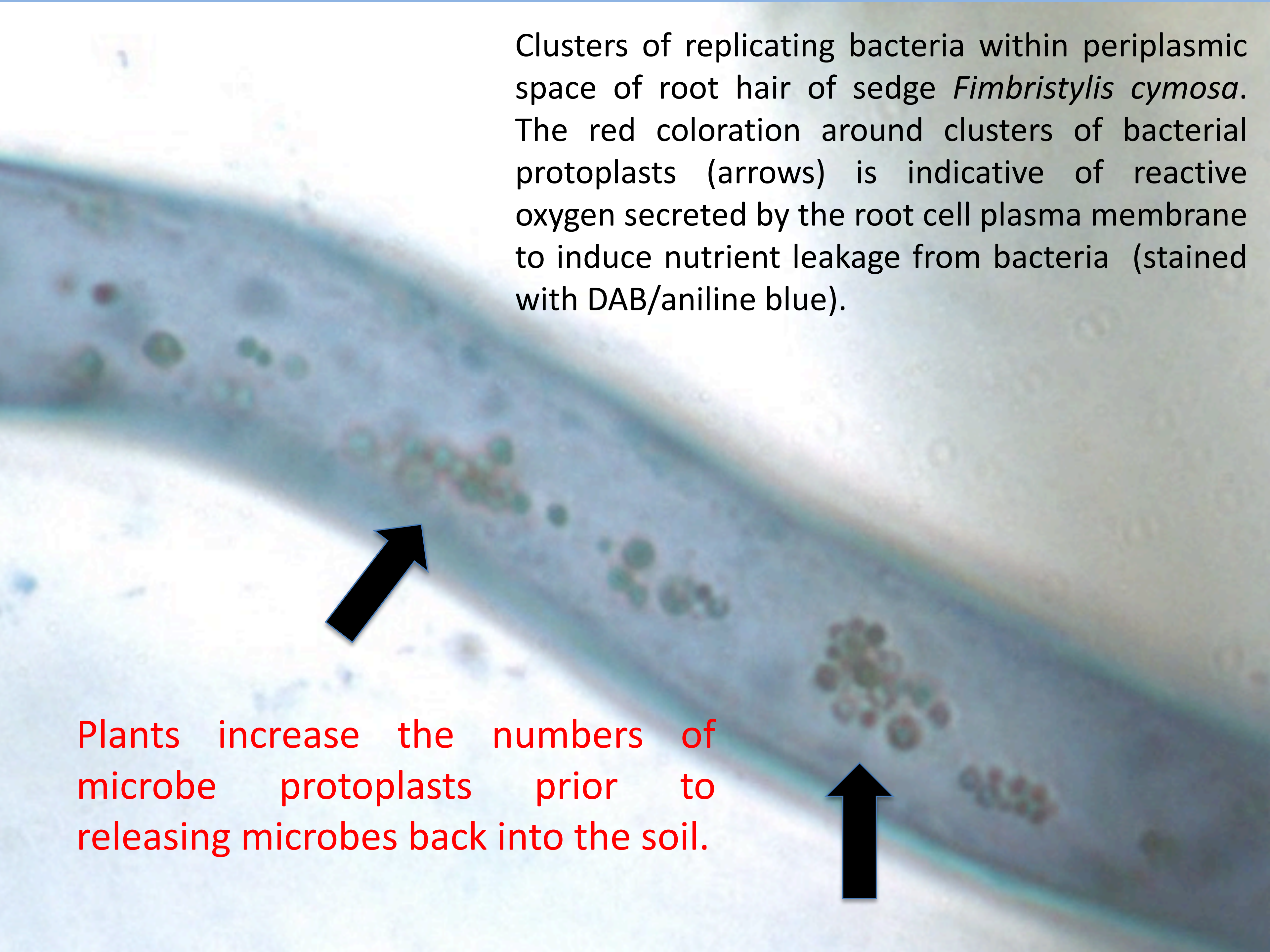
James White
Rutgers University
Feb. 23, 2019

Sedge (*Fimbristylis cymosa*)

Plant grows in pits and crevices of limestone or in sand along high salt Caribbean shore environments.


Root hair showing microbes circulating along interior of hair
Constant cyclosis of microbes enables efficient nutrient exchange between microbe and root cells by reducing gradient buildups.


Root hair stained to show microbes (arrows) in periplasmic space of hair



Clusters of replicating bacteria within periplasmic space of root hair of sedge *Fimbristylis cymosa*. The red coloration around clusters of bacterial protoplasts (arrows) is indicative of reactive oxygen secreted by the root cell plasma membrane to induce nutrient leakage from bacteria (stained with DAB/aniline blue).

Plants increase the numbers of microbe protoplasts prior to releasing microbes back into the soil.

Root hair of sedge *Fimbristylis cymosa*

Cyclosis was measured to move microbes at a rate of 8-11 micrometers/second in root hairs of the sedge *Fimbristylis cymosa*.

Microbes accumulating in hair tip.

Microbes circulating along length of root hair.

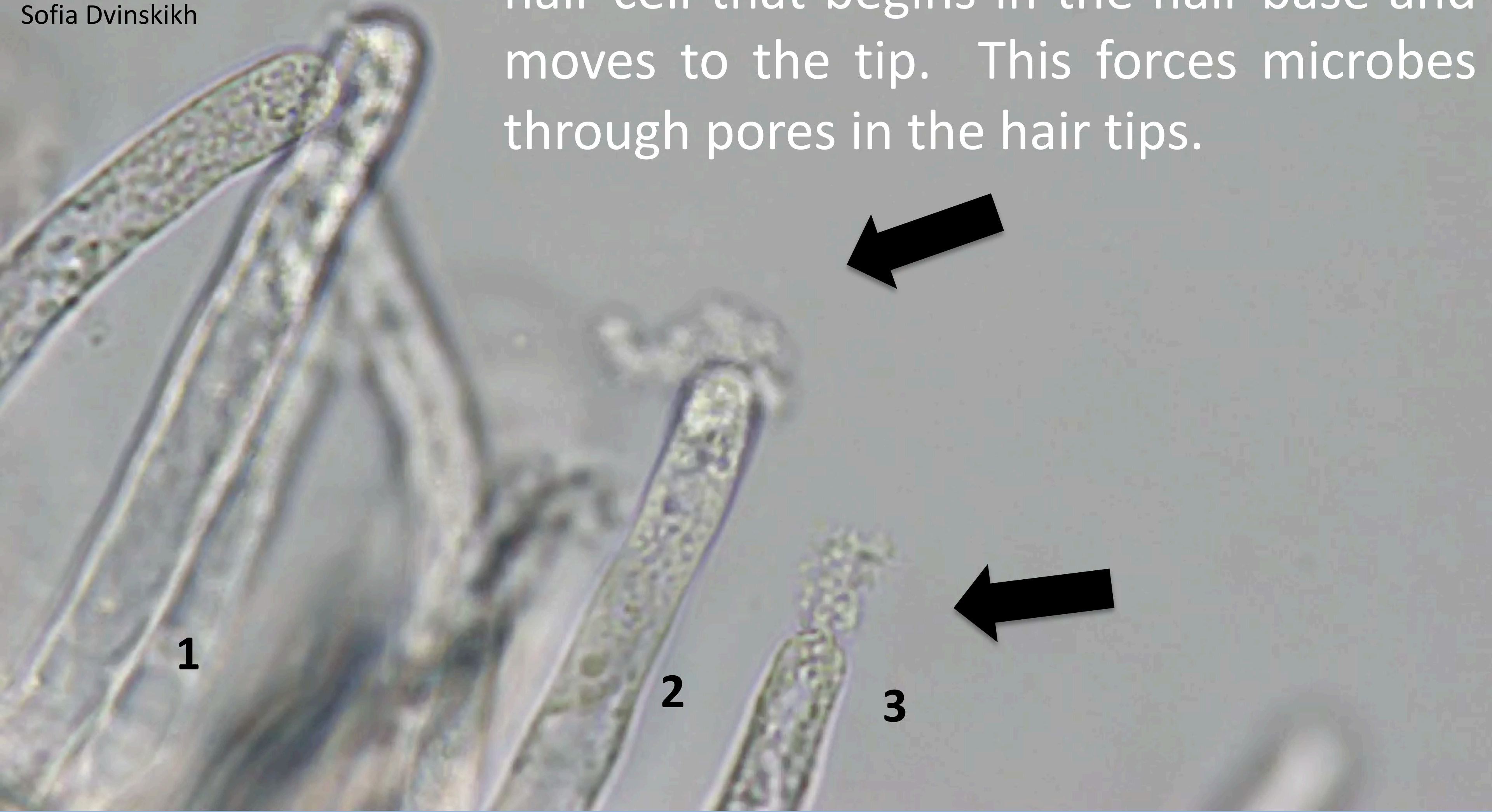
This constant circulation may be a way to induce replication in the microbe protoplasts.

Qiang Chen

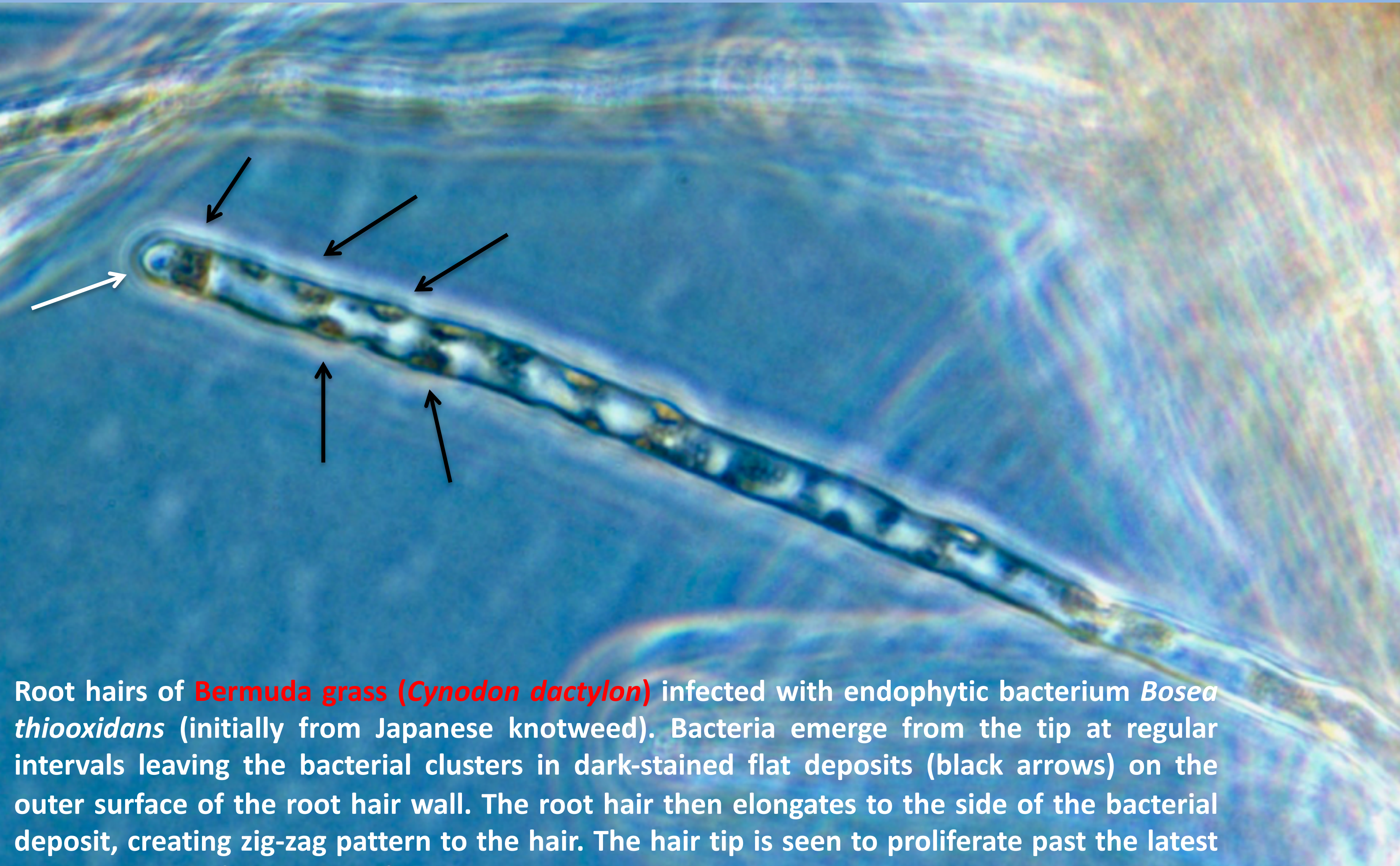
Root hair of sedge (*Fimbristylis cymosa*) showing expulsion of bacteria (large arrow) from the soft-walled hair tip. Red-staining bacterial protoplasts are seen in root hair. A wave of expansion of the hair protoplast propagates from base to tip of hair and this wave forces microbes through pores that form in the hair tip.

Sofia Dvinskikh

This ejection of microbes (arrows) occurs rapidly with a wave of expansion in the hair cell that begins in the hair base and moves to the tip. This forces microbes through pores in the hair tips.

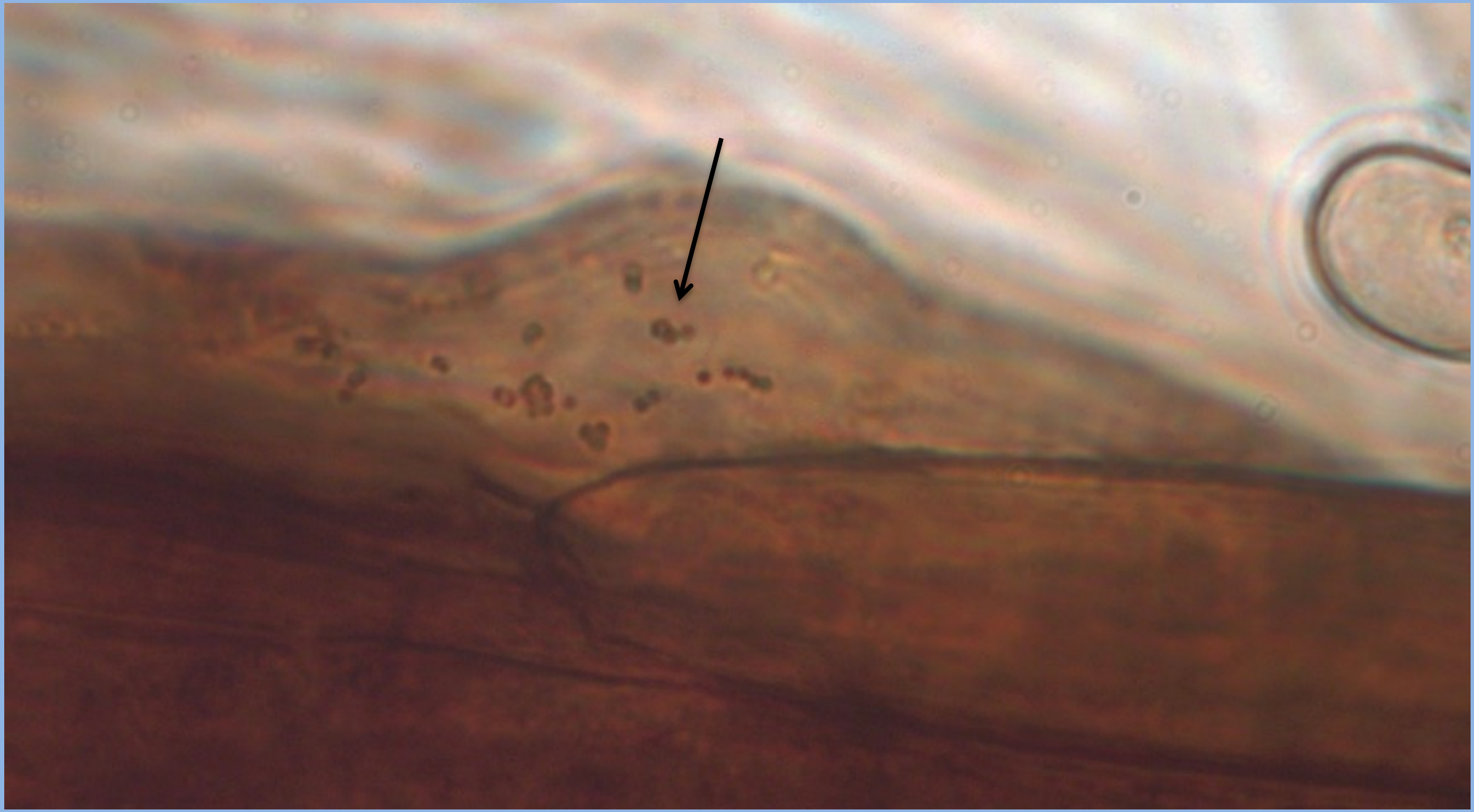


Microbe ejection appears to be periodic rather than continuous. Microbes may be ejected in clusters rather than 1 at a time. It is unknown what causes the periodicity.

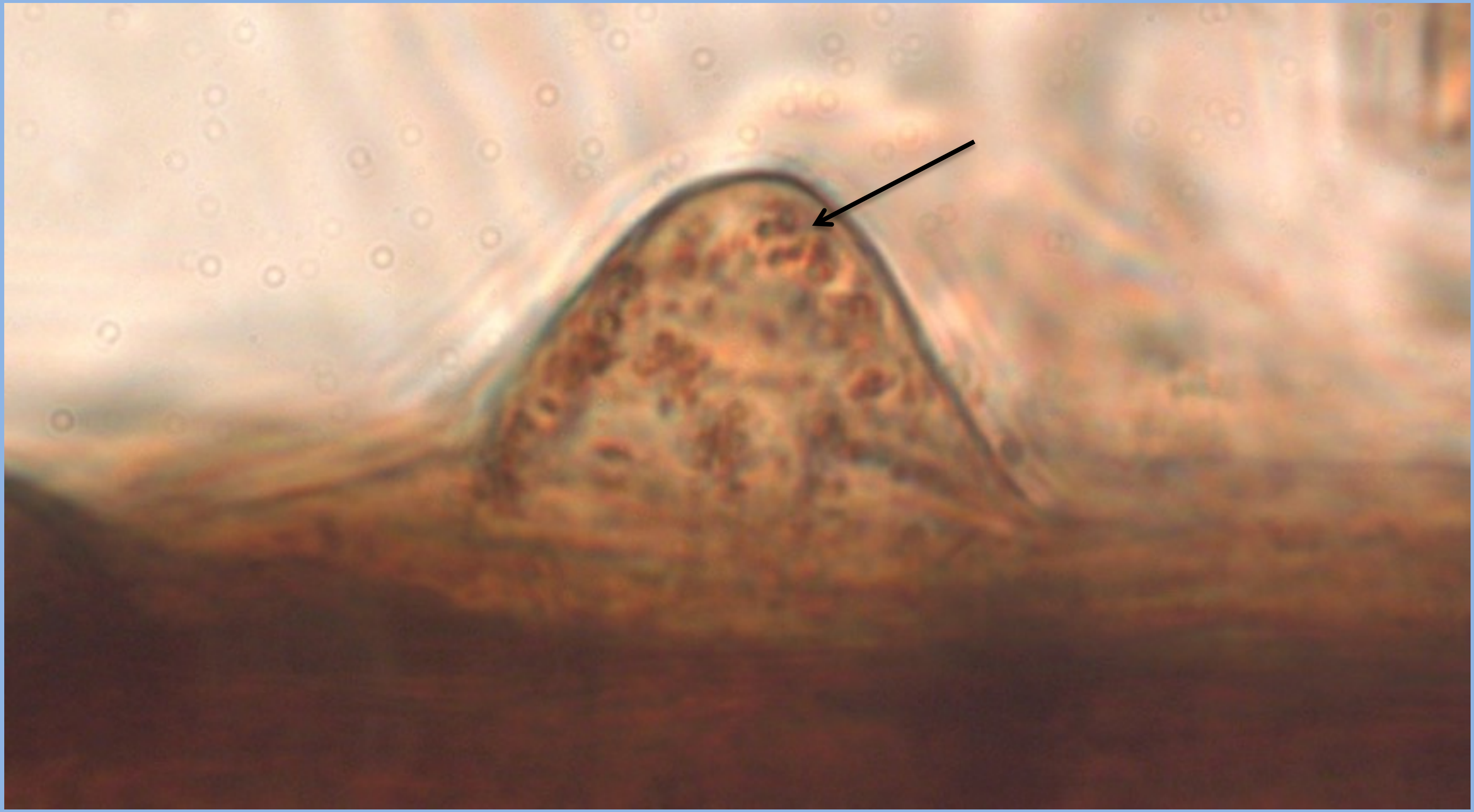


Root hairs of Bermuda grass (*Cynodon dactylon*) infected with endophytic bacterium *Bosea thiooxidans* (initially from Japanese knotweed). Bacteria emerge from the tip at regular intervals leaving the bacterial clusters in dark-stained flat deposits (black arrows) on the outer surface of the root hair wall. The root hair then elongates to the side of the bacterial deposit, creating zig-zag pattern to the hair. The hair tip is seen to proliferate past the latest

Root hair Initials of tomato seedling showing internal bacteria (arrow). The papillae accumulate microbes that continue to replicate. This papilla permits microbes to immobilize and accumulate.

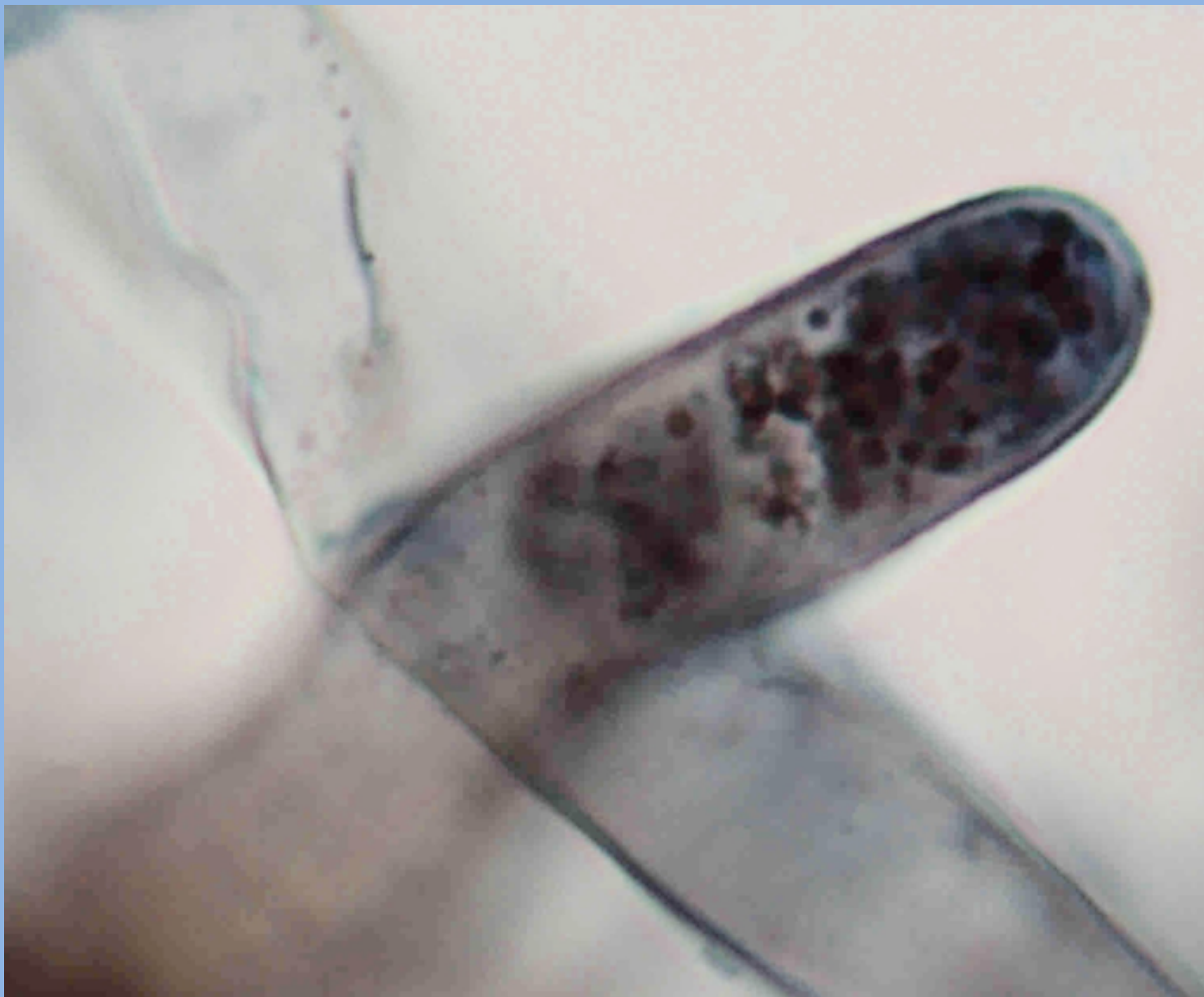


Tomato seedling hair initial with internal replicating bacteria (arrow).



Bacteria (arrow) in tip of young root hair of tomato seedling. Brown color shows where reactive oxygen is concentrating in the cell.

Bacterial concentration in root hairs appears to increase as hairs elongate. This image is of a tomato seedling root hair (stained with DAB/aniline blue).

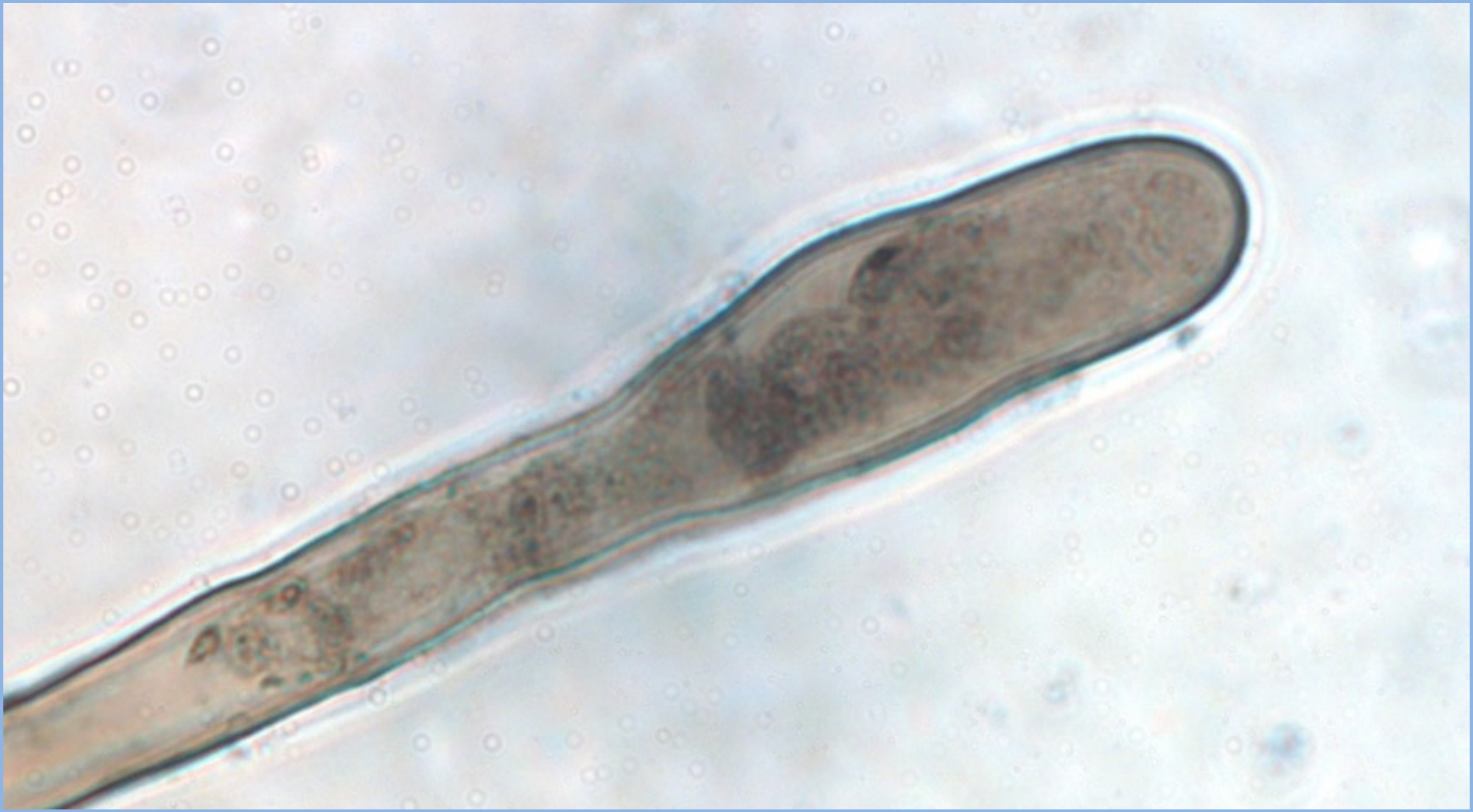


Secretion of reactive oxygen around bacteria at tip (brown color) likely causes the microbe and the root hair to produce nitric oxide to reduce oxidative damage. This may result in continued elongation at the hair tip.

The elongation continues until all microbes have been ejected from the hair and no more reactive oxygen is produced at the hair tip.

Tomato seedling root hairs containing bacteria showing intense hydrogen peroxide staining (brown) around bacteria. Often root hair tips with internal bacteria appear swollen. This shows the elasticity of the wall at the hair tip. Microbes continue to replicate in the hair.

Tomato seedling root hair showing swollen tip containing microbes.



What stimulates the plant root hairs to elongate?

Nitric Oxide/Ethylene Signaling Hypothesis

Arginine
(Produced by microbe)

Microbial
Ethylene Forming
Enzyme

Ethylene → Root Hair Elongates

(Produced by microbes
Accumulating in hair tip)

Microbial
Nitric Oxide + O_2
Synthase

Nitric Oxide → Root Hair Elongates

(Produced by microbes
Accumulating in hair tip)

Nitric oxide protects microbe from oxidation by inhibiting destructive hydroxyls by scavenging iron and preventing the Fenton reaction.

Sharpe et al., 2003. Nitric oxide and Feton/Haber-Weiss chemistry: nitric oxide is a potent antioxidant at physiological concentartions. J. Neurochem. 87: 386-394.

Lombardo, M. C., Graziano, M., Polacco, J. C., & Lamattina, L. (2006). Nitric oxide functions as a positive regulator of root hair development. *Plant signaling & behavior*, *1*(1), 28-33.

Gusarov I, Shatalin K, Starodubtseva M, Nudler E. Endogenous nitric oxide protects bacteria against a wide spectrum of antibiotics. Science. 2009;325:1380–1384.

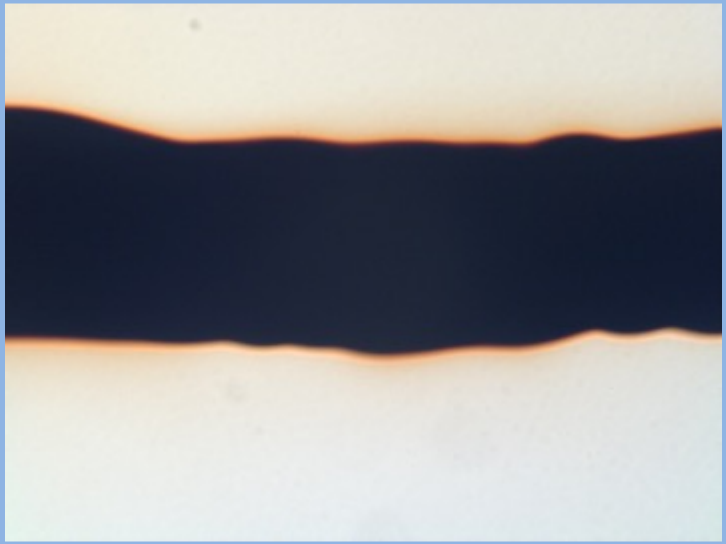
How do the microbes inside root hairs trigger root hair elongation?

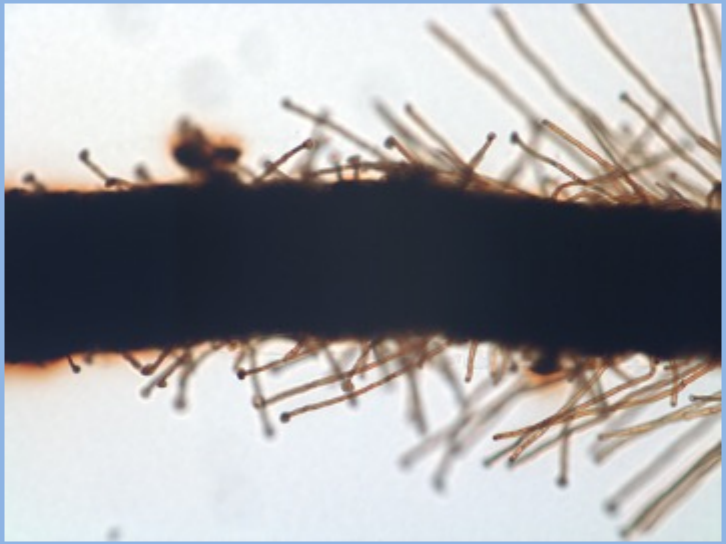
Nitric Oxide Hypothesis

Microbes in the tips of hairs secrete nitric oxide that acts as a hormone and stimulates elongation at the tip of the root hair.

Xiaoqian (Ivy) Chang

Experiments to test the 'Nitric Oxide Hypothesis'





No microbes in seedlings

(Seeds disinfected rigorously.)

Bacterium present

(Pseudomonads fluorescens inoculated onto disinfected seeds.)

Nitric oxide probe: DAF-FM diacetate

DAF-FM Diacetate (4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate)

Where nitric oxide is present, fluorescence will occur.

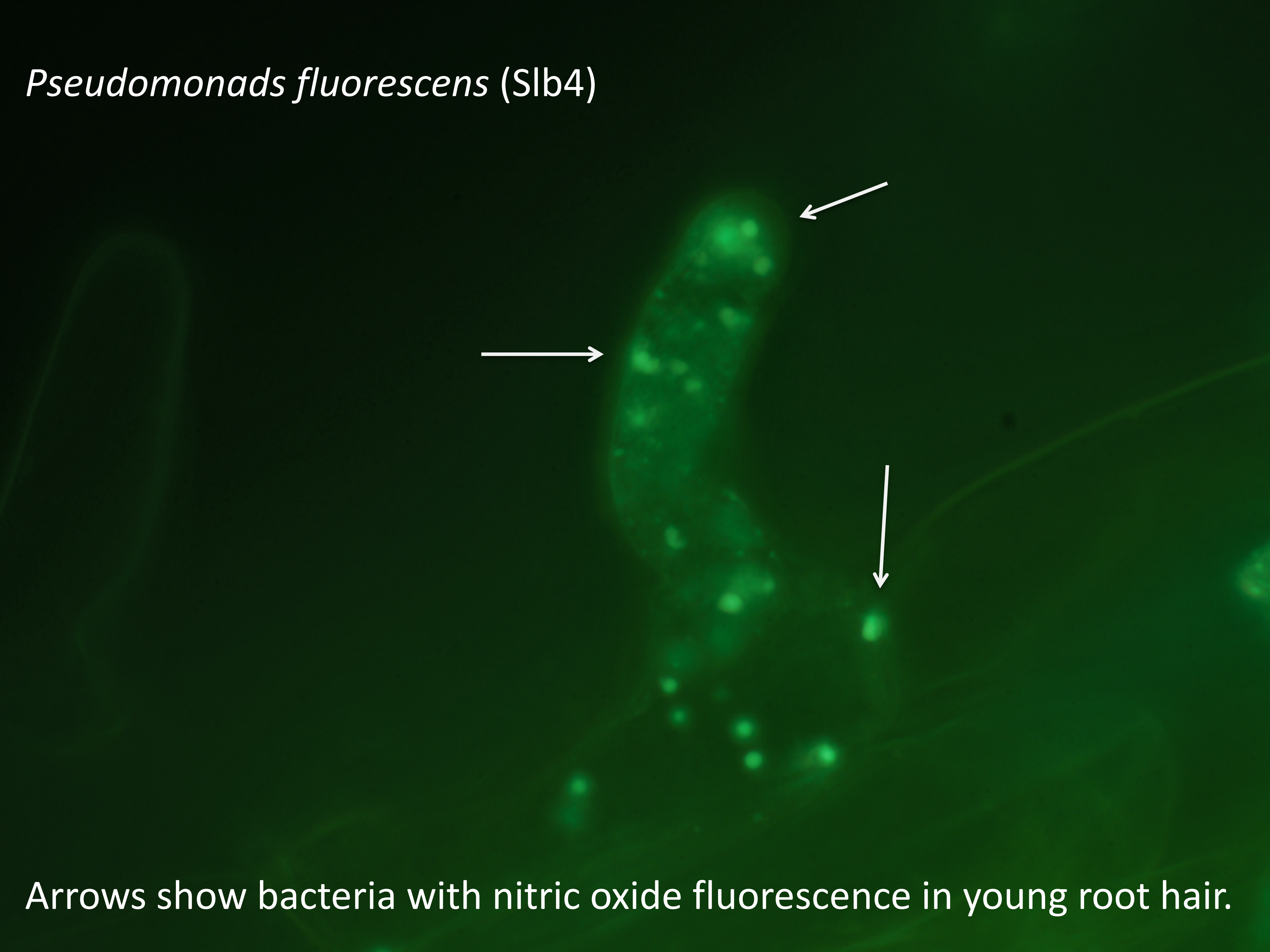
Methods:

Roots placed in a PCR tube containing 1ml of loading buffer (10mM Tris/KCl, pH 7.2) and DAF-2DA at a final concentration of 10uM (added from a 5mM stock in high-quality anhydrous DMSO). Maintained in the dark for 10 min. The roots were then removed and transferred to a dish of fresh loading buffer (without probe) to wash off excess fluorophore. After 10-20 min, roots were removed and placed on a slide with buffer. Slides were examined by fluorescence microscopy.

Results:

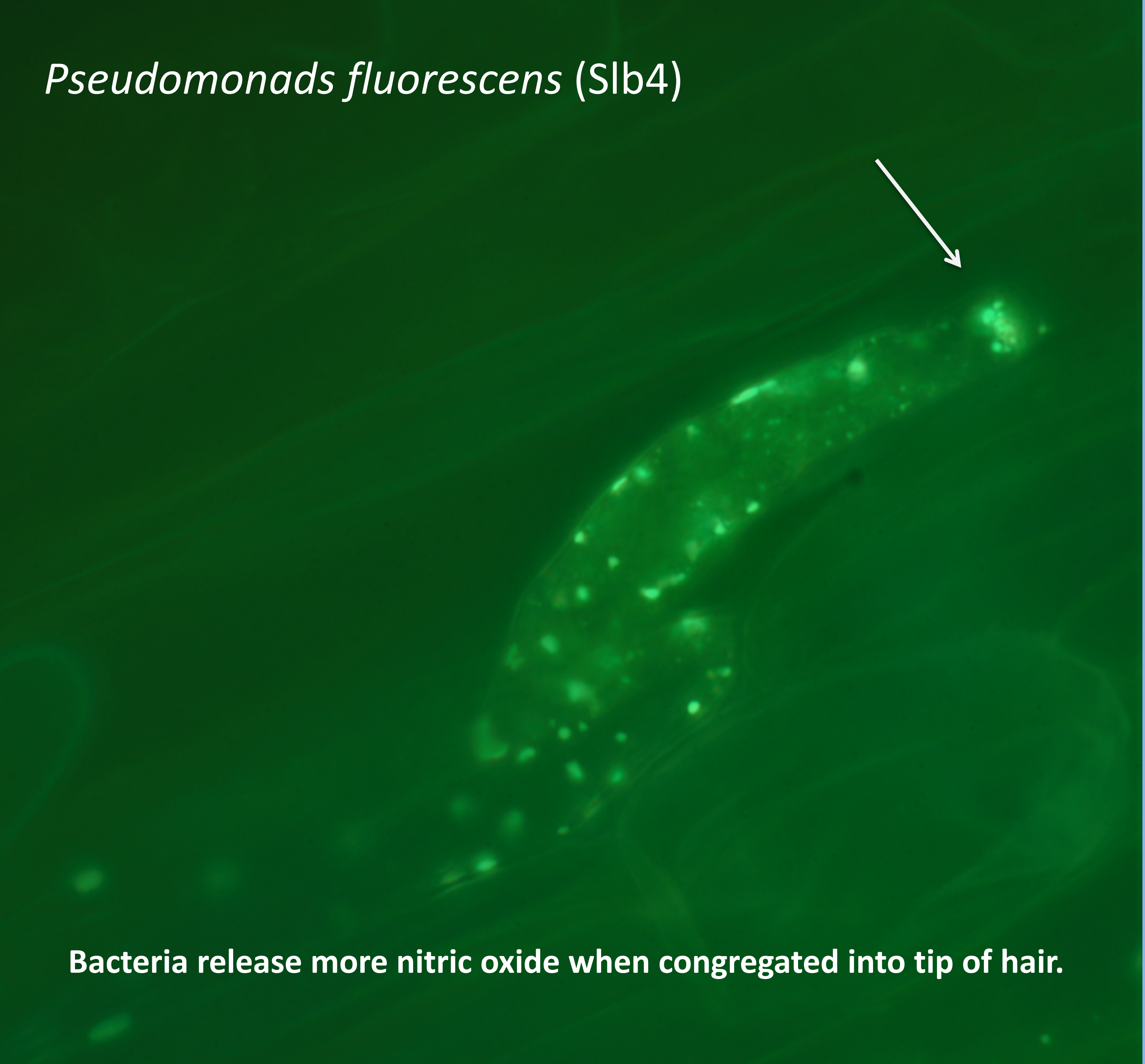
Both Gram-negative *Pseudomonads fluorescens* (Slb4) and Gram-positive *Bacillus amyloliquefaciens* (M4) showed strong fluorescence, suggesting that bacterial endophytes produce nitric oxide.

Pseudomonads fluorescens (Slb4)

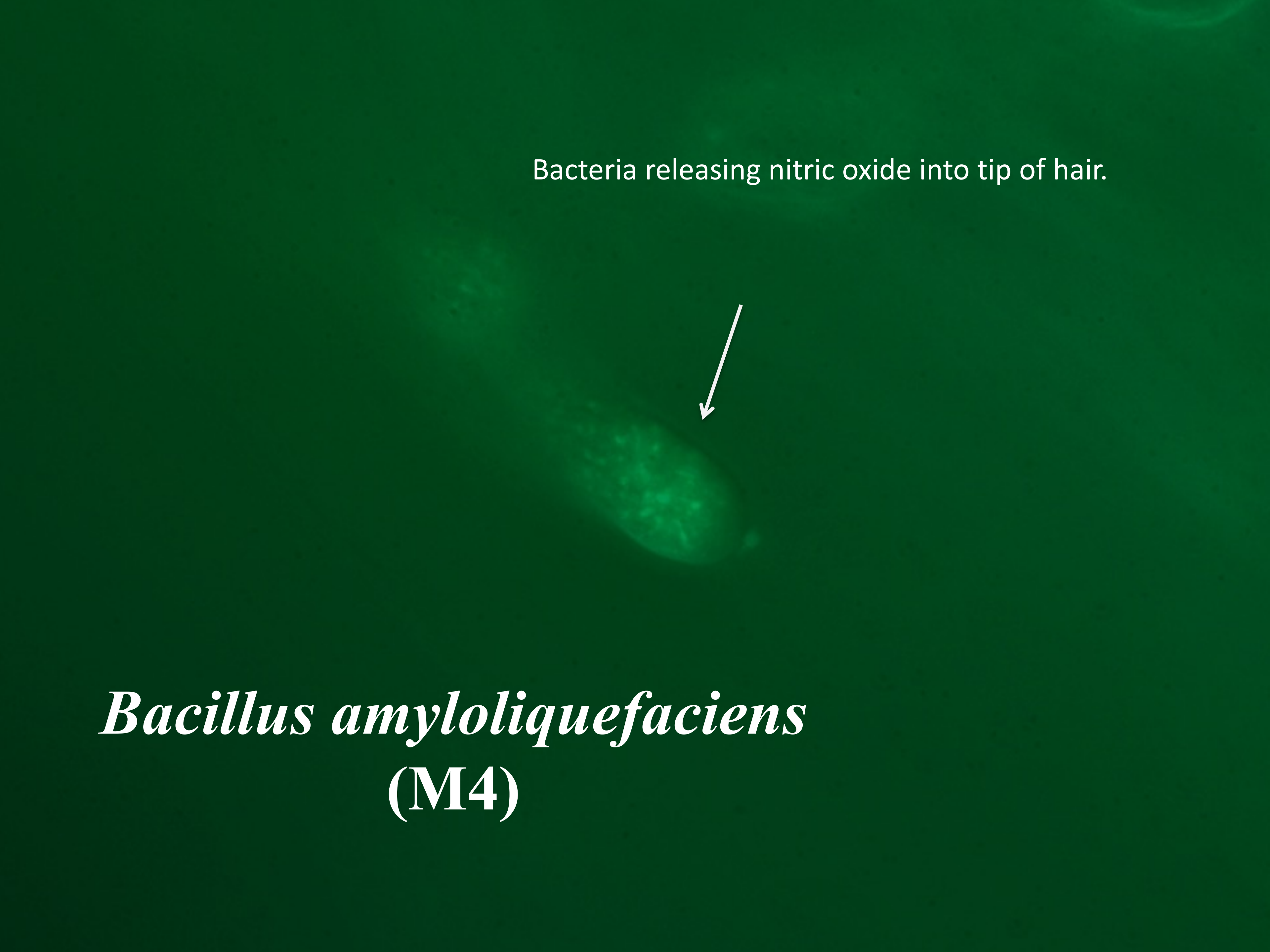


Arrows show bacteria with nitric oxide fluorescence in young root hair.

Pseudomonads fluorescens (Slb4)



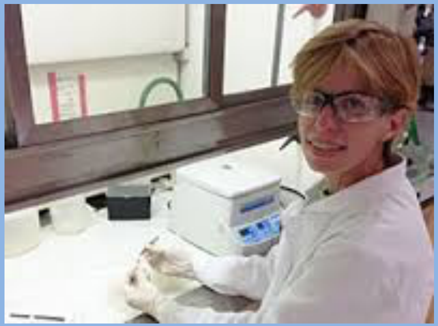
Bacteria release more nitric oxide when congregated into tip of hair.



Bacillus amyloliquefaciens
(M4)

What happens to plants without the rhizophagy cycle?

Satish K. Verma

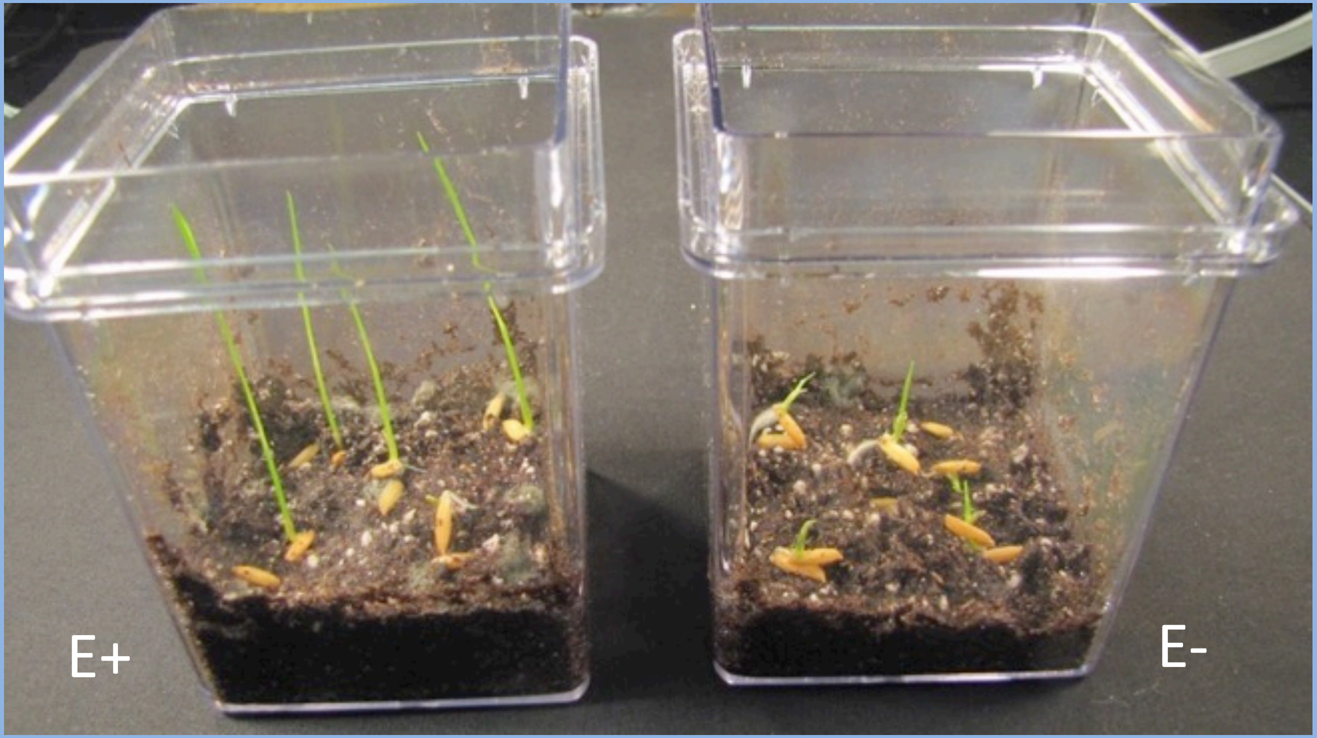


Kate Kingsley

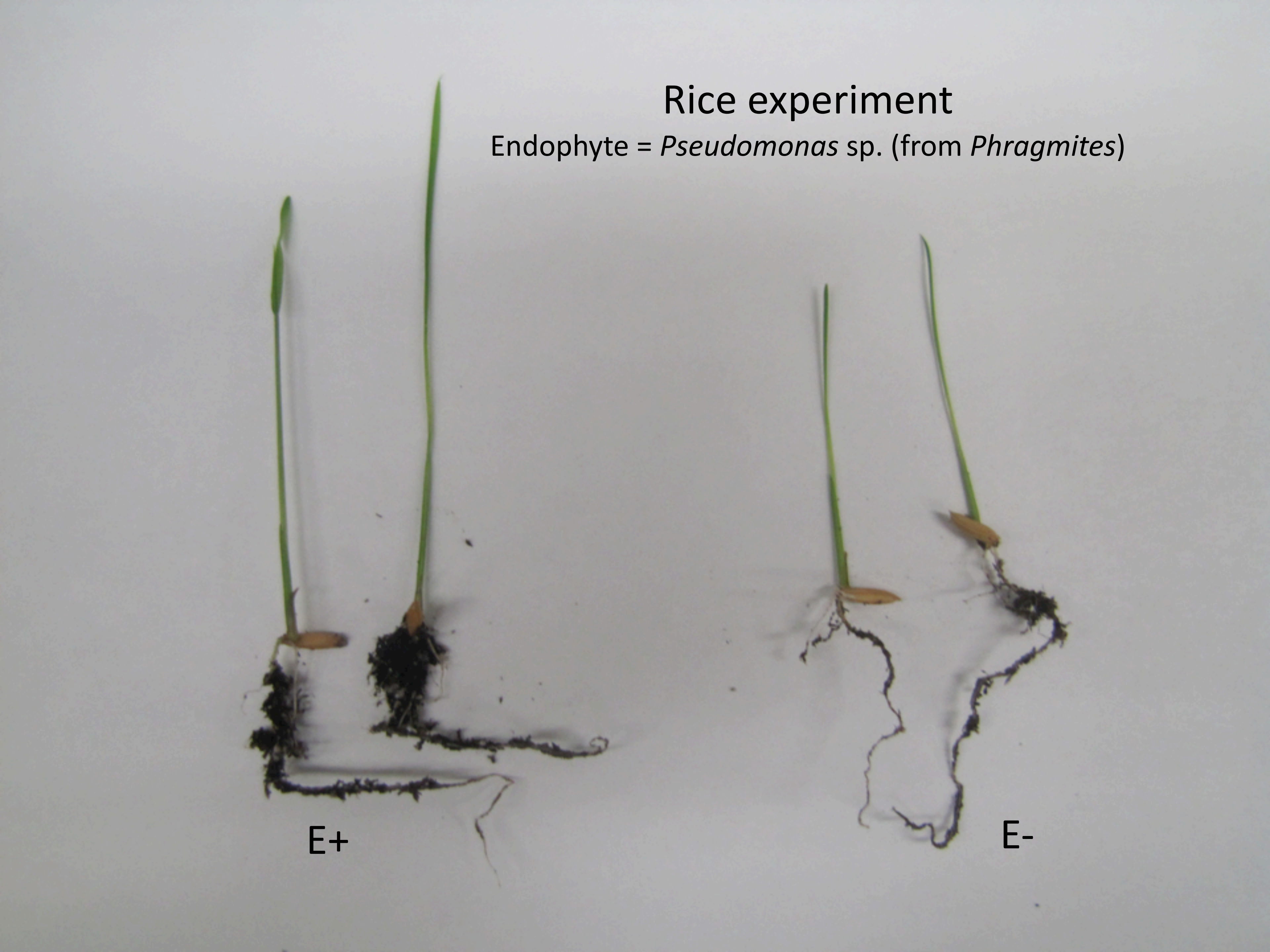
Kurt Kowalski

Rice:

Growth
Promotion!



1. Endophytes removed from rice by surface sterilization.
2. Endophytes (*Pseudomonas* spp.) isolated from *Phragmites australis* inoculated onto seeds to restore development.


Rice experiment
Endophyte = *Pseudomonas* sp. (from *Phragmites*)
E+
E-

Nutrient Absorption Function of the Rhizophagy Cycle

Nutrient Analysis in Winter Wheat With and Without Endophytic Bacteria

Dr. Shuai Zhao

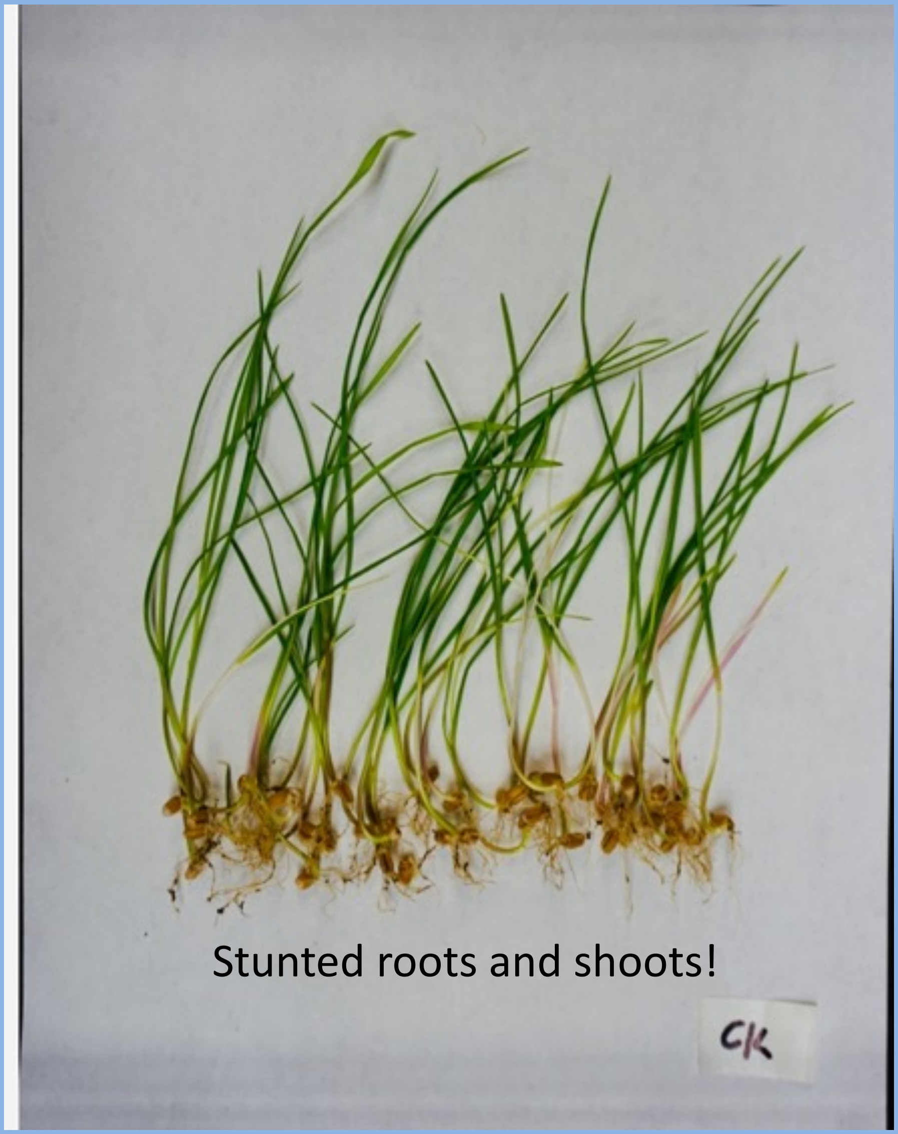
1. **Bacteria isolated from winter wheat seedlings.**

2. **Bacteria removed from seeds by surface disinfection of seeds (50% Chlorox for 50 mins.).**

3. **Seeds with and without bacteria germinated and seedlings grown in sterile potting mix.**

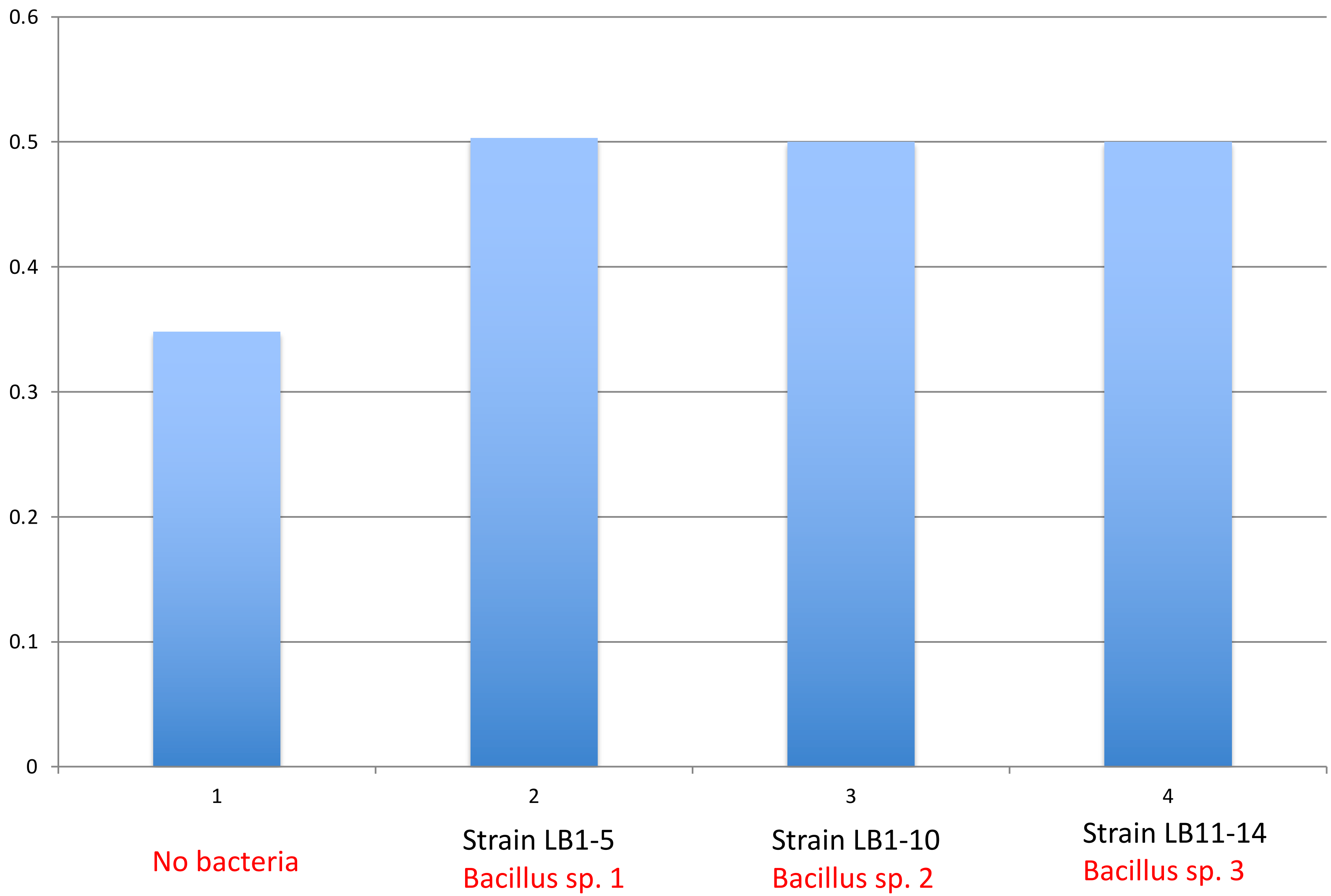
4. **Shoots and roots of wheat dried, weighed, triturated and sent for macronutrient and macronutrient analysis.**

No bacterial control



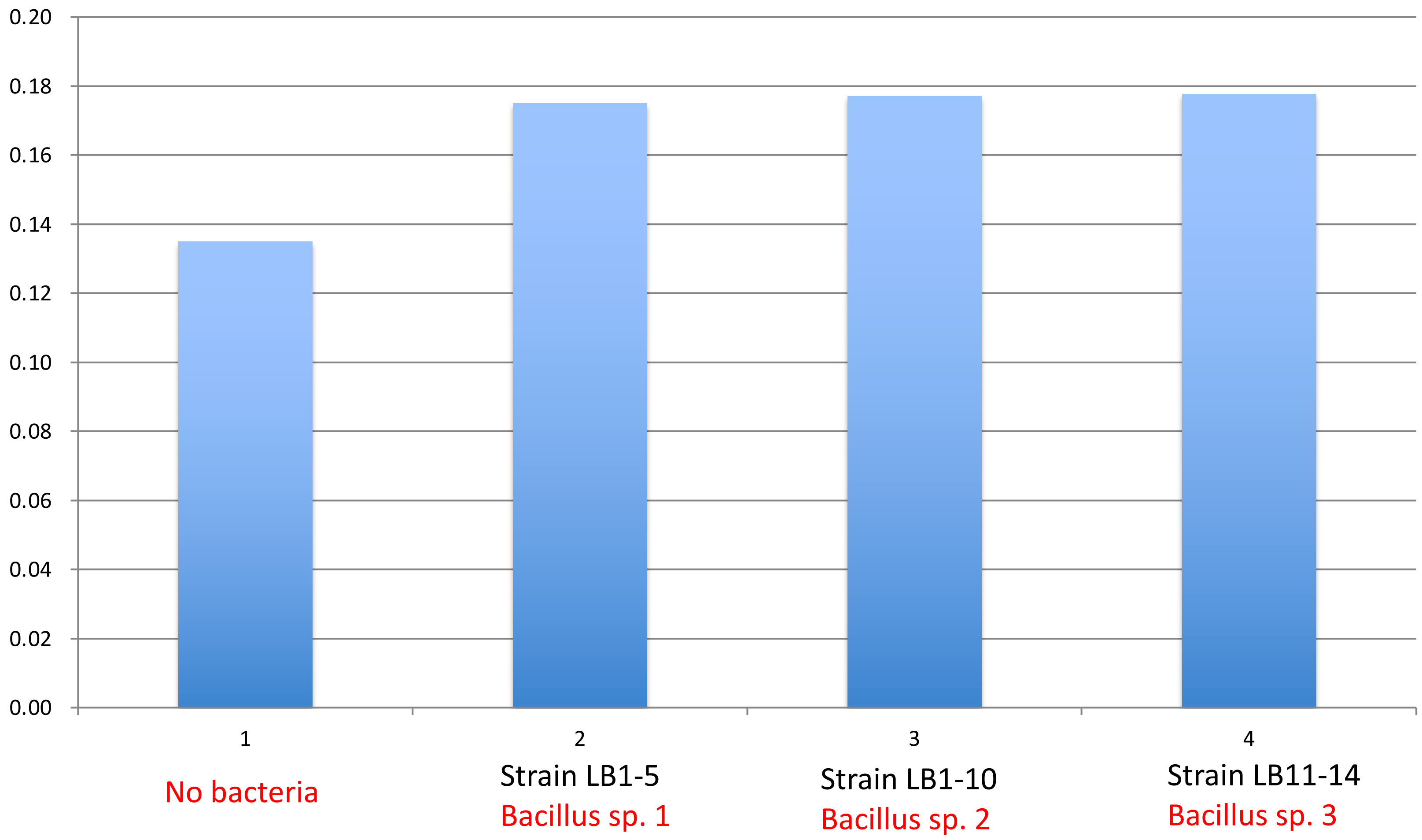
Bacillus sp. Treatment

Nitrogen



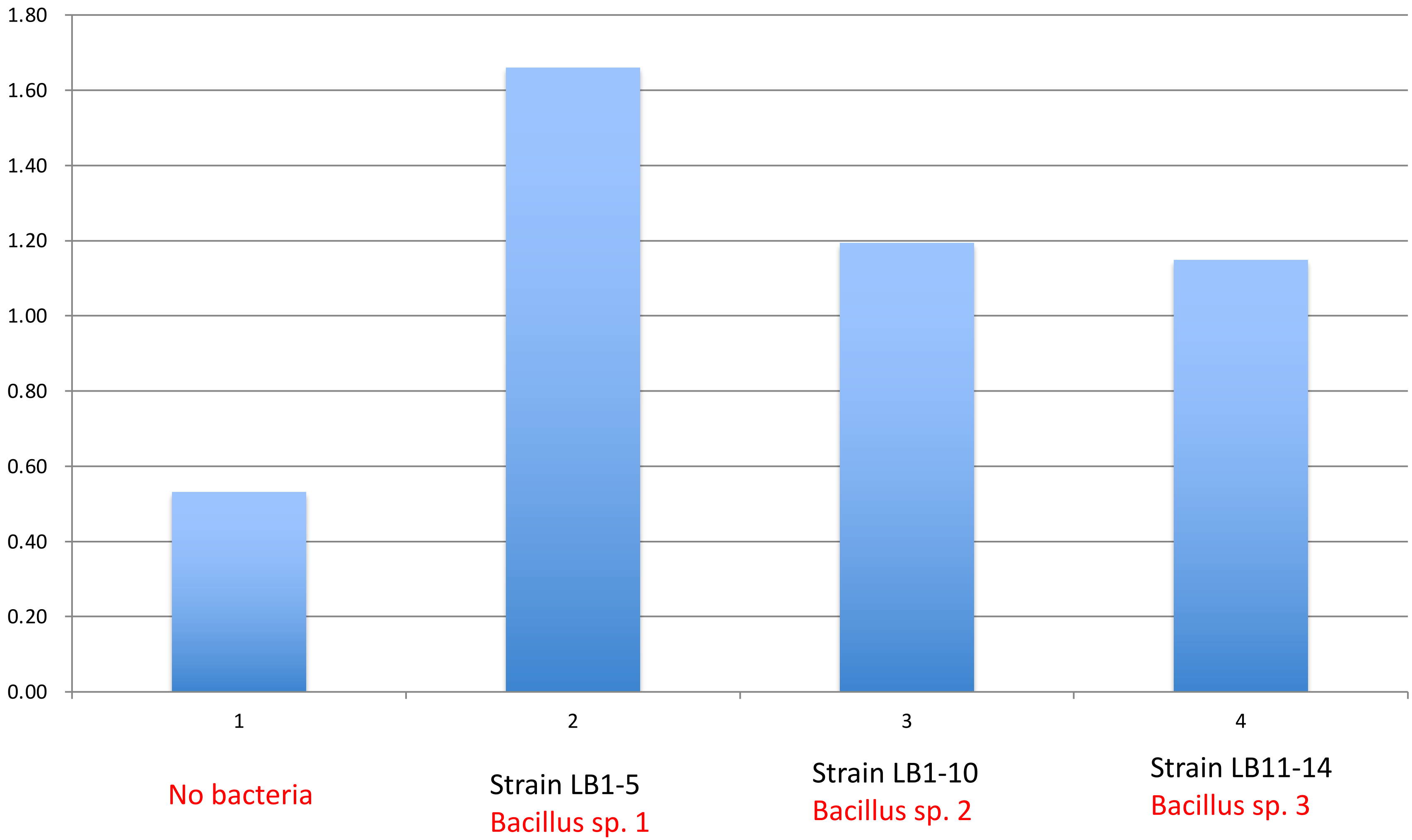
Nitrogen content (% dry weight of wheat seedling shoot tissues) in seedlings with and without rhizophagy bacteria/5

Phosphorus



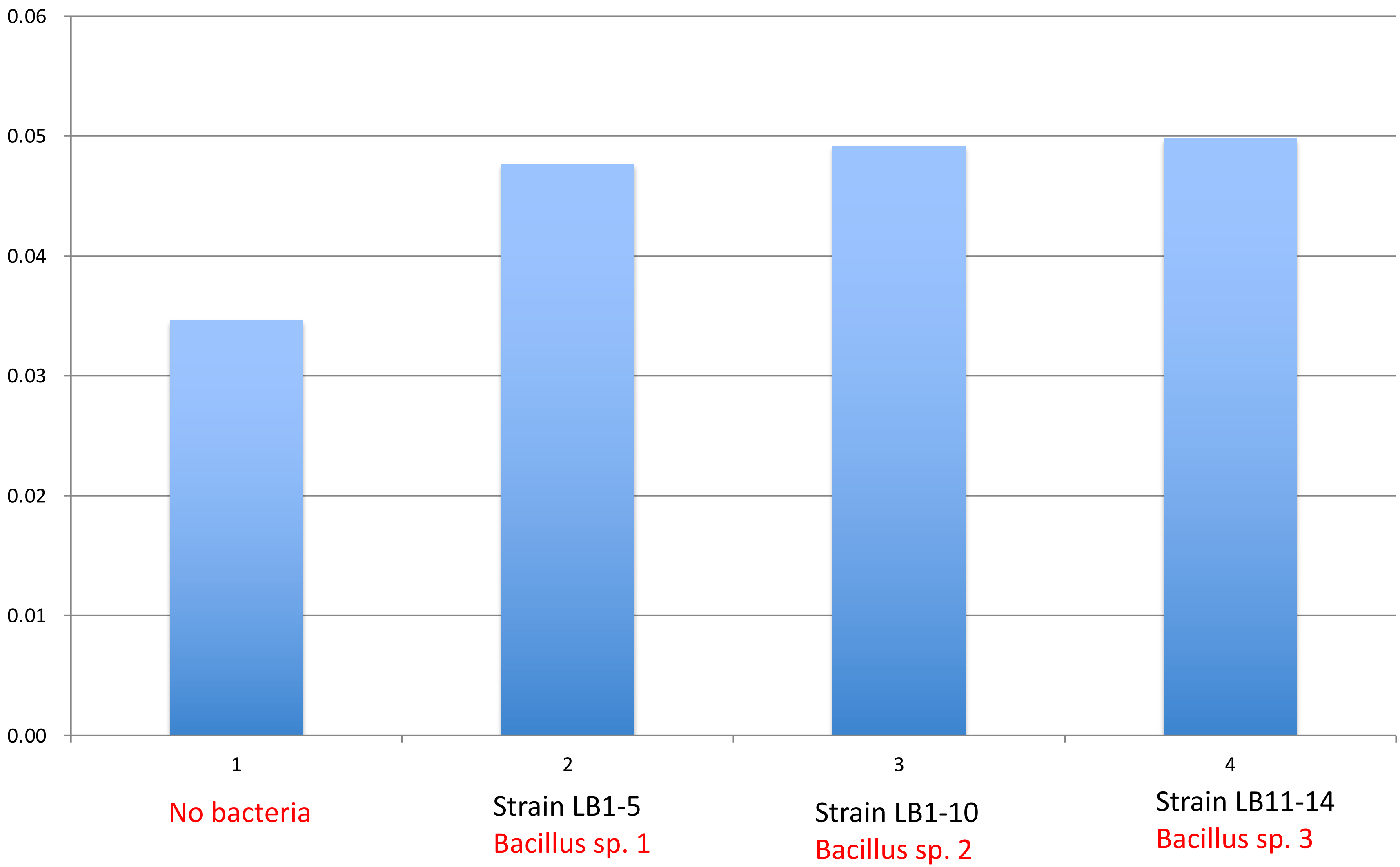
Phosphorus content (percent content of wheat seedling shoot tissues) in seedlings with and without rhizophagy bacteria/5

Potassium



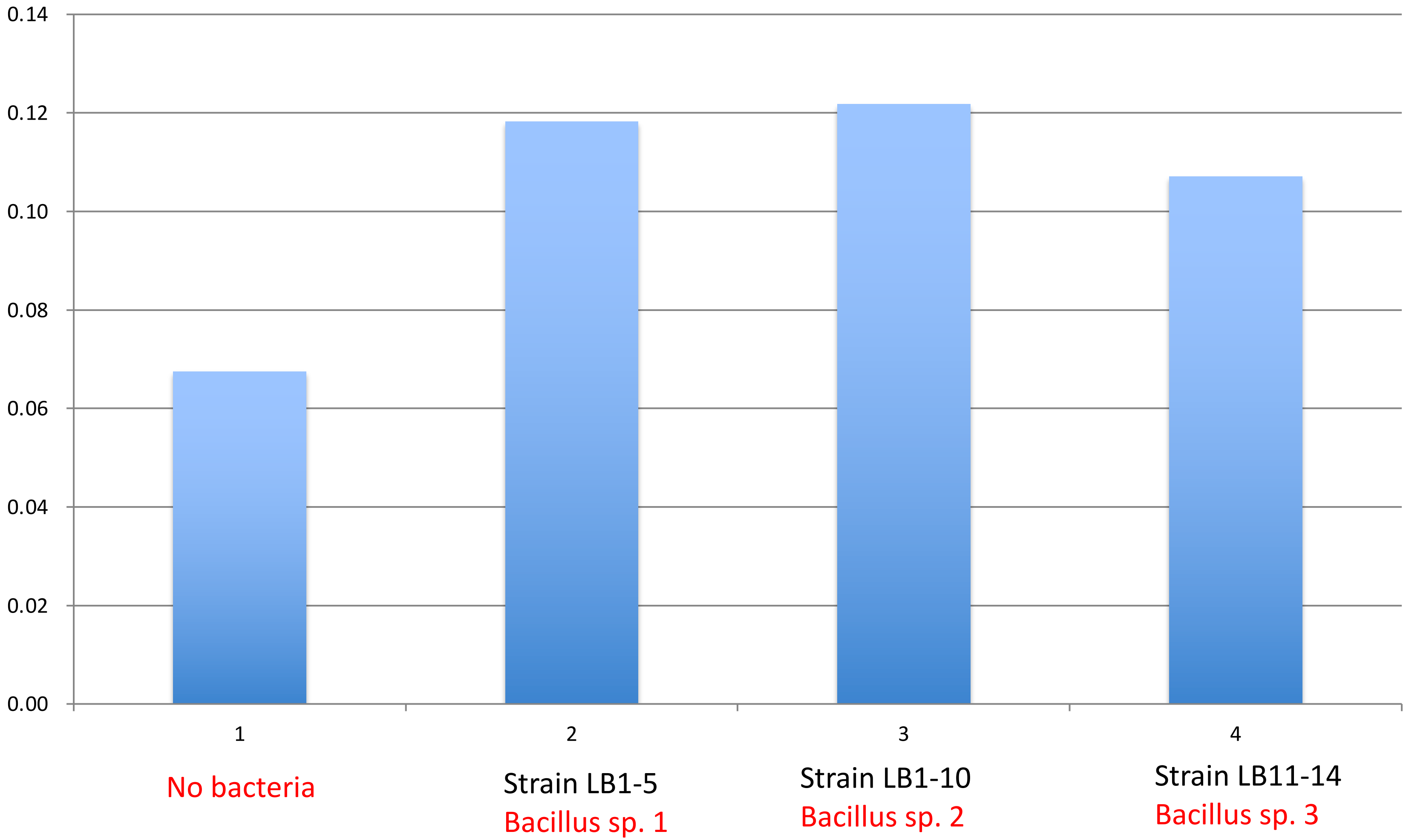
Potassium content (percent dry weight of wheat seedling shoot tissues) in seedlings with and without rhizophagy bacteria/5

Calcium



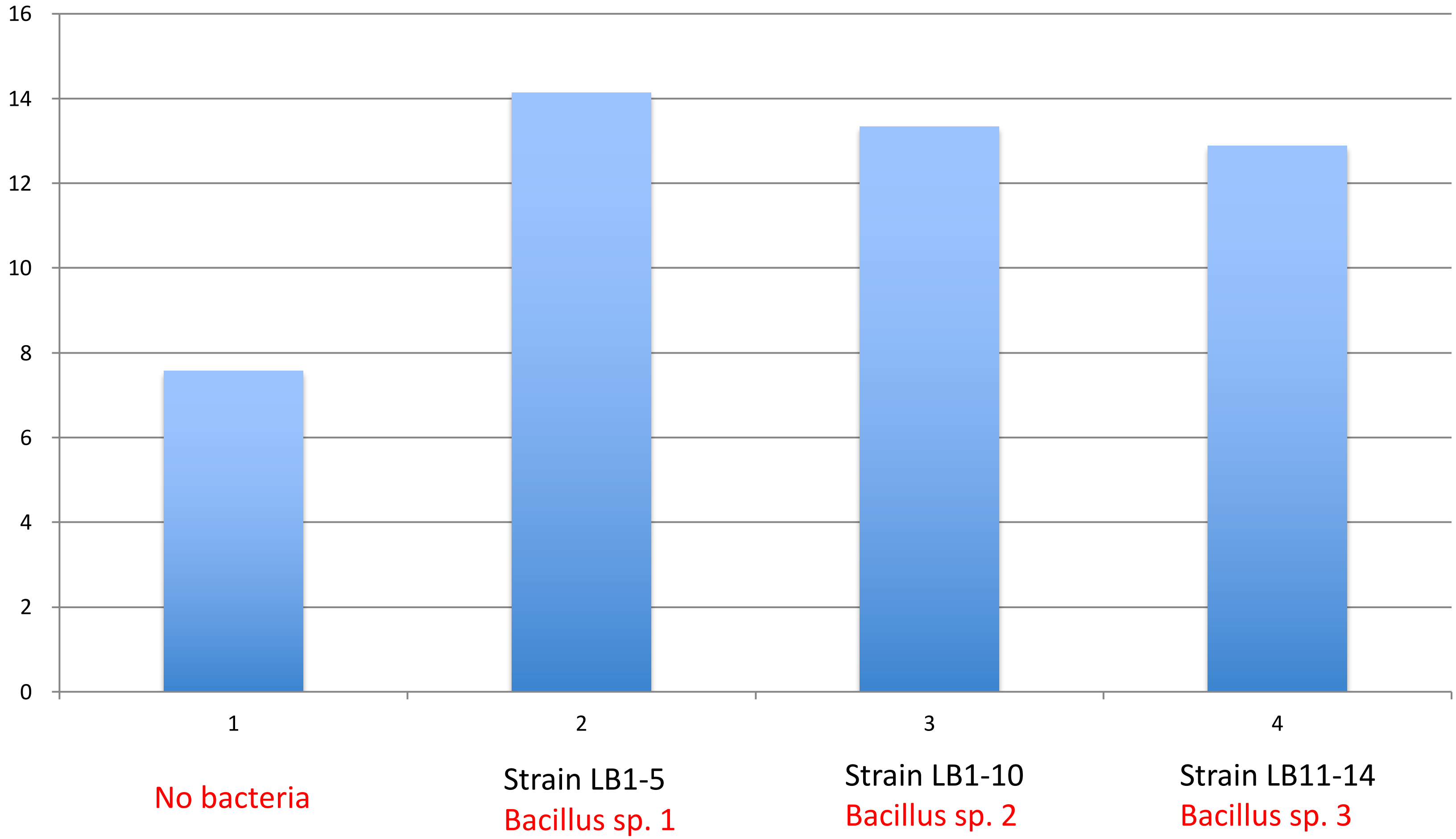
Calcium content (percent of wheat seedling shoot tissues) in seedlings with and without rhizophagy bacteria/5

Sulfur



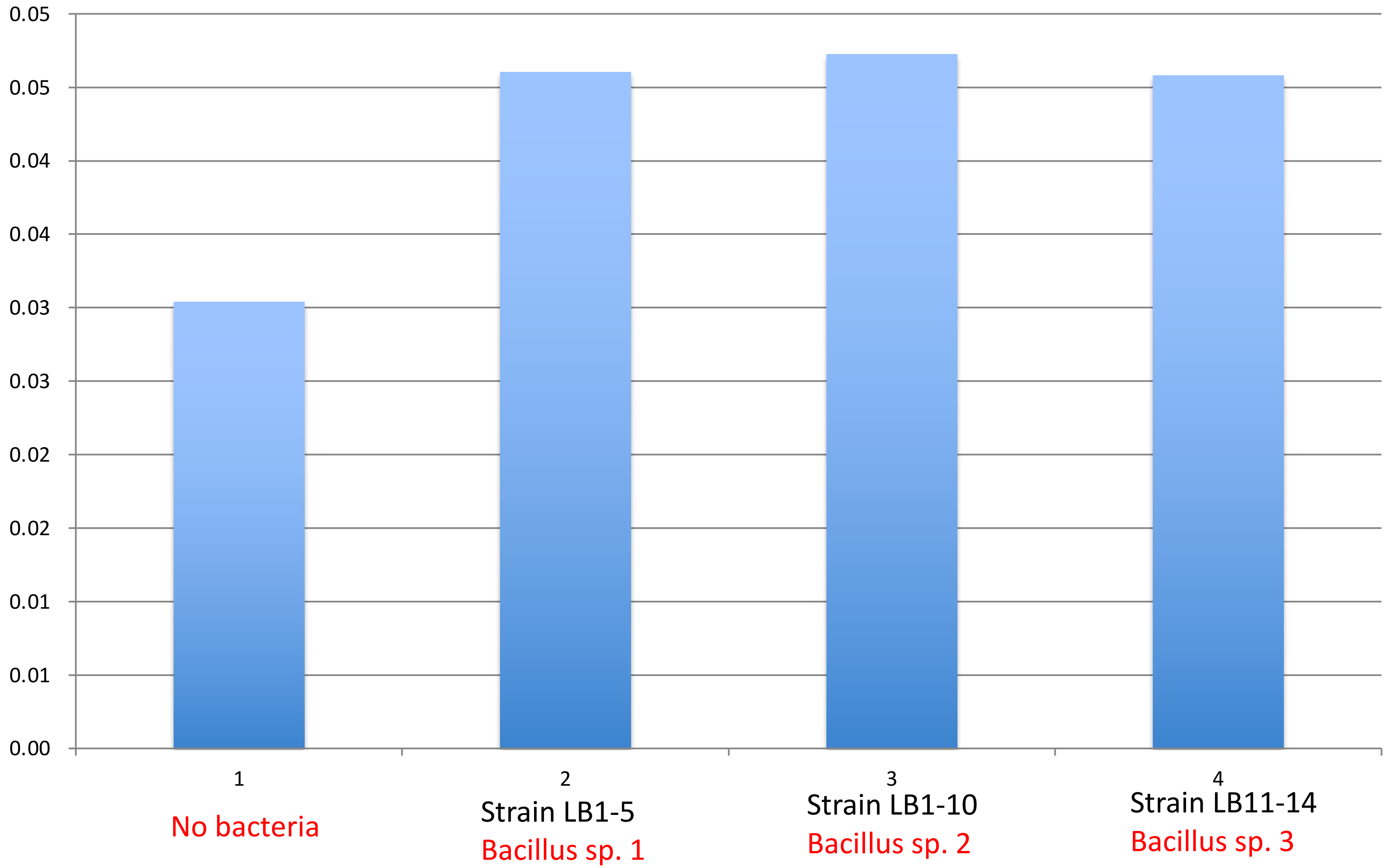
Sulfur content (percent content of shoot tissues) in seedlings with and without rhizophagy bacteria/5

Manganese



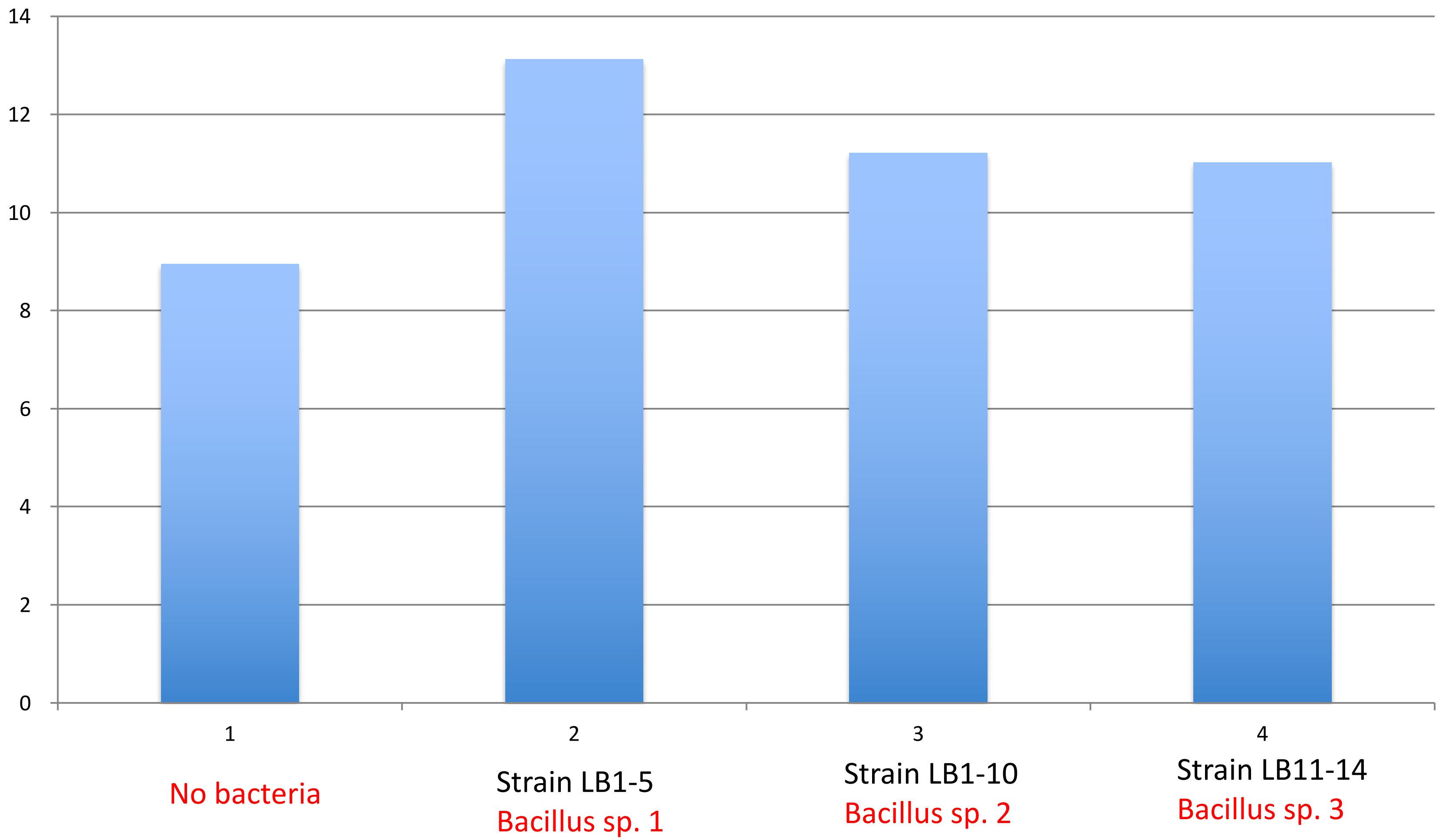
Manganese content (mg/kg of wheat seedling shoot tissues) in seedlings with and without rhizophagy bacteria/10

Magnesium



Magnesium content (mg/kg of wheat seedling shoot tissues) in seedlings with and without rhizophagy bacteria/10

Zinc



Zinc content (mg/kg of wheat seedling shoot tissues) in seedlings with and without rhizophagy bacteria/10

Strains of bacteria are unique in terms of the nutrients they provide to the plant!


Xiaoqian (Ivy) Chang

Can we add commercial biostimulant microbes to crops?

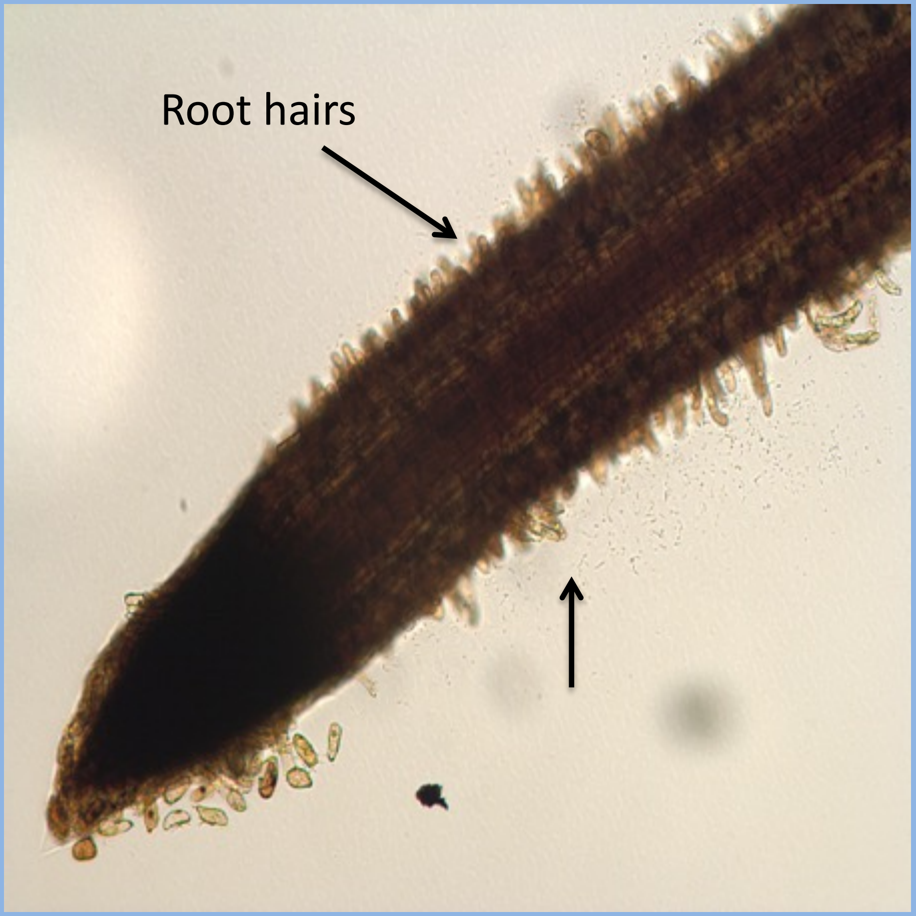
Celery (*Apium graveolens*) Experiments

Methodology

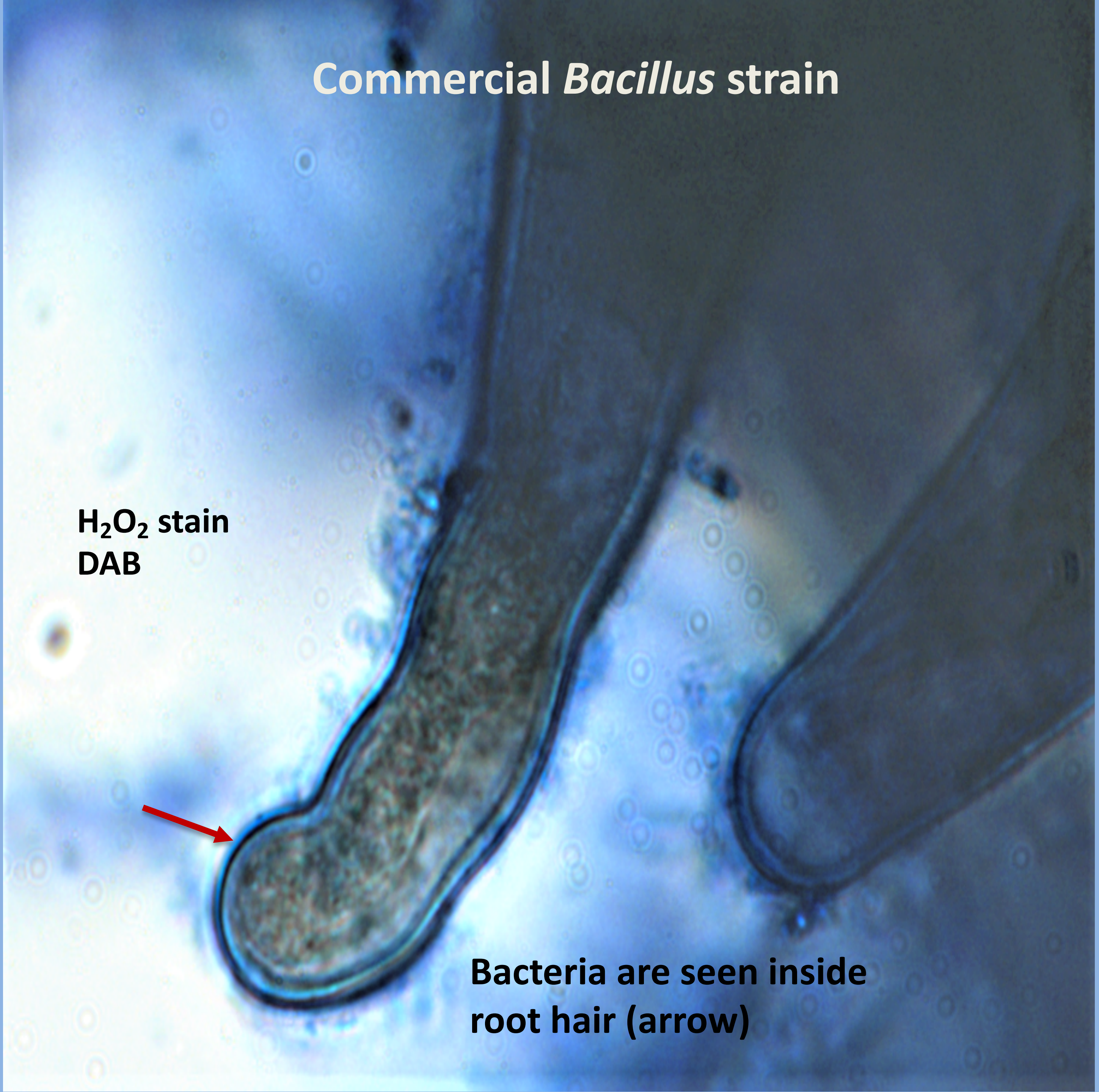
- Seeds of *Apium graveolens* were heat treated for 24 hr (50-55℃) in oven.
- Seeds were surface disinfected for 1 hr in 3% sodium hypochlorite.
- Seeds were plated onto agarose.
- Microbes were applied to the surfaces of seeds.
- Seedlings grown for 15 days.
- Seedlings were stained for reactive oxygen using 3,3-diaminobenzidine.
- Seedlings were assessed and ranked for 2 criteria: 1) root hair elongation, and 2) stimulation of exudation around the root tip.

Commercial Biostimulant Added

Control (No microbe)



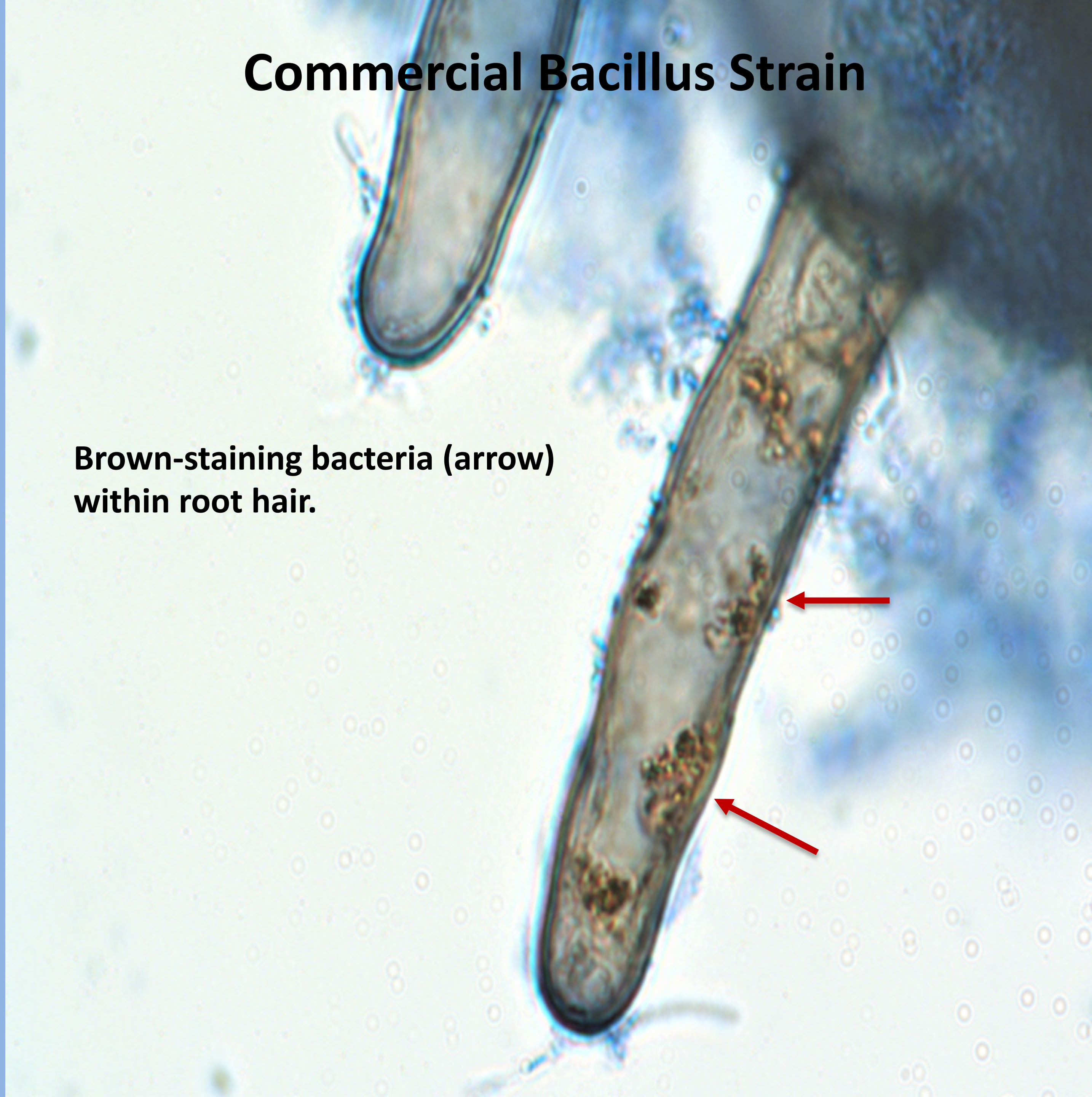
Commercial biostimulant microbe
Bacillus amyloliquefaciens


Commercial *Bacillus* strain
H_2O_2 stain
DAB
Bacteria are seen inside
root hair (arrow)

H_2O_2 stain


Commercial Bacillus Strain
Brown-staining bacteria (arrow) within root hair.

Commercial Bacillus Strain



Brown-staining bacteria (arrow) within root hair.

Always test products!

Microbes alter chemical constituents of plants!!

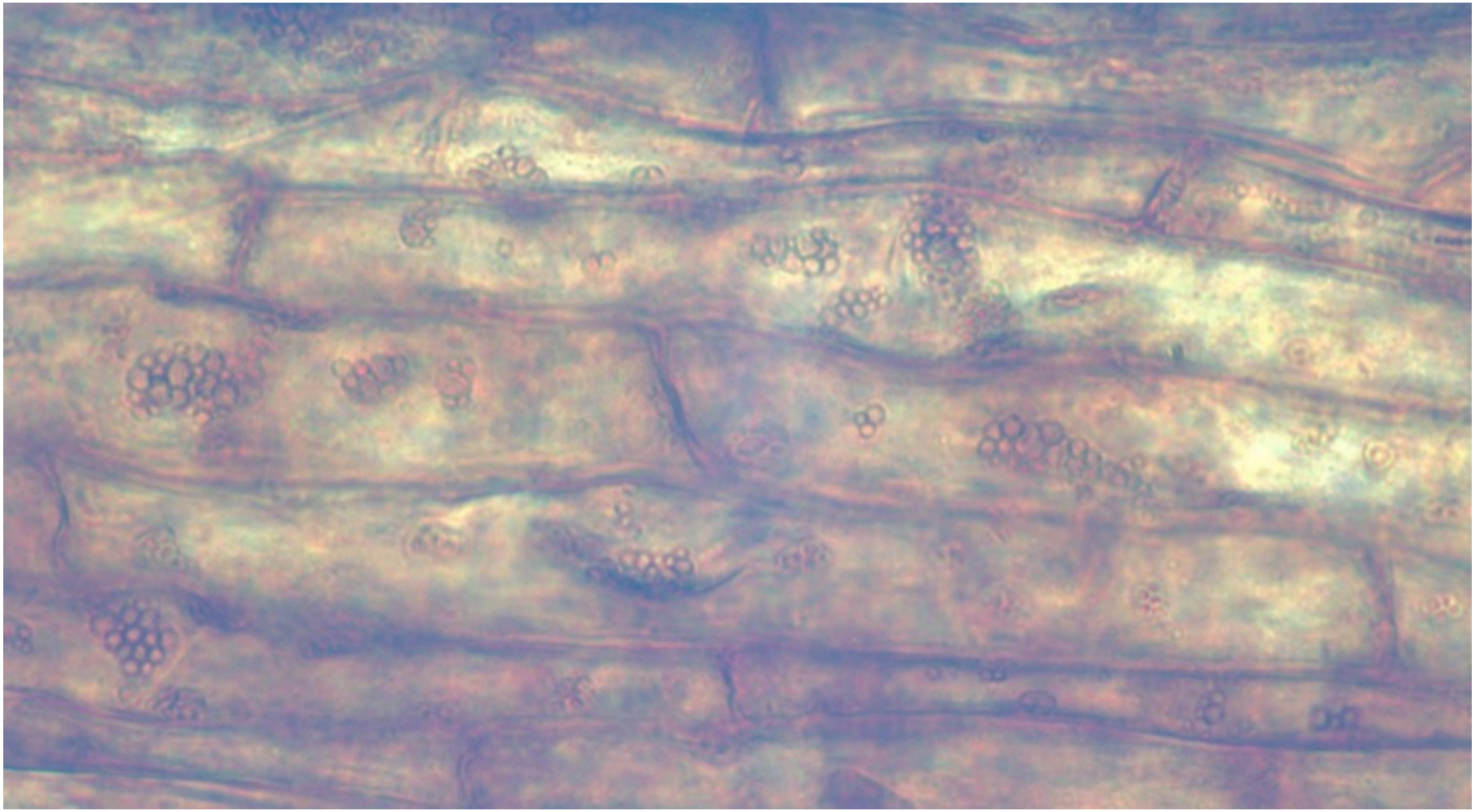
Carotenoid study using carrot seedlings

Methodology

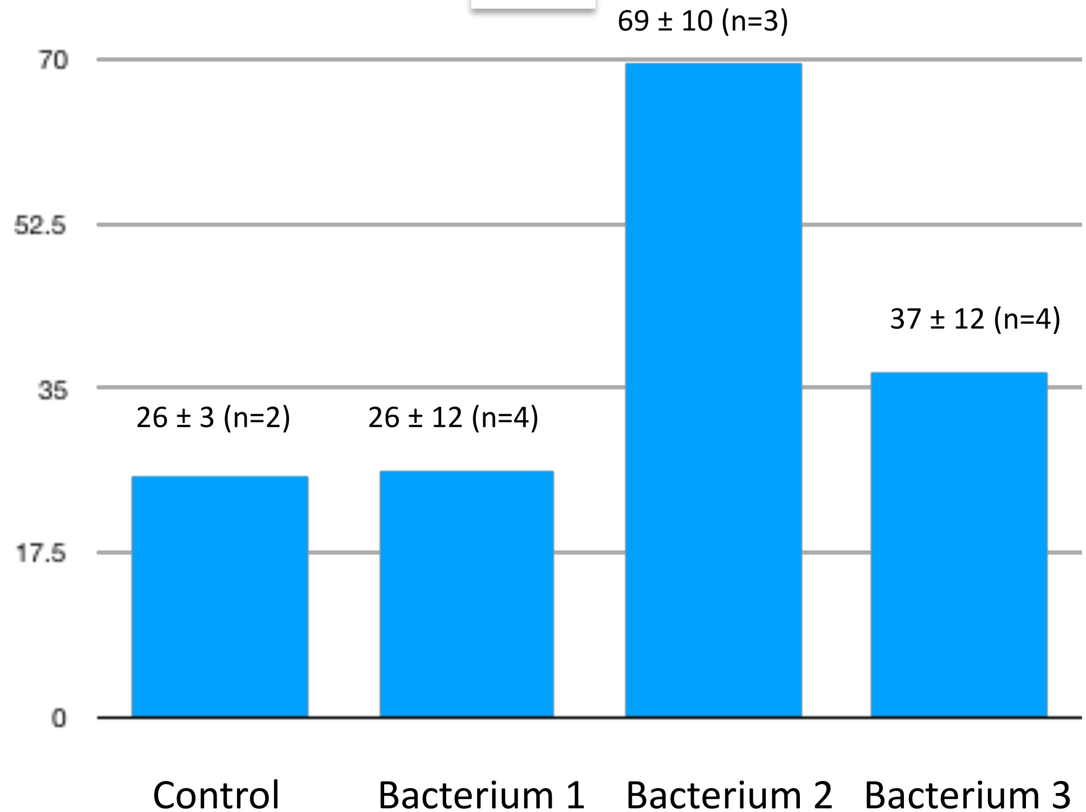
- Bacteria isolated from species of family Apiaceae, including seedlings of celery (Bacterium 1), cumin (Bacterium 2) and parsley (Bacterium 3).
- Carrot seeds surface disinfected for 50 min to remove native microbes.
- Axenic carrot seeds germinated in sterile potting with bacteria added to seed.
- After 4 weeks roots harvested, dried and analyzed using HPLC for carotenoids.

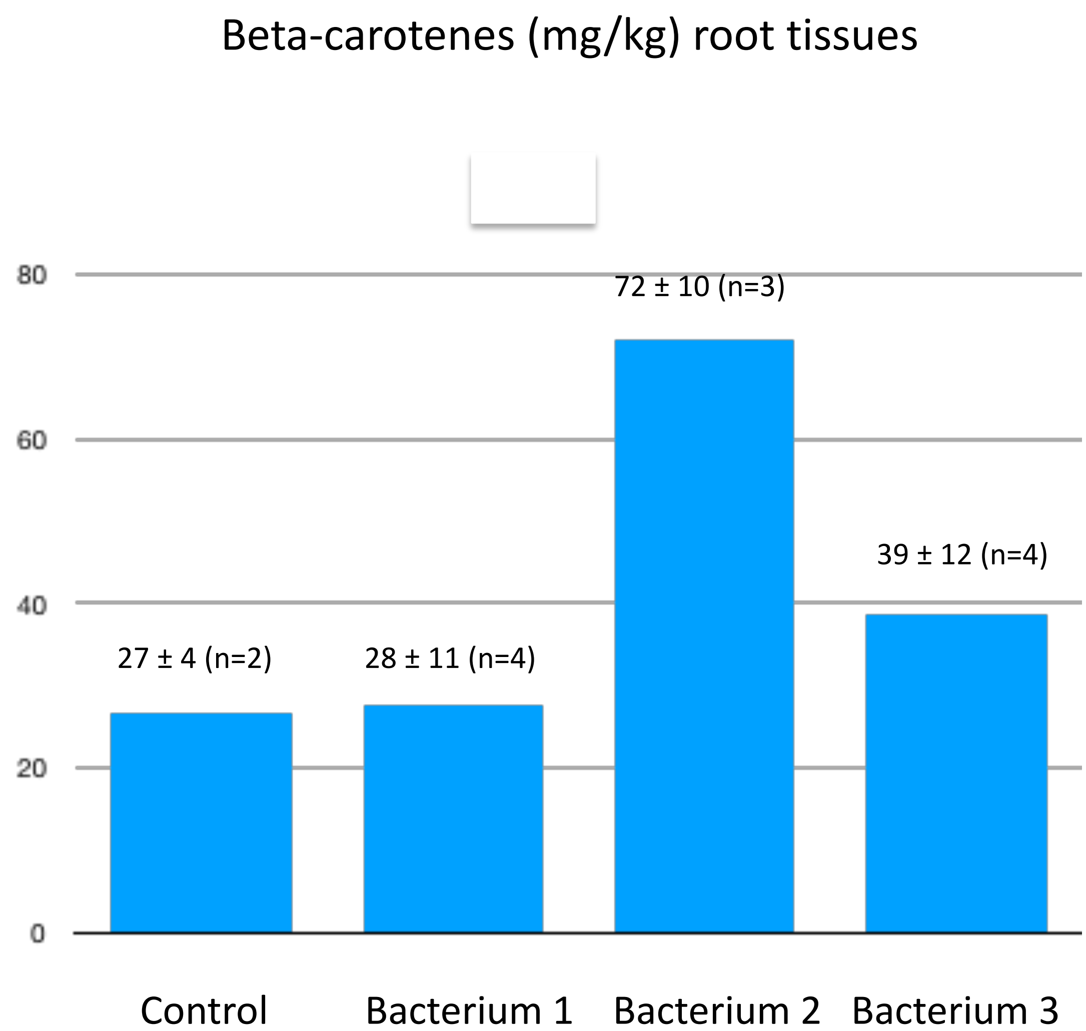
Bacteria in carrot root hairs

Bacteria in carrot root epidermis cells



Alpha-carotenes (mg/kg) root tissues




Beta-carotenes (mg/kg) root tissues
80
60
40
20
0
72 ± 10 (n=3)
39 ± 12 (n=4)
27 ± 4 (n=2)
28 ± 11 (n=4)
Control
Bacterium 1
Bacterium 2
Bacterium 3

3

A. Three Beneficial Outcomes of Rhizophagy Symbiosis

1. Plants absorb nutrients from microbes

Rhizophagy microbes enter plant roots with nutrients

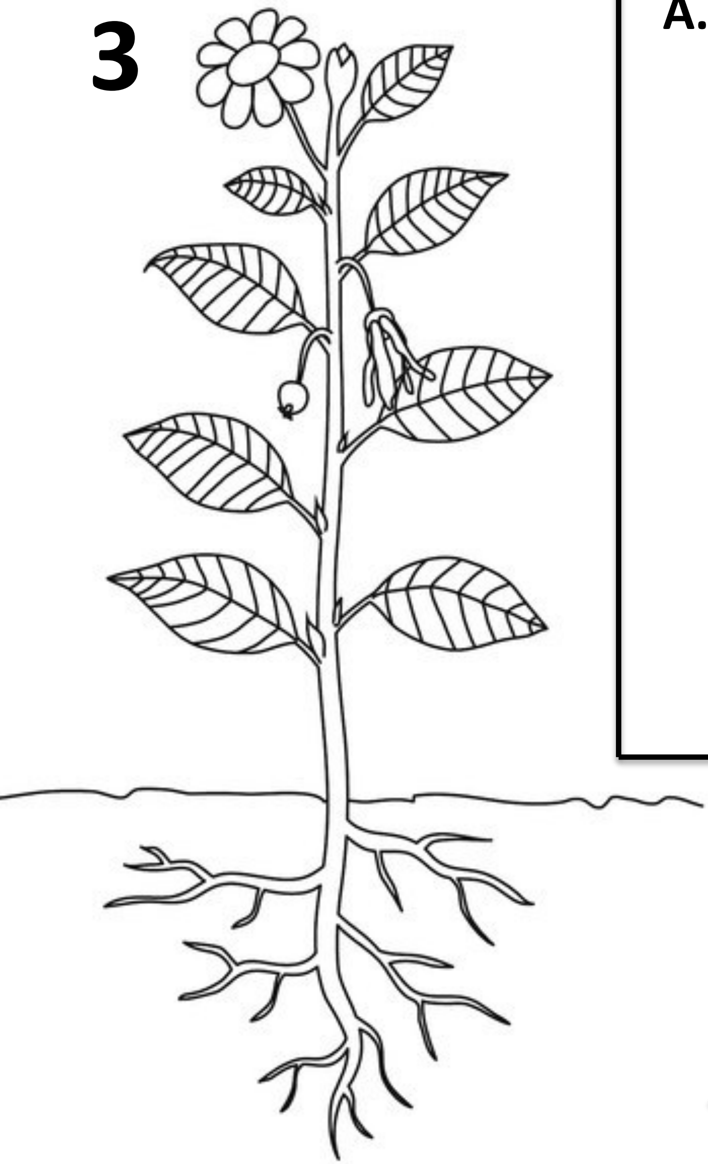
2. Increased oxidative stress tolerance in plants

Increased reactive oxygen activity in root cells

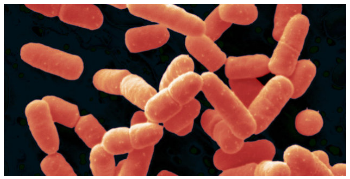
3. Soil fungal pathogens have reduced virulence

Soil fungi drained of nutrients by rhizophagy cycle microbes

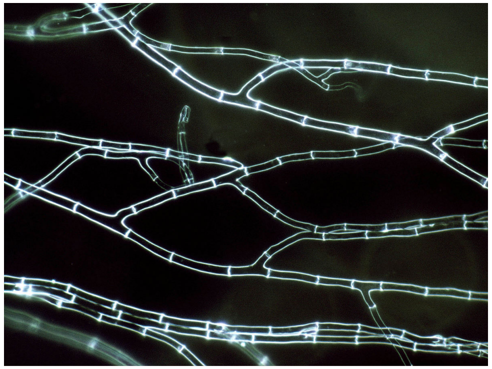
B. Nutrient Flow



The plant takes nutrients from rhizophagy cycle microbes, and provides photosynthate to soil microbes.



Rhizophagy cycle microbes take nutrients from microbial community.



The soil microbial community liberates and absorbs nutrients from soil.

Concluding Remarks:

- **Native microbes on seeds should be preserved (i.e., no antimicrobials on seeds, and no removal of seed tissues (de-husking)).**

- **Manage soils and seeds to build up microbial communities.**

- **Biostimulant microbes may be added; these are generally endophytes that enter into root cells and participate in the rhizophagy cycle but the correct microbes need to be employed for optimal results.**

References

White, J.F.; Kingsley, K.L.; Verma, S.K.; Kowalski, K.P. **2018**. Rhizophagy Cycle: An Oxidative Process in Plants for Nutrient Extraction from Symbiotic Microbes. *Microorganisms* 2018, *6*, 95. **https://doi.org/10.3390/microorganisms6030095**

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