RUTGERS

THE STATE UNIVERSITY
OF NEW JERSEY

A & L Canada Labs Presentation:

How plants cultivate soil microbes and extract nutrients from them in roots

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September 25, 2019

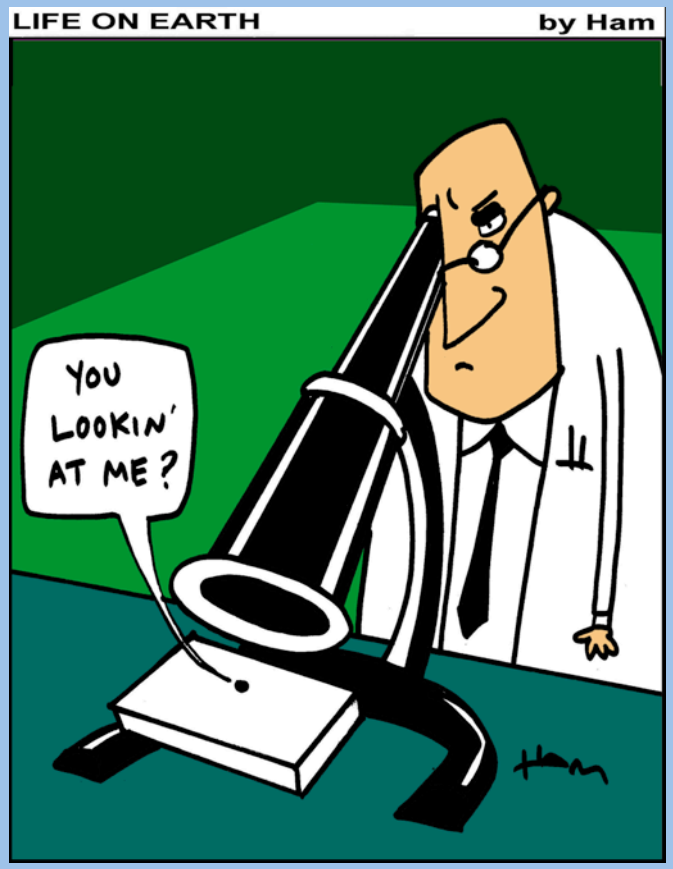
What are endophytes?

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

(Medical definition): A tumor that grows like a parasite into other tissues.

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

All plants naturally have endophytes!



Endophytes are everywhere!

Every plant contains multiple endophytes!

Microbial Endophytes

- Fungi and bacteria
- Common in all plants
- Intracellular and intercellular
- Improve plant stress tolerance
- Suppress plant pathogenic fungi
- Modulate root development
- Improve nutrient absorption

‘Cadushy’ cactus: *Subpilocereus repandus* in Bonaire

Seeds

Cadushy seedling

Reactive Oxygen Staining Technique

Assay for bacterial endophytes involves growth on agarose. Then staining with Diaminobenzidine tetrahydrochloride (DAB) overnight.

Reactive oxygen secretion is used by all eukaryotes to kill endoparasitic bacteria. It is part of the innate Defensive system of all Eukaryotes.

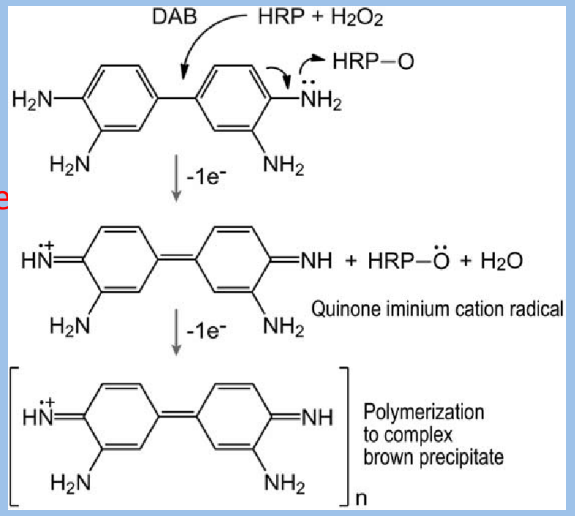
How DAB Works:

1) Plant cells secrete superoxide Onto intracellular bacteria to degrade them.

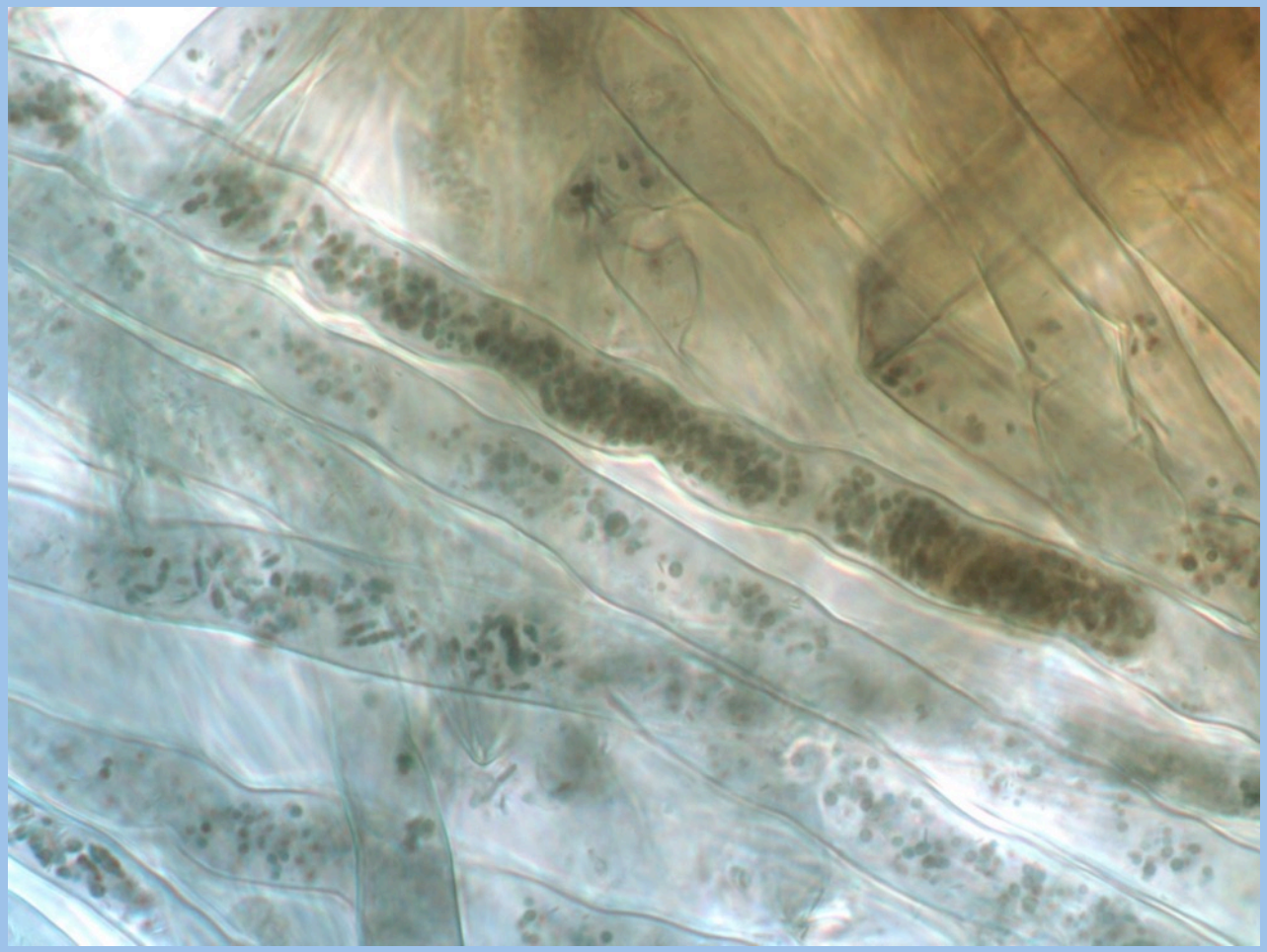
2) Plant uses superoxide dismutase to transform Superoxide to water and Hydrogen peroxide.

3) DAB reacts with Hydrogen peroxide to form Brown/red coloration

HRP is peroxidase



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



Bacteria in root hairs showing recently divided pairs

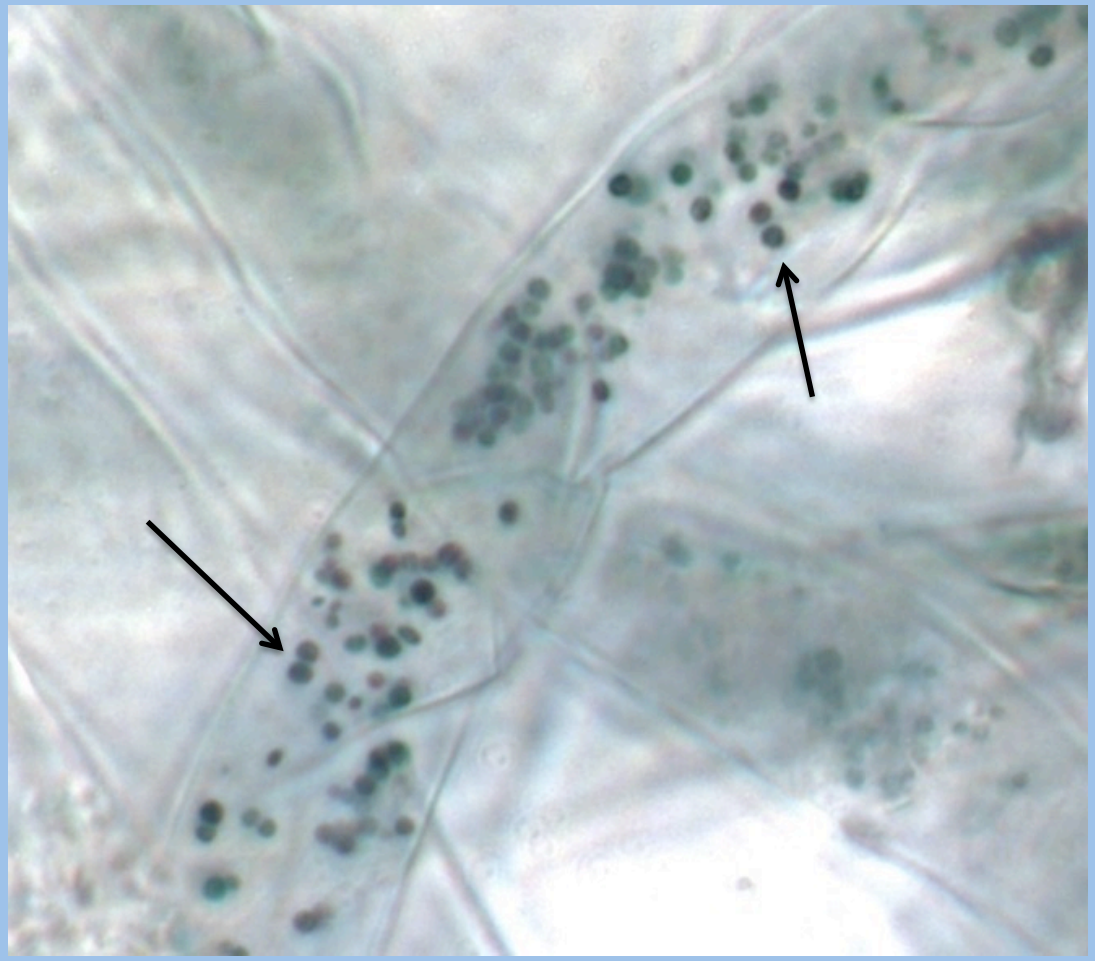
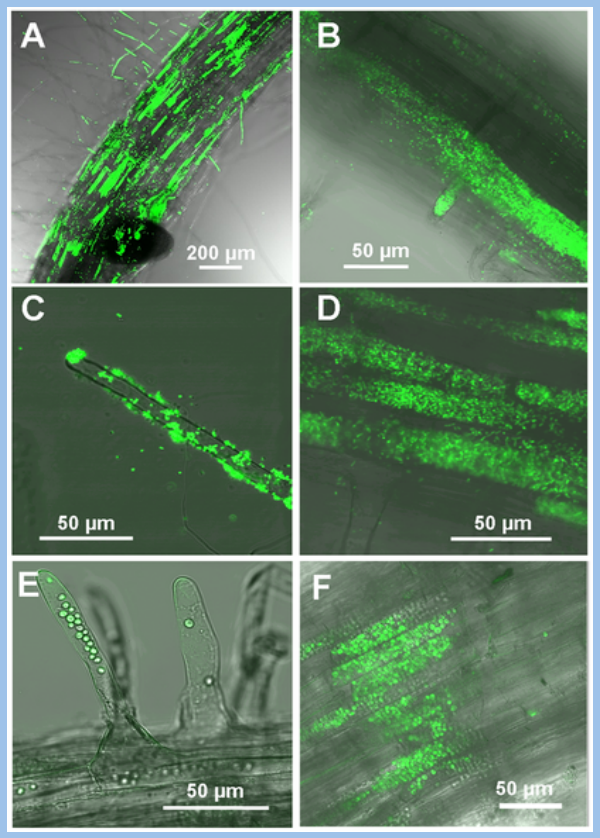


Figure 1. Roots of axenically grown Arabidopsis and tomato were incubated with E coli or yeast expressing green fluorescent protein (GFPE. coli or GFPyeast).

“Rhizophagy”

Do plant roots consume bacteria to obtain nutrients?



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

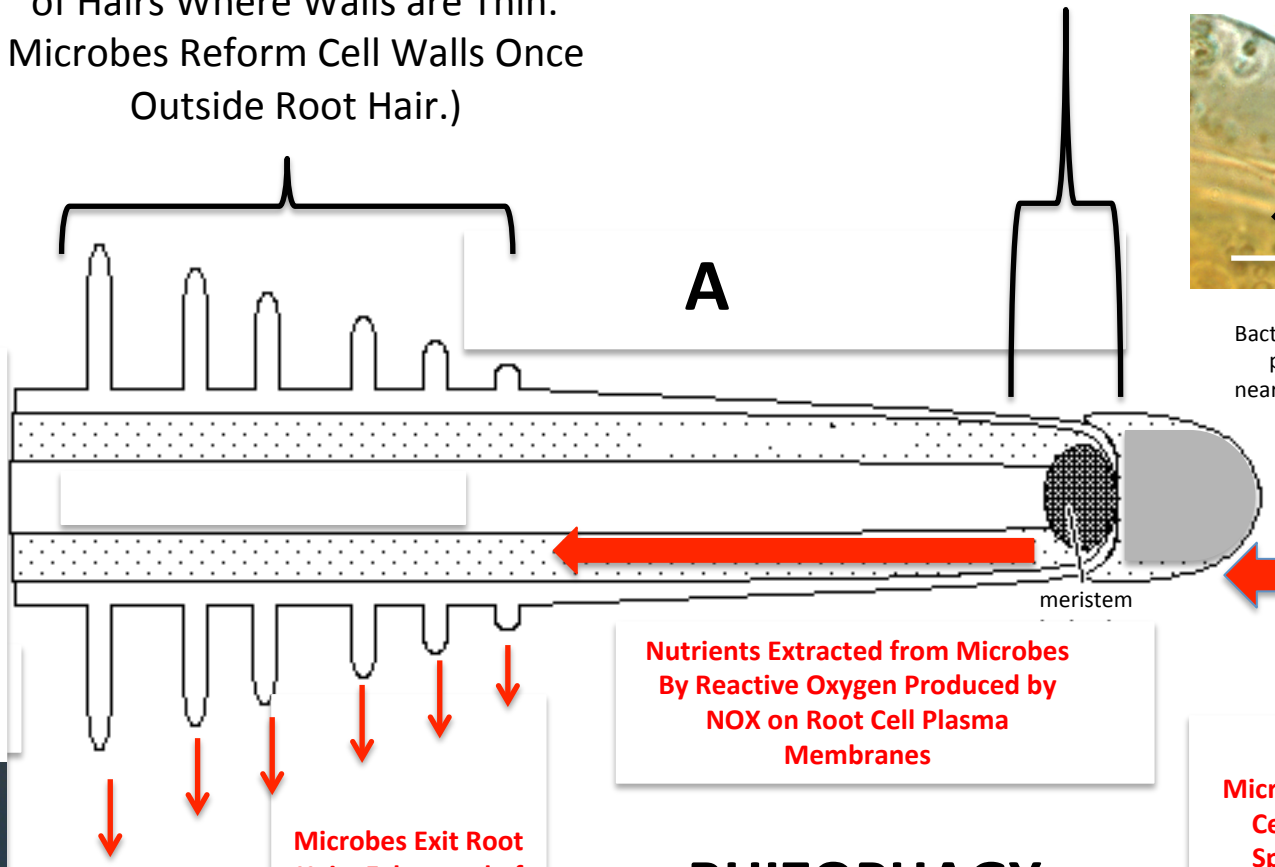
Chany Paungfoo-Lonhienne

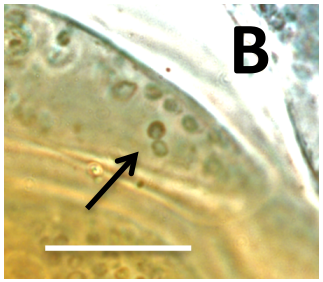
Paungfoo-Lonhienne C et al. 2010.
Turning theTable: Plants Consume Microbes as a Source of Nutrients.
PLoS ONE 5(7): e11915, doi:10.1371/journal.pone.0011915

1

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.)

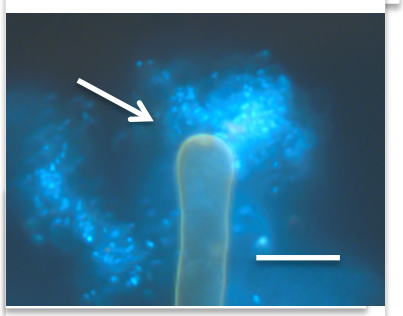
Plant Cell Entry Zone
(Microbes Become Intracellular in Meristem Cells as Wall-less Protoplasts.)





Bacteria (arrow) in root parenchyma cell near root tip meristem.

C



Bacteria (arrow) emerging from root hair tip of millet seedling.

Nutrients Extracted from Microbes By Reactive Oxygen Produced by NOX on Root Cell Plasma Membranes

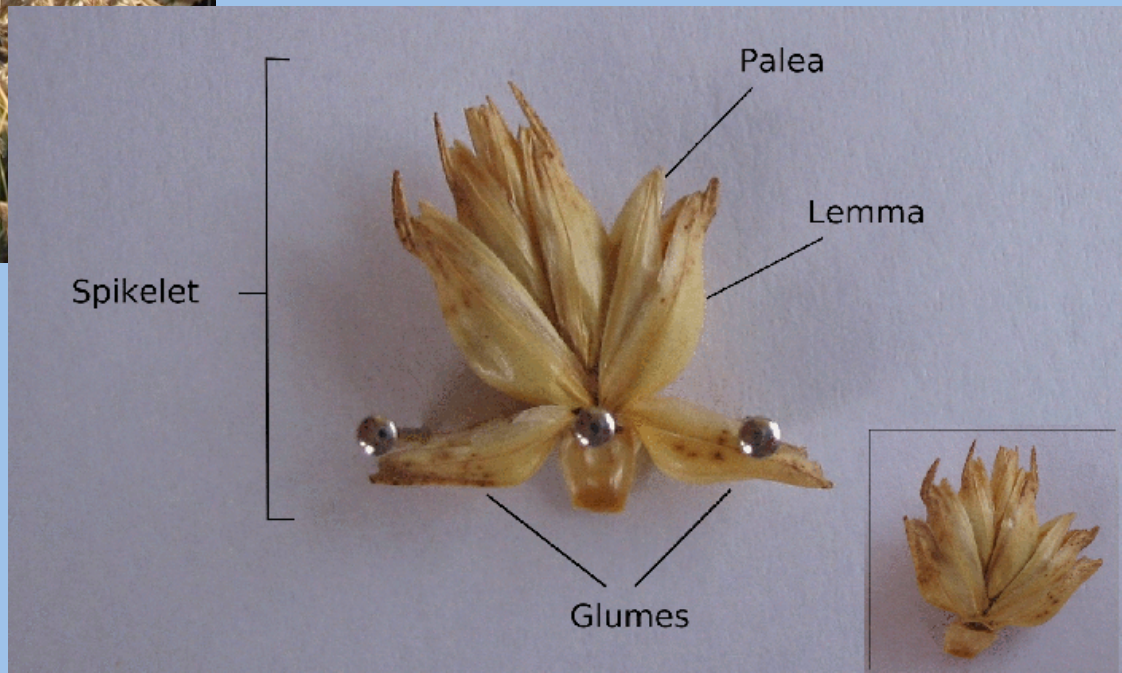
Microbes Enter Root Cell Periplasmic Spaces Carrying Nutrients From Soil

Microbes Exit Root Hairs Exhausted of Nutrients

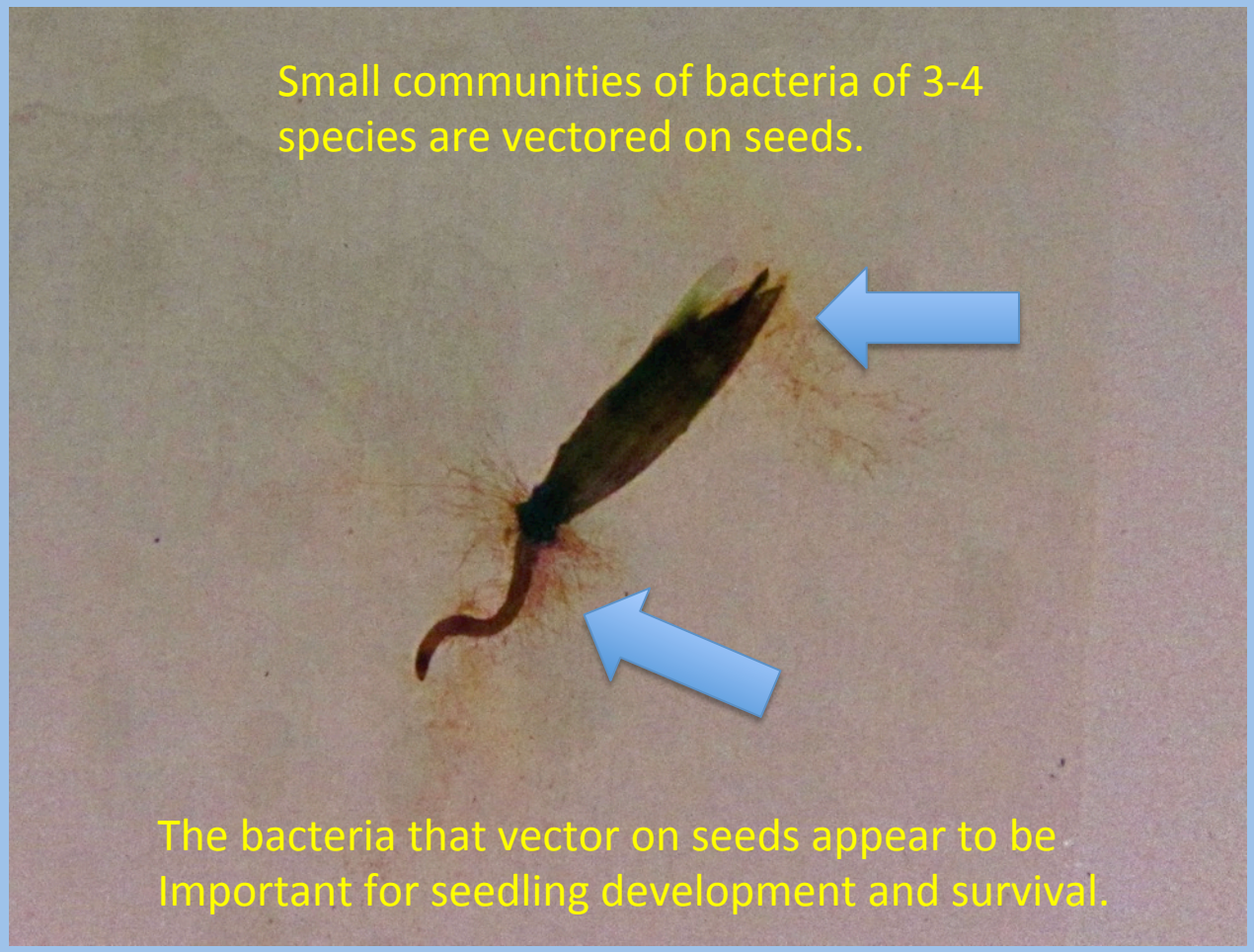
RHIZOPHAGY CYCLE

Microbes Recharge with Nutrients in the Rhizosphere

Grasses vector symbiotic microbes on paleas and lemmas



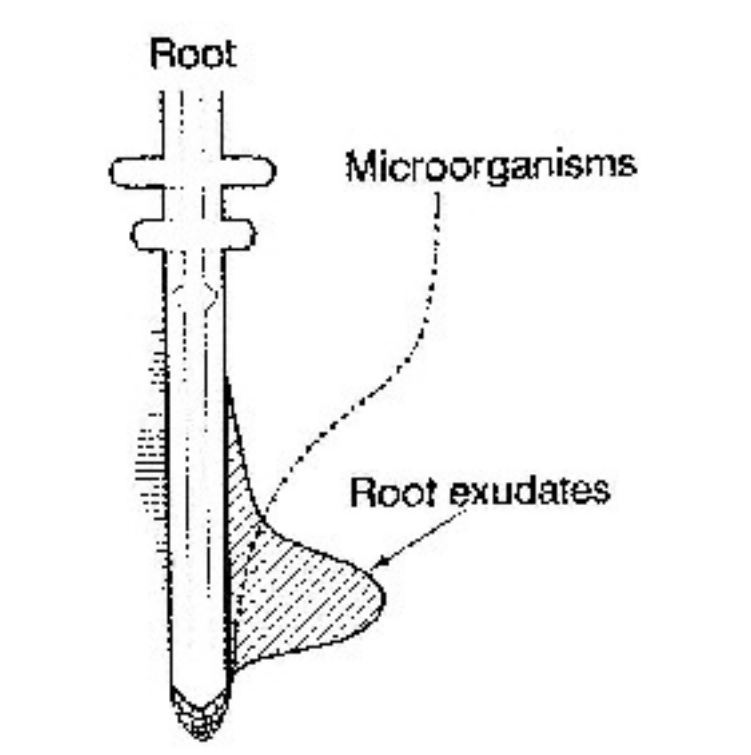
Bacterial symbiosis: germinating tall fescue seed showing seed-transmitted bacteria (*Pantoea agglomerans* and *Pseudomonas* sp.; both gamma-Proteobacteria)

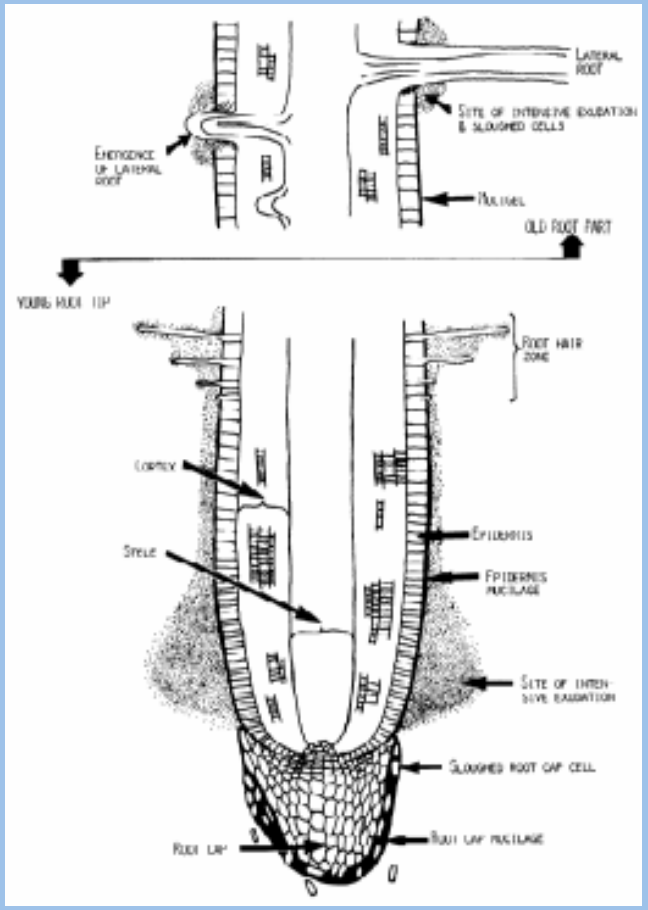


*Bacteria are ‘Proteobacteria’; Perhaps mostly gamma- and beta-Proteobacteria.

Root exudation zones determined by 14C experiments.

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



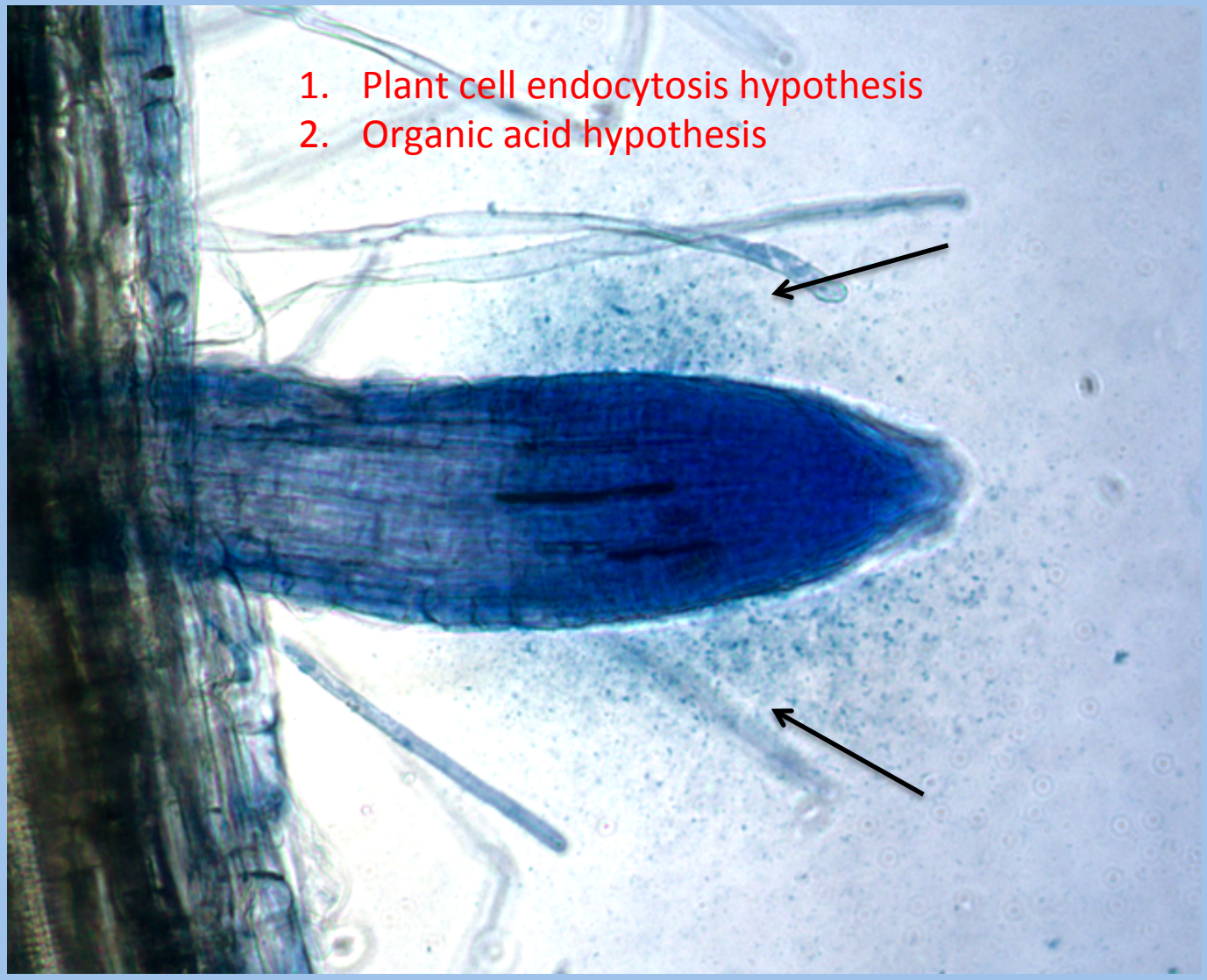


Secretion of exudates in a zone proximal to root tip meristems facilitates microbe entry into cells of the plant meristem.

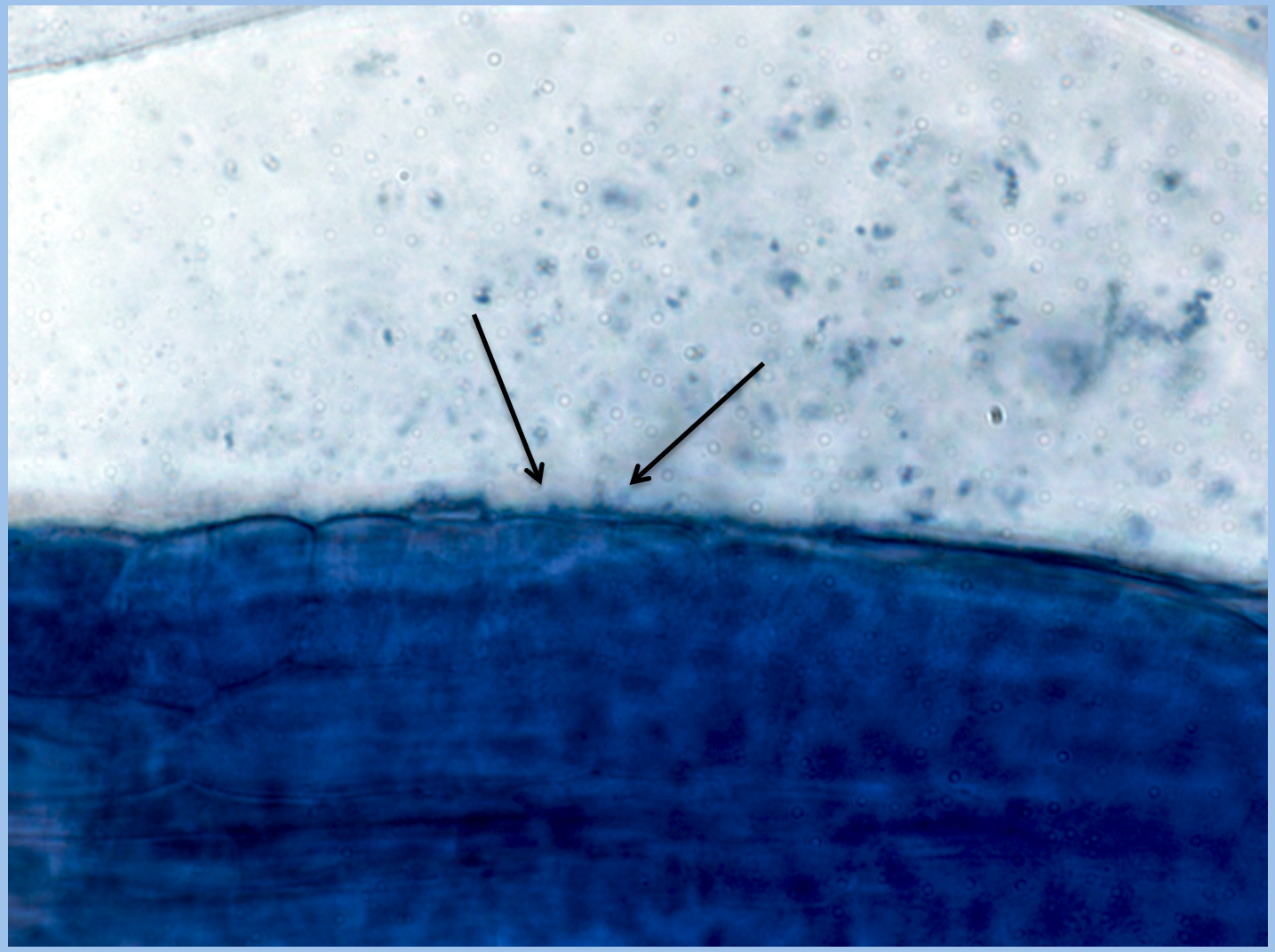
Marschner, H., 1995. Mineral Nutrition of Higher Plants, 2nd edn., Academic Press, London.

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.



Bacteria (arrows) colonizing the epidermal cells in the zone of intracellular colonization. Bacteria will enter cells as walled bacteria—but soon lose cell walls after exposure to reactive oxygen (superoxide produced on root cell plasma membranes).



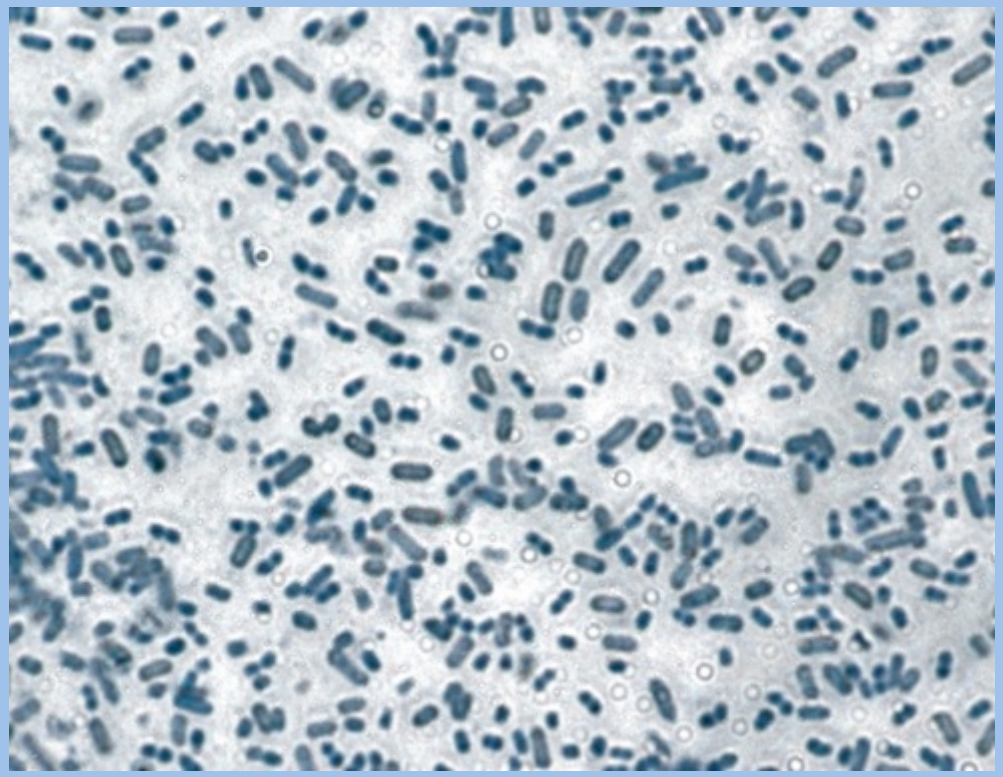
BERMUDA GRASS SEEDLING ROOT TIP IN AGAROSE WITHOUT MICROBES

CONSTITUTIVE SECRETION OF REACTIVE OXYGEN IN ROOT TIPS TRIGGER INFECTING MICROBES TO LOSE CELL WALLS.

The brown coloration is due to presence of reactive oxygen. This tissue was stained For 13 hours in diaminobenzidine tetrachloride (DAB)

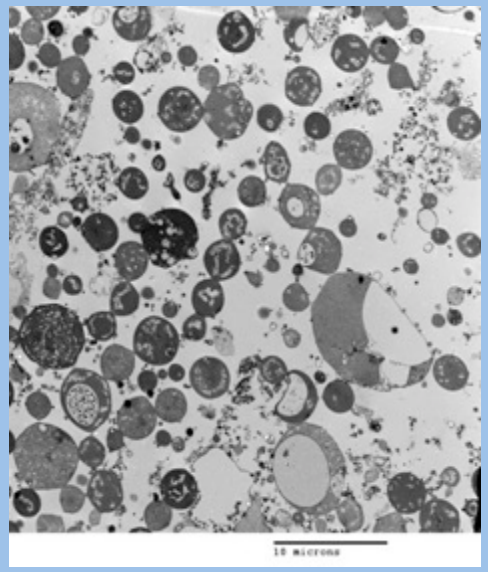
Bacterium *Bacillus subtilis*

Bacteria with cell walls (rods)



Reactive oxygen
(superoxide)

Spherical bacterial protoplasts
(no cell walls)

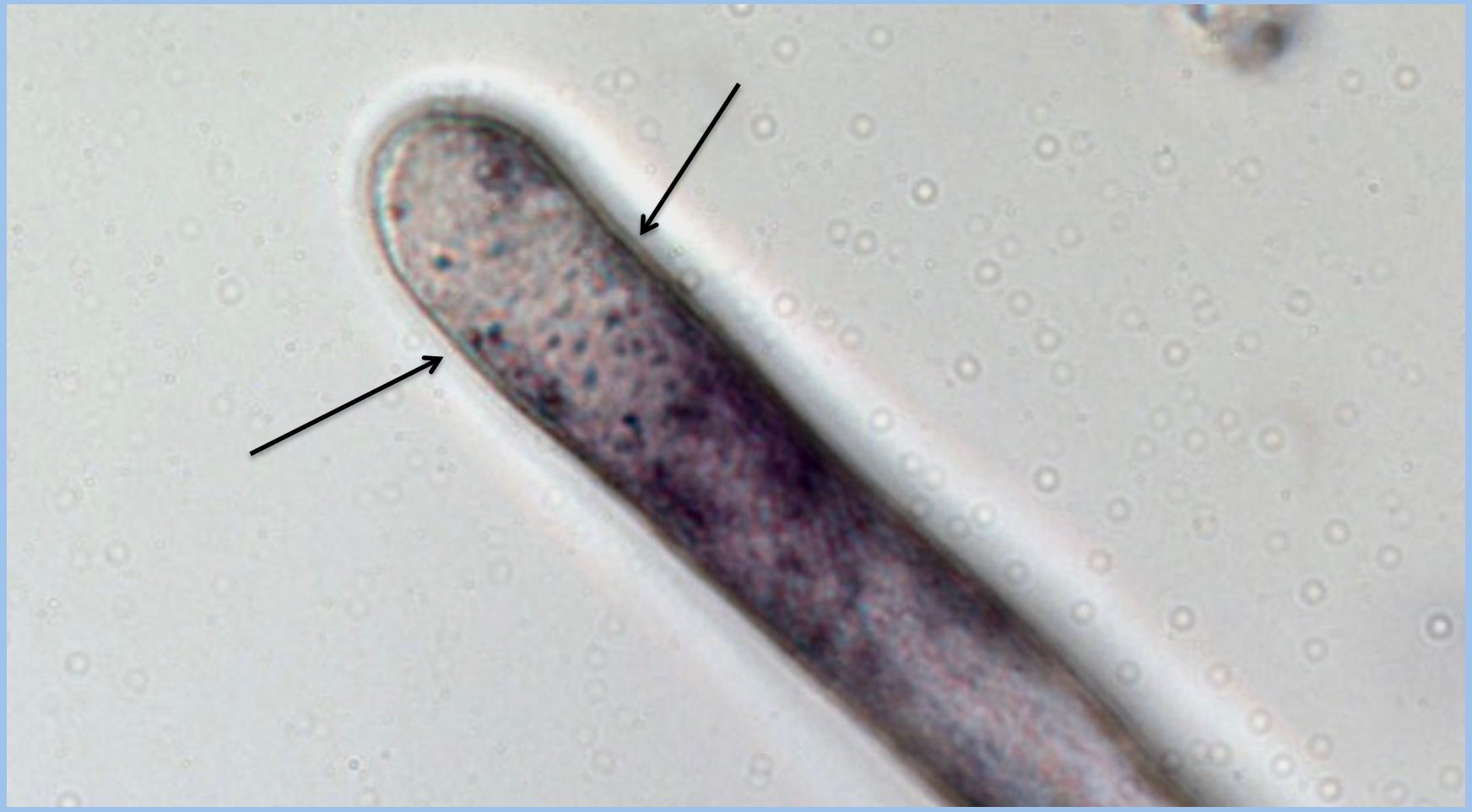


Bacterial protoplasts
are called L-forms.

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

NBT stained tall fescue seedling root showing superoxide at tips of root hairs (arrows) where microbe protoplasts accumulate.

NBT stained tall fescue root hair tip from the 0.04% CO_2 treatment showing purple stained superoxide around microbes.



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

Reactive Oxygen Staining Technique

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Reactive oxygen secretion is used by all eukaryotes to kill endoparasitic bacteria. It is part of the innate Defensive system of all Eukaryotes.

How DAB Works:

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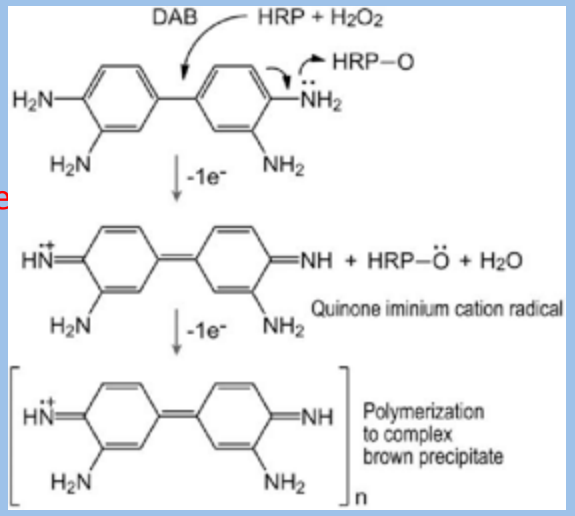
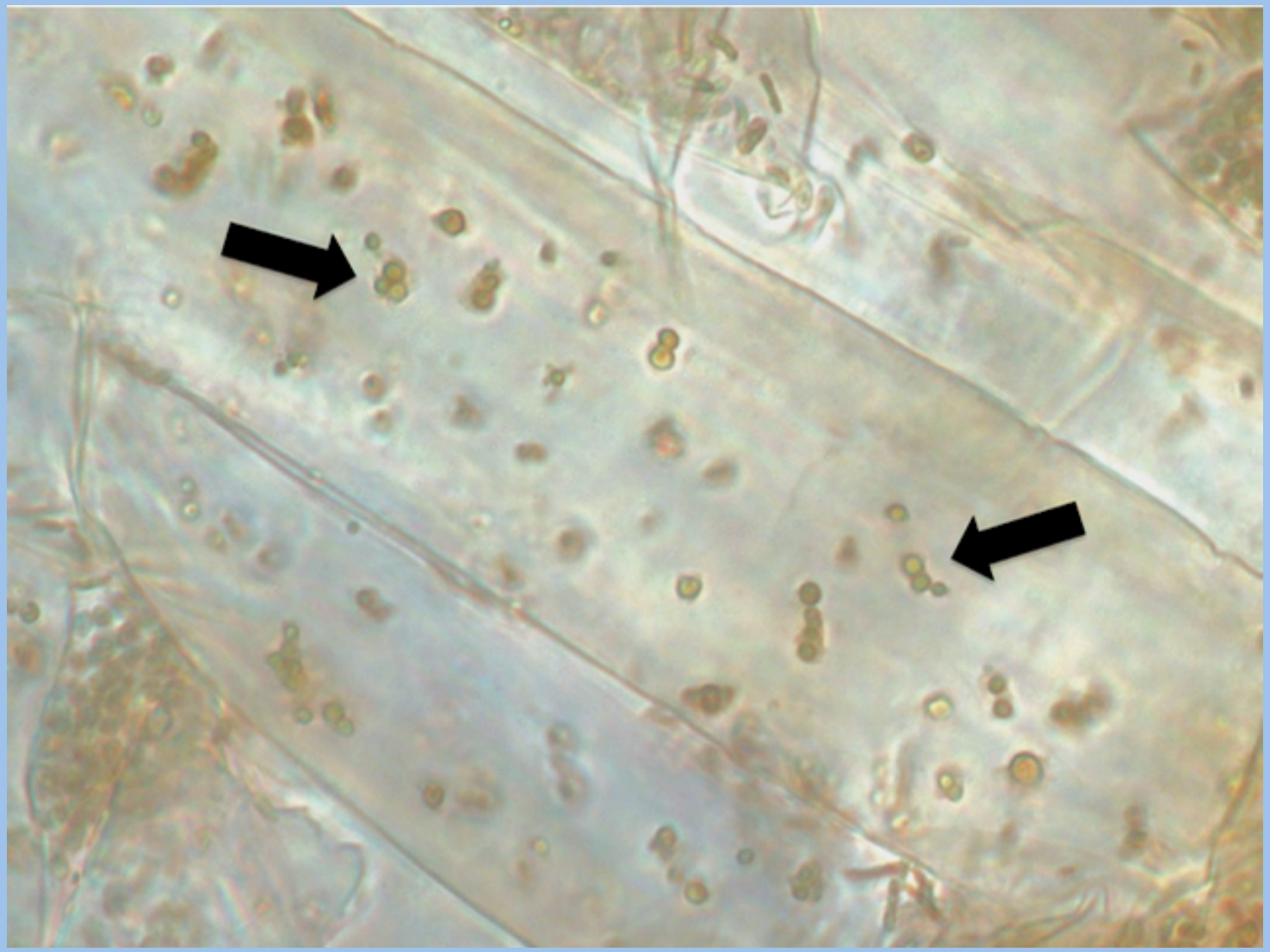
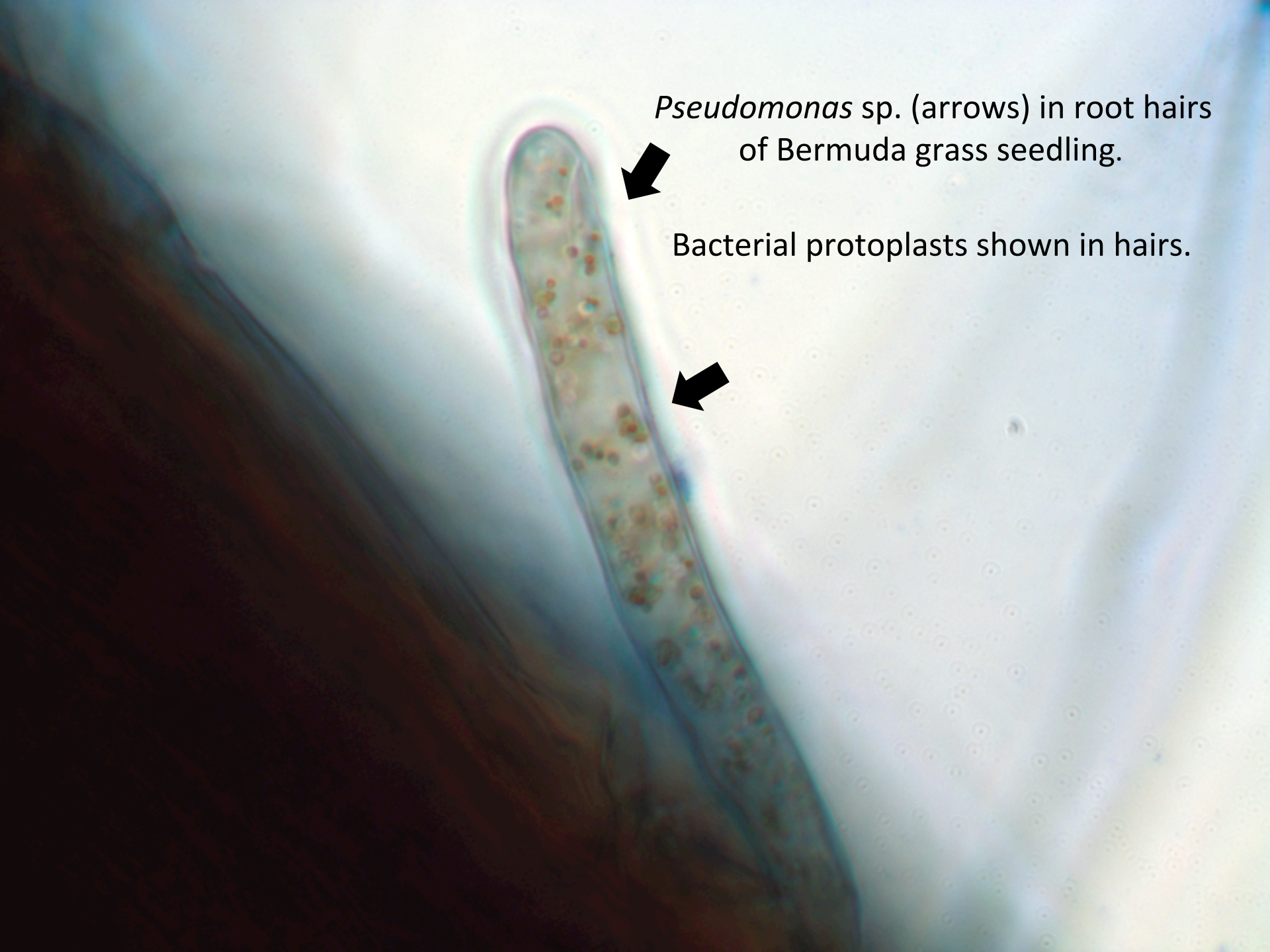


Phragmites root stained with diaminobenzidine DAB to visualize reactive oxygen around bacterial protoplasts (arrows). Reactive oxygen is visualizable as brown or red coloration around bacteria. The reactive oxygen is the result of superoxide produced by NADPH oxidases on the root cell plasma membranes.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



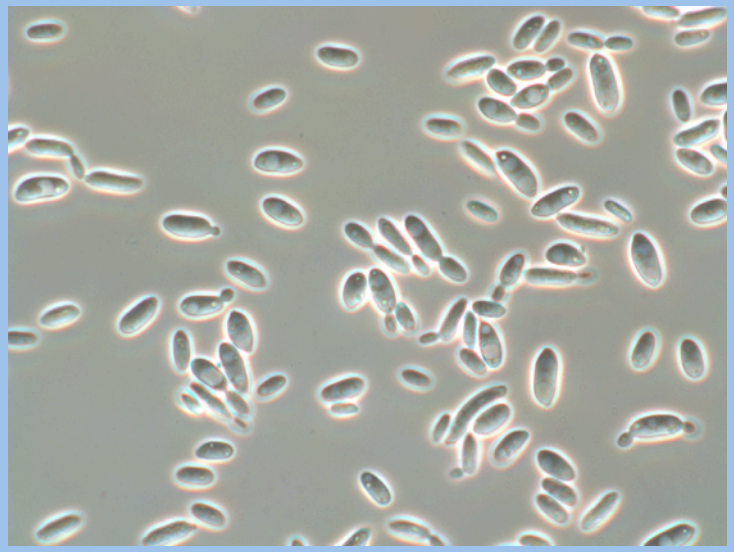

Pseudomonas sp. (arrows) in root hairs of Bermuda grass seedling.
Bacterial protoplasts shown in hairs.

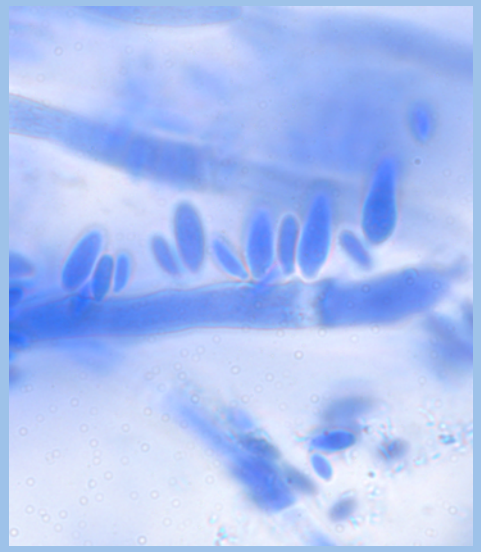
How Superoxide Affects Microbe Protoplasts

1. NADPH oxidase on plant cell plasma membrane converts O_2 to superoxide O_2^{--}.
2. Superoxide breaks down the microbial cell walls.
3. Superoxide damages proteins in the microbe plasma membrane.
4. Superoxide causes leakage in the microbe plasma membrane.
5. Superoxide enters microbe cells and damages proteins and nucleic acids.
6. Microbe protoplasts swell and lose internal proteins as contents are oxidized.

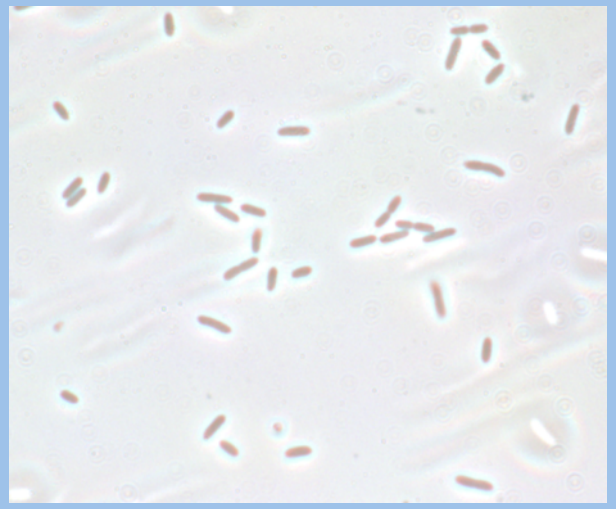
Snakecotton *(Froelichia gracilis)* is an invasive weed in many parts of North America; seeds were sourced from wild populations in New Jersey.

Seed-vectored endophytes from snakecotton seedlings include:





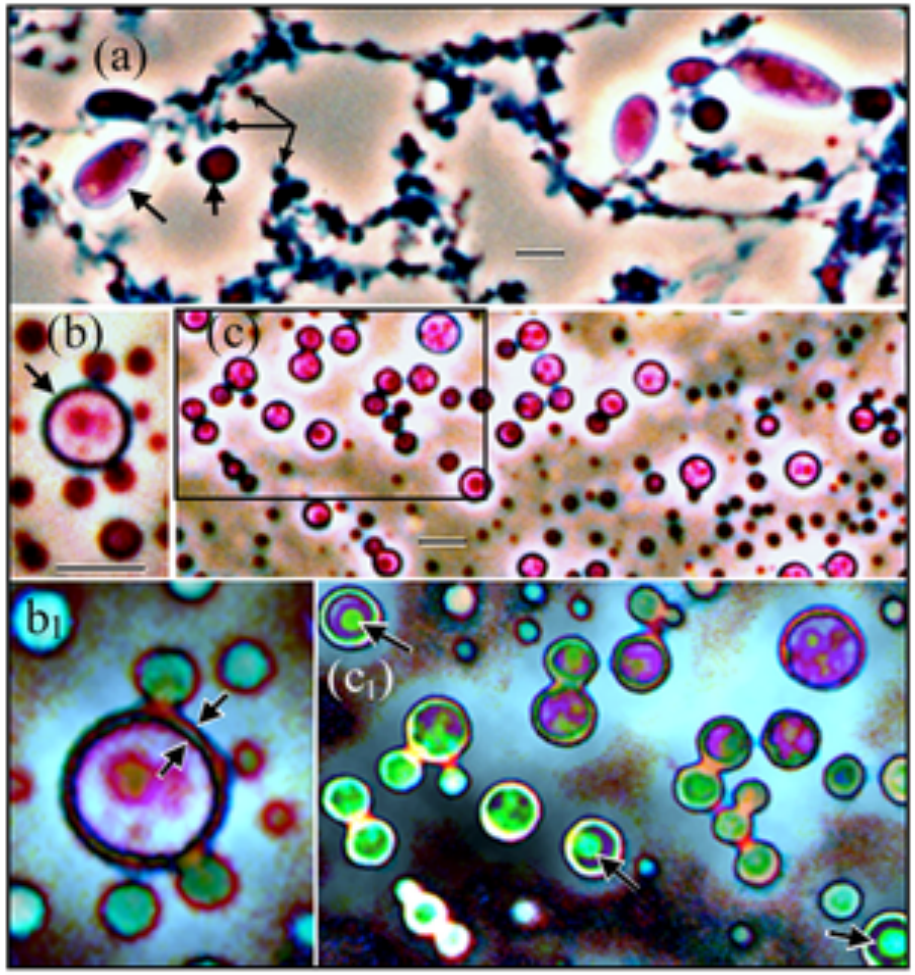
Yeast: *Aureobasidium pullulans* (Froelichia # 2)

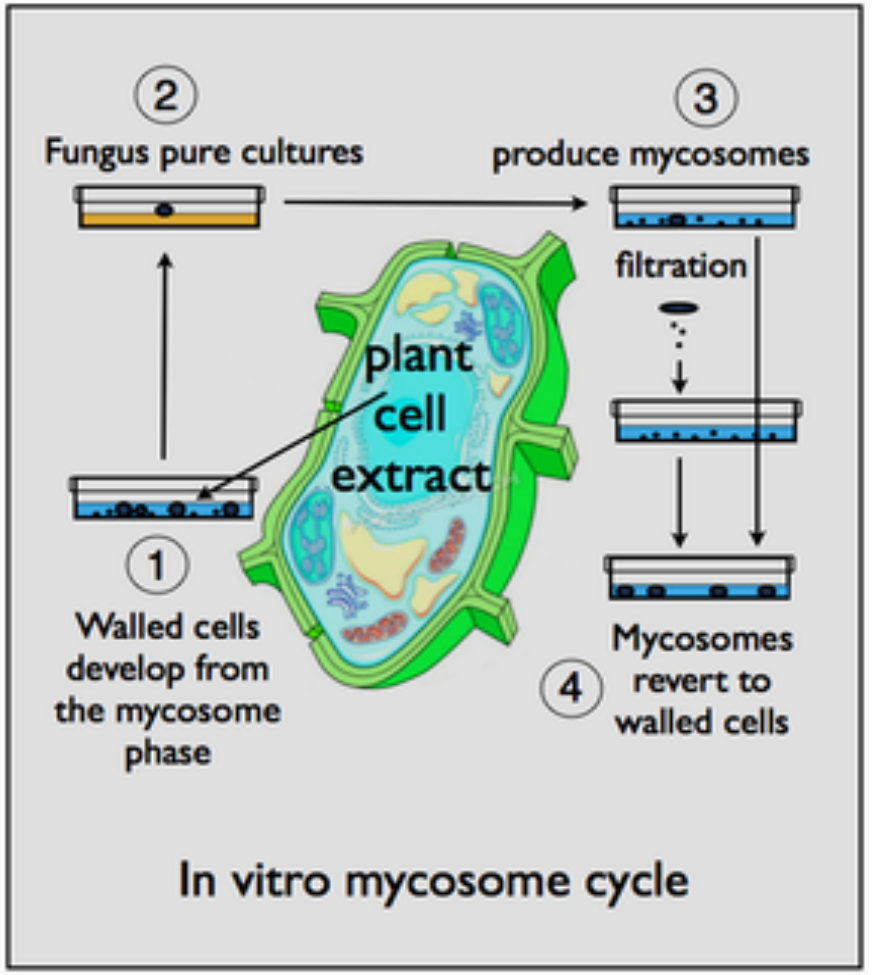


Identifications of bacteria made using Sequences of rDNA and blast to NCBI database. Aureobasidium pullulans identified using morphological features in culture.

Bacterium: *Curtobacterium* sp. (Froelichia #4)

Intracellular phases of fungi may form protoplasts called ‘mycosomes’.

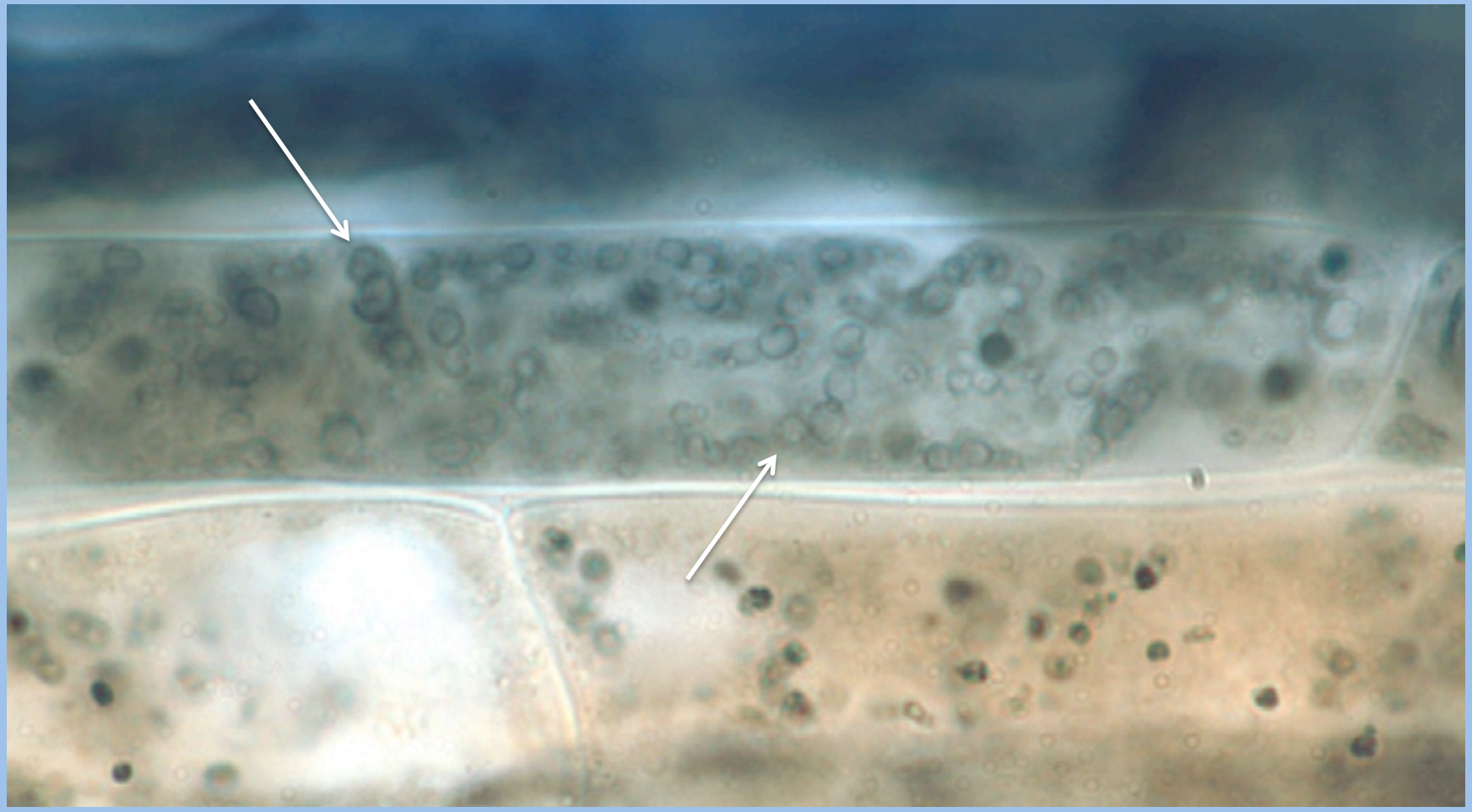




Atsatt PR, Whiteside MD (2014) Novel Symbiotic Protoplasts Formed by Endophytic Fungi Explain Their Hidden Existence, Lifestyle Switching, and Diversity within the Plant Kingdom. PLOS ONE 9(4): e95266. https://doi.org/10.1371/journal.pone.0095266
http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0095266

PLOS ONE

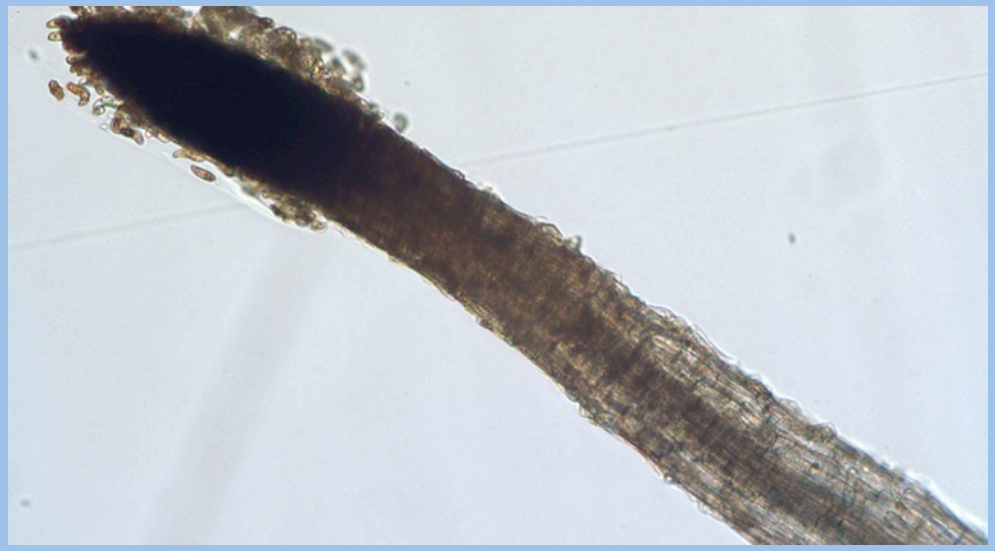
Mycosomes (arrows) in snakecotton seedling root parenchyma.



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)

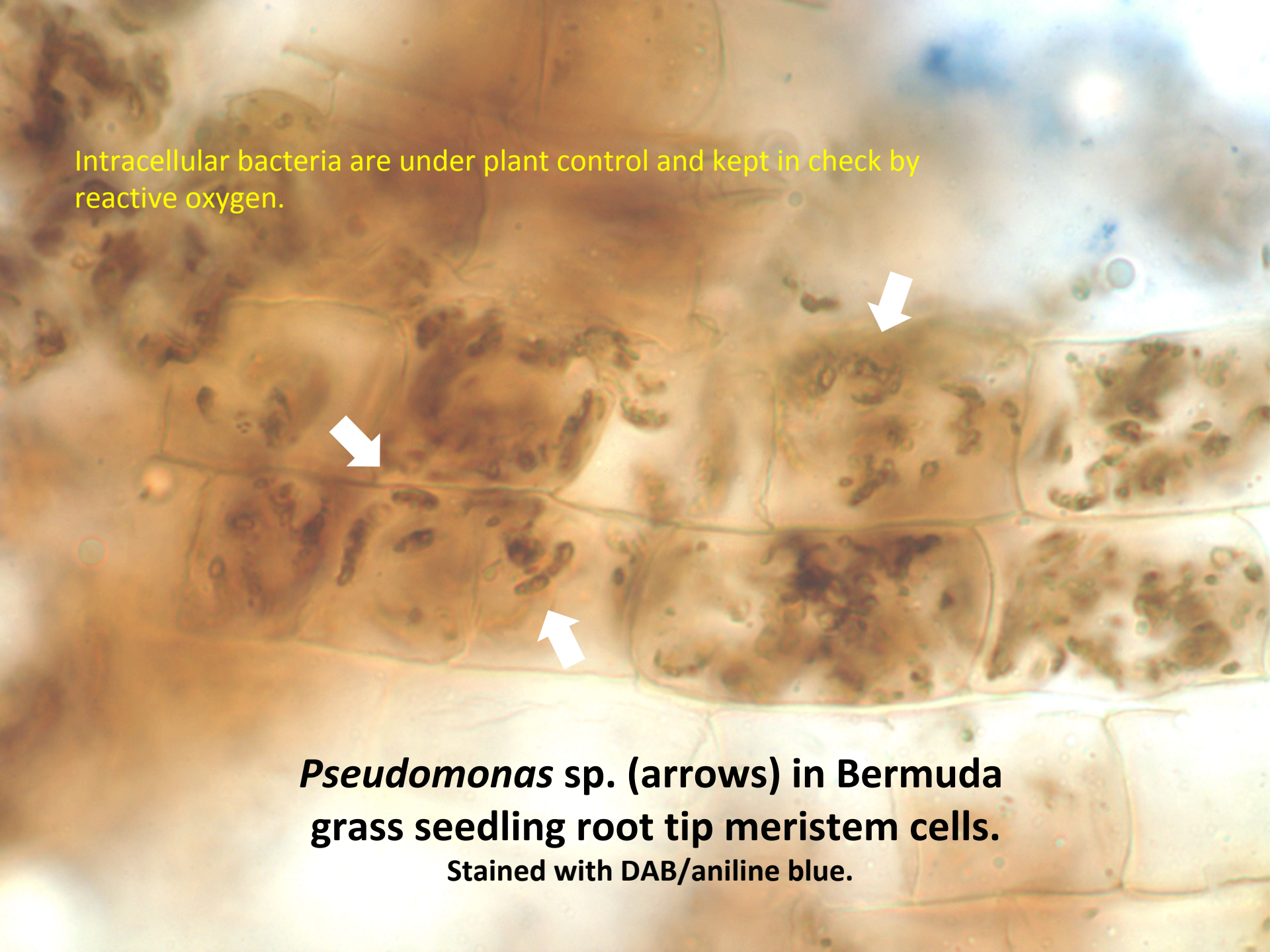
Intracellular in root parenchyma

Bacteria stimulate root hair formation In root epidermis

Bacteria emerge to surface of hair as the hair elongates

Bacteria colonize soil rhizosphere

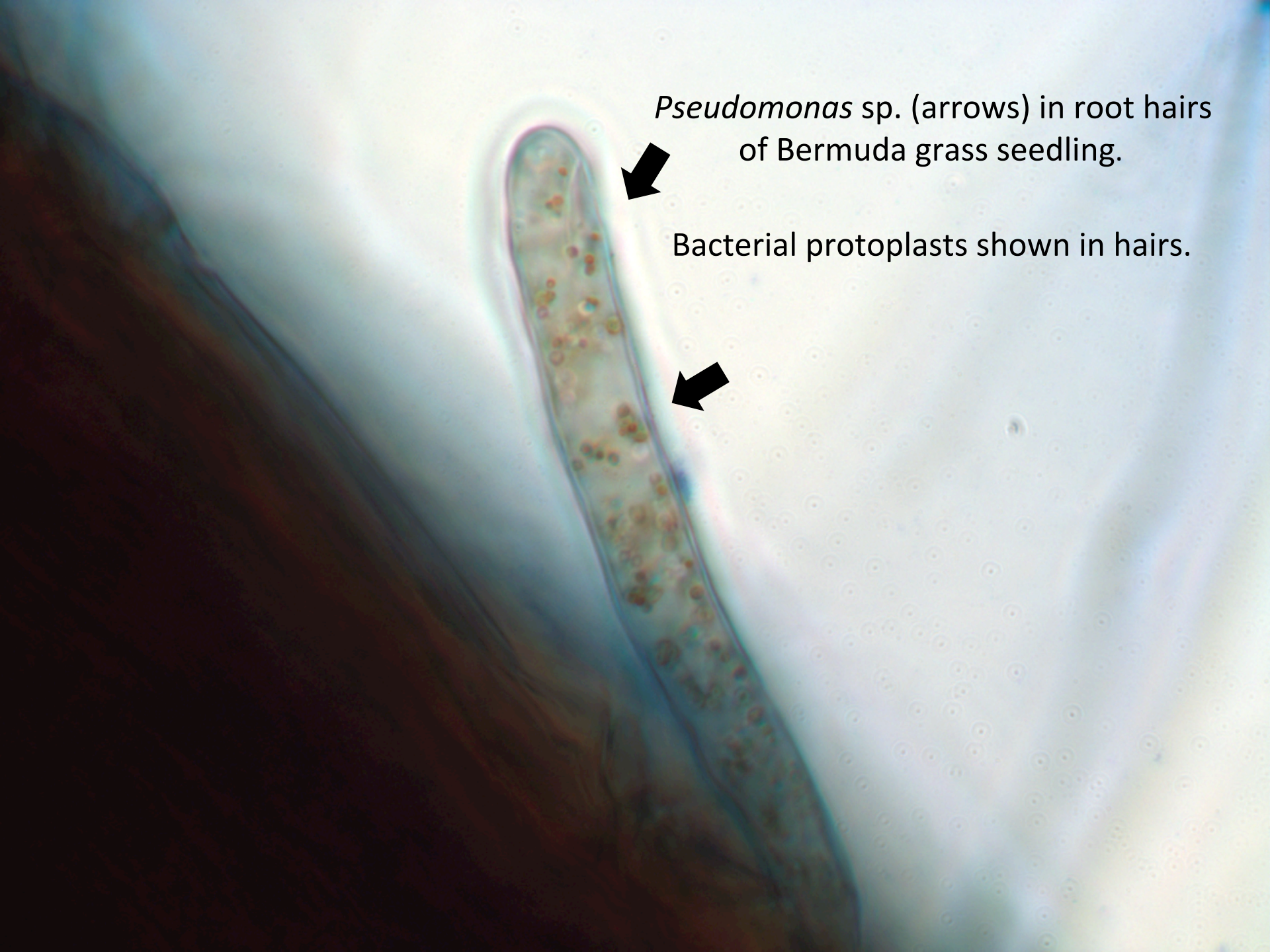
Bacteria acquire nutrients in rhizosphere



***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.

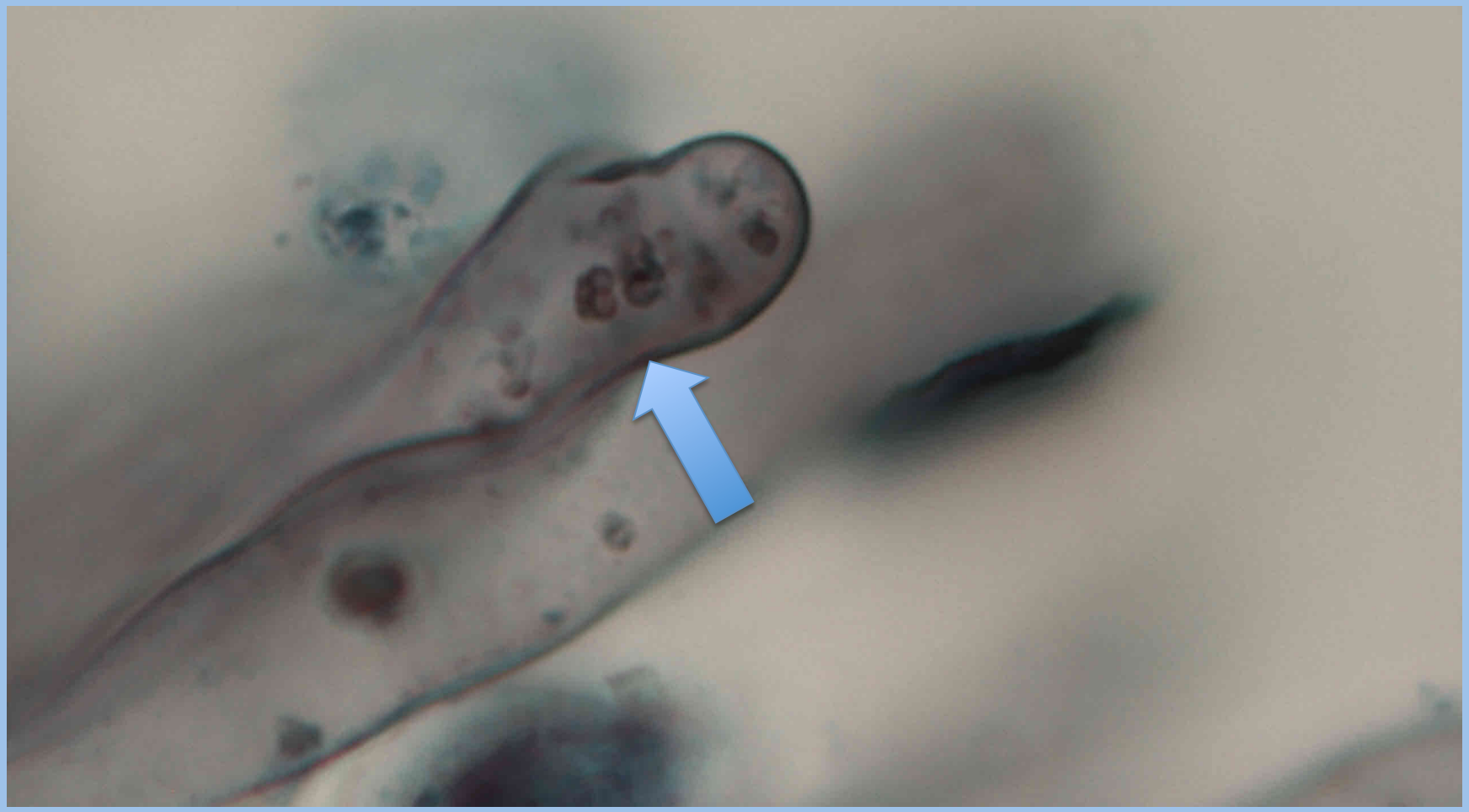

Pseudomonas sp. (arrows) in root hairs of Bermuda grass seedling.
Bacterial protoplasts shown in hairs.

Lara Brindisi

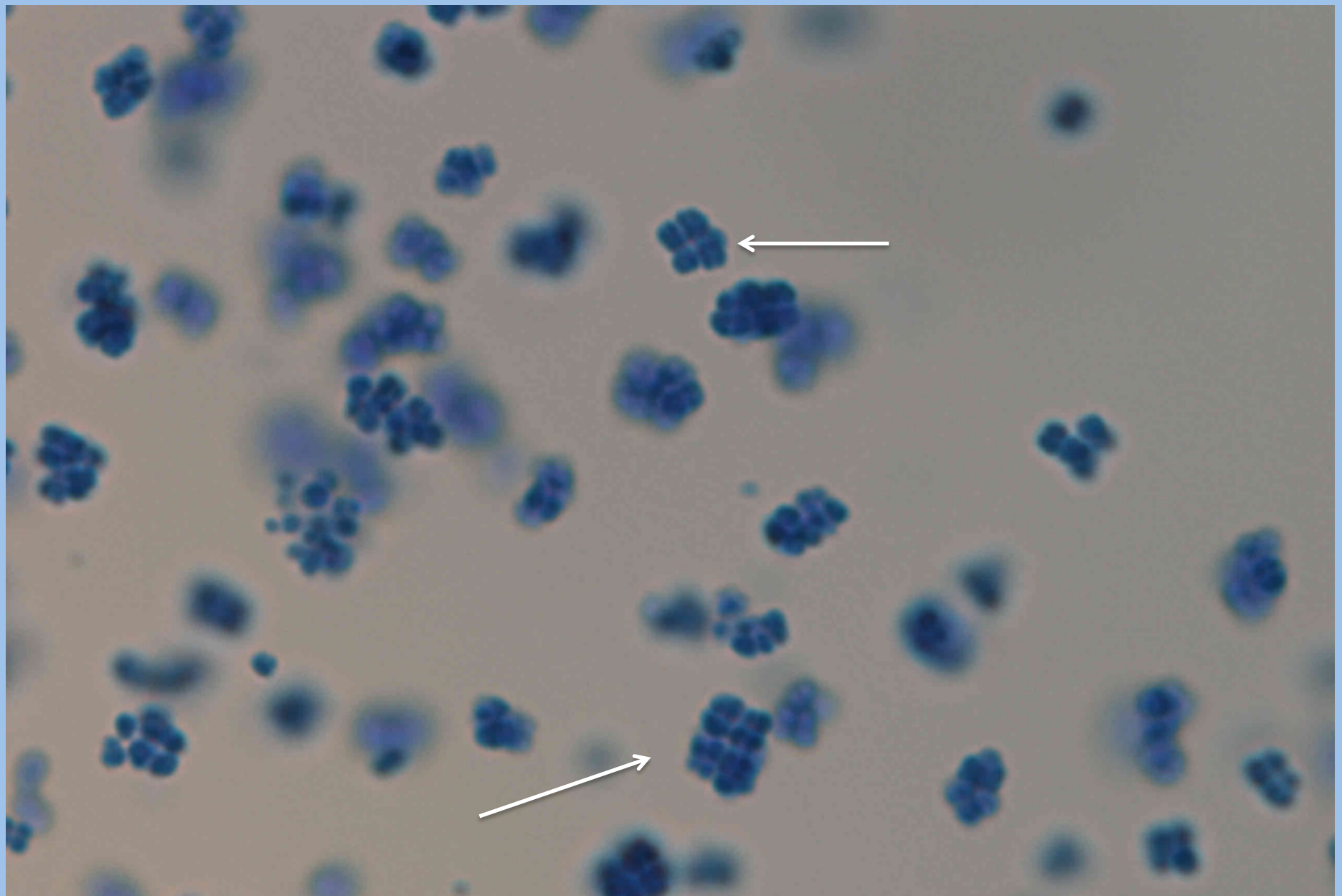
Tomatoes harbor endophytic microbes that stimulate growth of roots and root hairs in tomato seedlings. *Micrococcus luteus* is one of the tomato endophytes.

Satish K. Verma

Endophyte is visible intracellularly as singlets to packets of cells (arrow)



Micrococcus luteus from culture showing tetrads (arrows).



Curley dock (*Rumex crispus;* Polygonaceae) endophyte colonization experiment

Procedure:

1. Seeds of Rumex crispus were surface disinfected to remove native bacteria.

2. Seeds placed onto agarose.

3. ½ seeds inoculated with suspension of *Micrococcus luteus* in water.

4. Seeds incubated for 7 days.

5. Seedlings stained in diaminobenzidine tetrachloride for 15 hours.

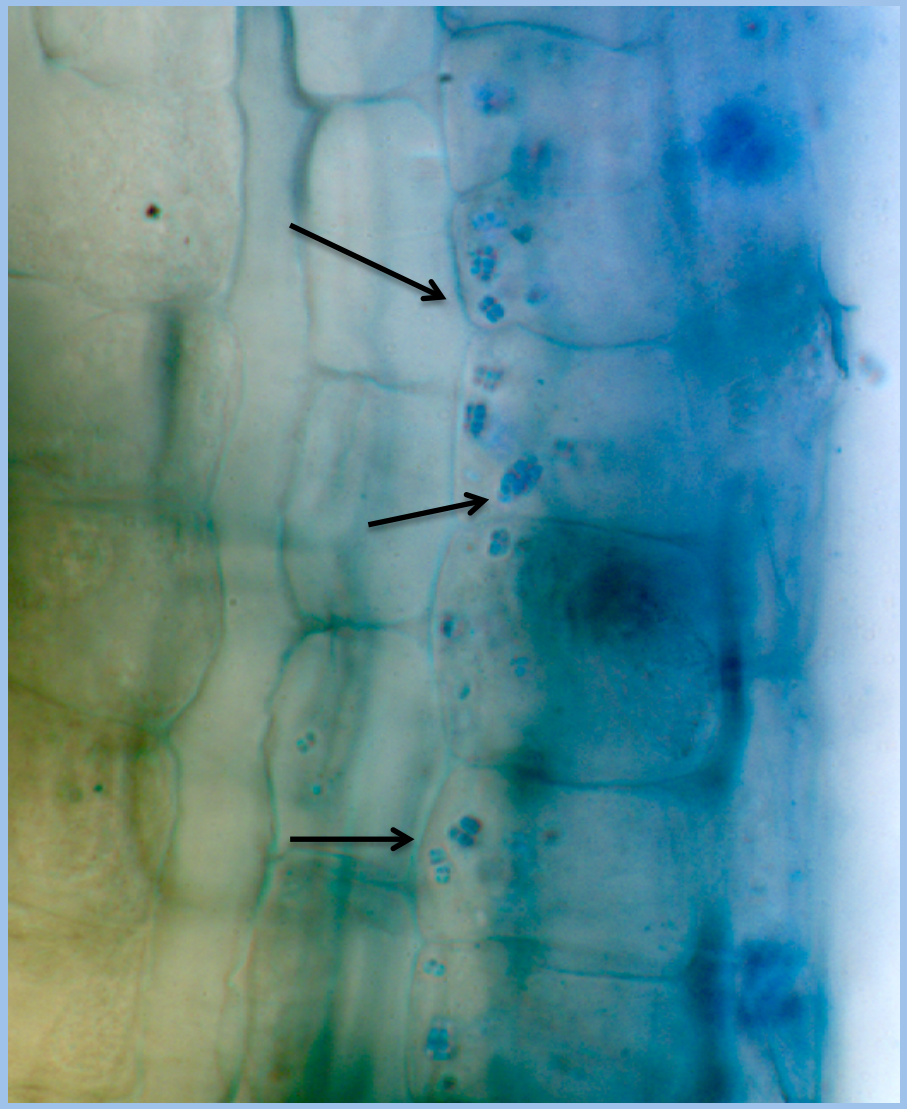
6. Slides prepared using aniline blue and examined microscopically.

7. All evidence of bacteria in seedling tissues photographed.

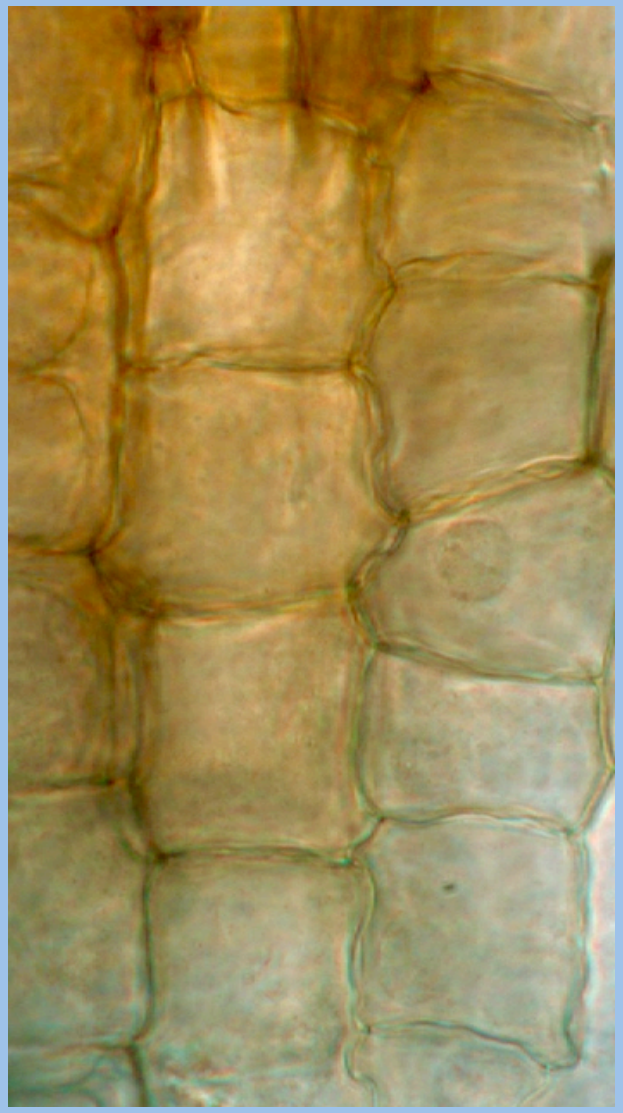
James White 5/26/2018
Rutgers University

Micrococcus luteus in *Rumex crispus*: *Path of bacteria in the rhizophagy cycle*

Micrococcus enters the outer two cell layers of the root tip meristem as walled tetrads (left photo, arrows). The bacterium is present only in the periplasmic space between cell wall and plasma membrane. The image to the right shows root meristematic cells of a seedling that was not inoculated with *Micrococcus luteus*. All tissues were stained with DAB followed by aniline blue.

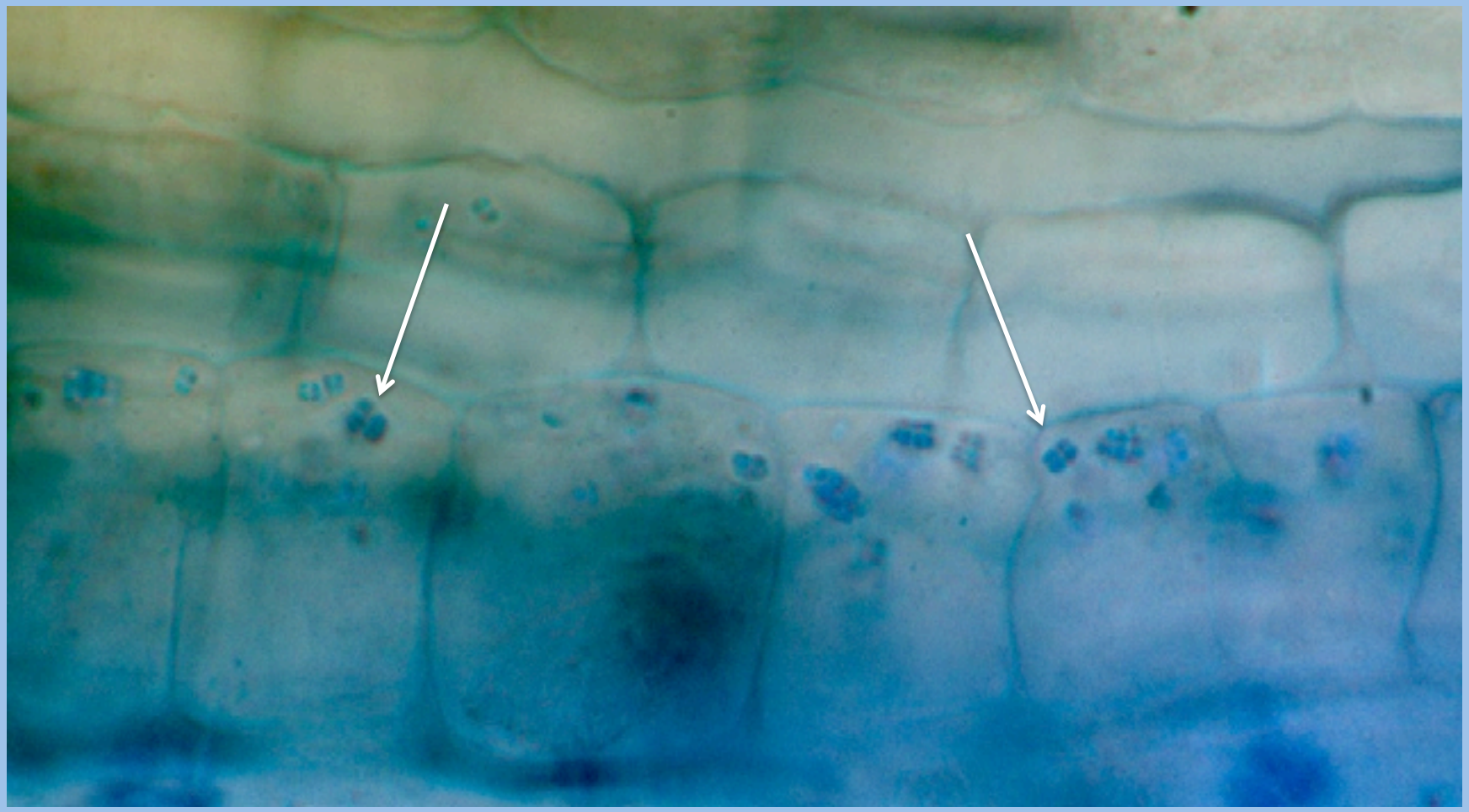


Seedling inoculated *with Micrococcus luteus*

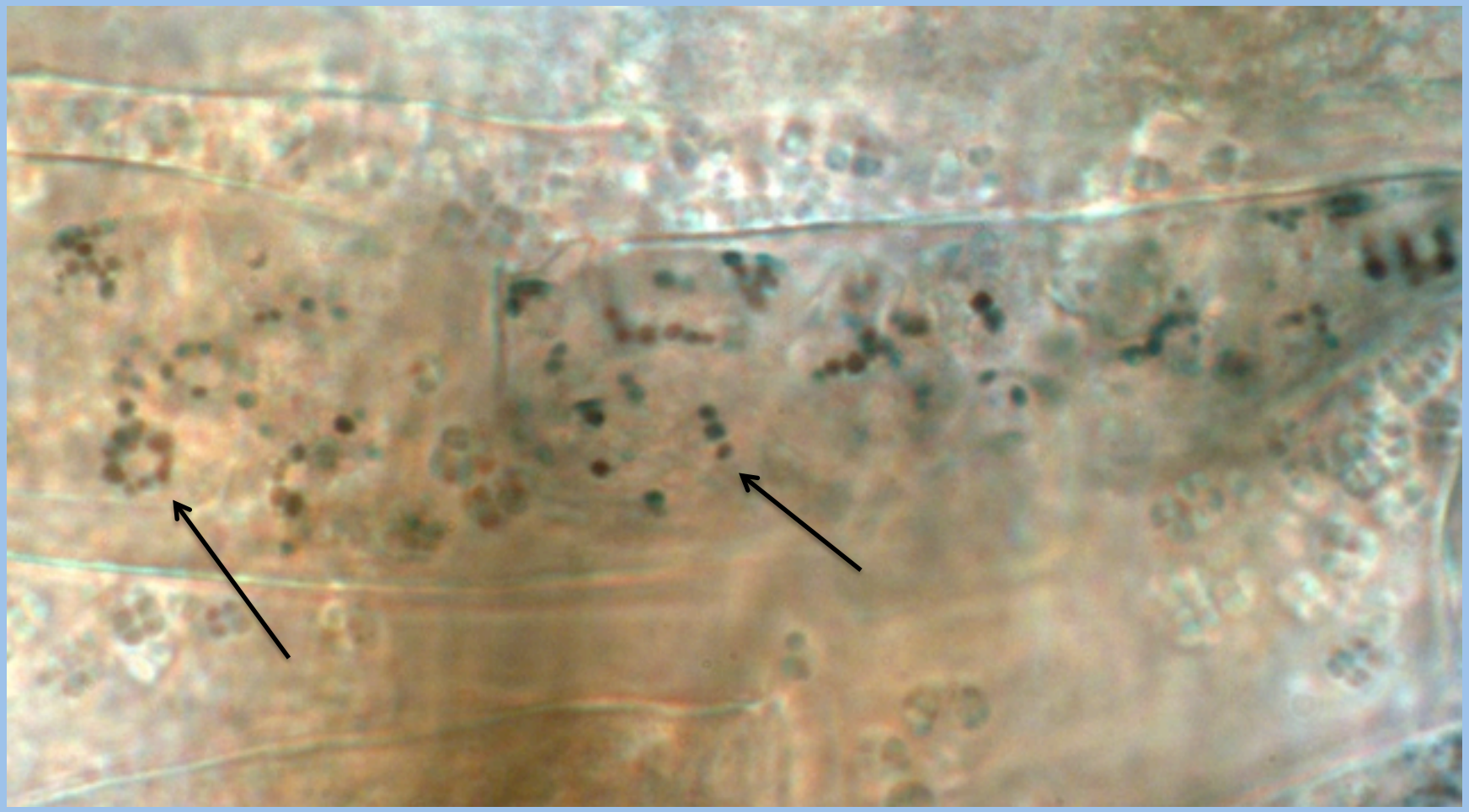


Seedling not inoculated

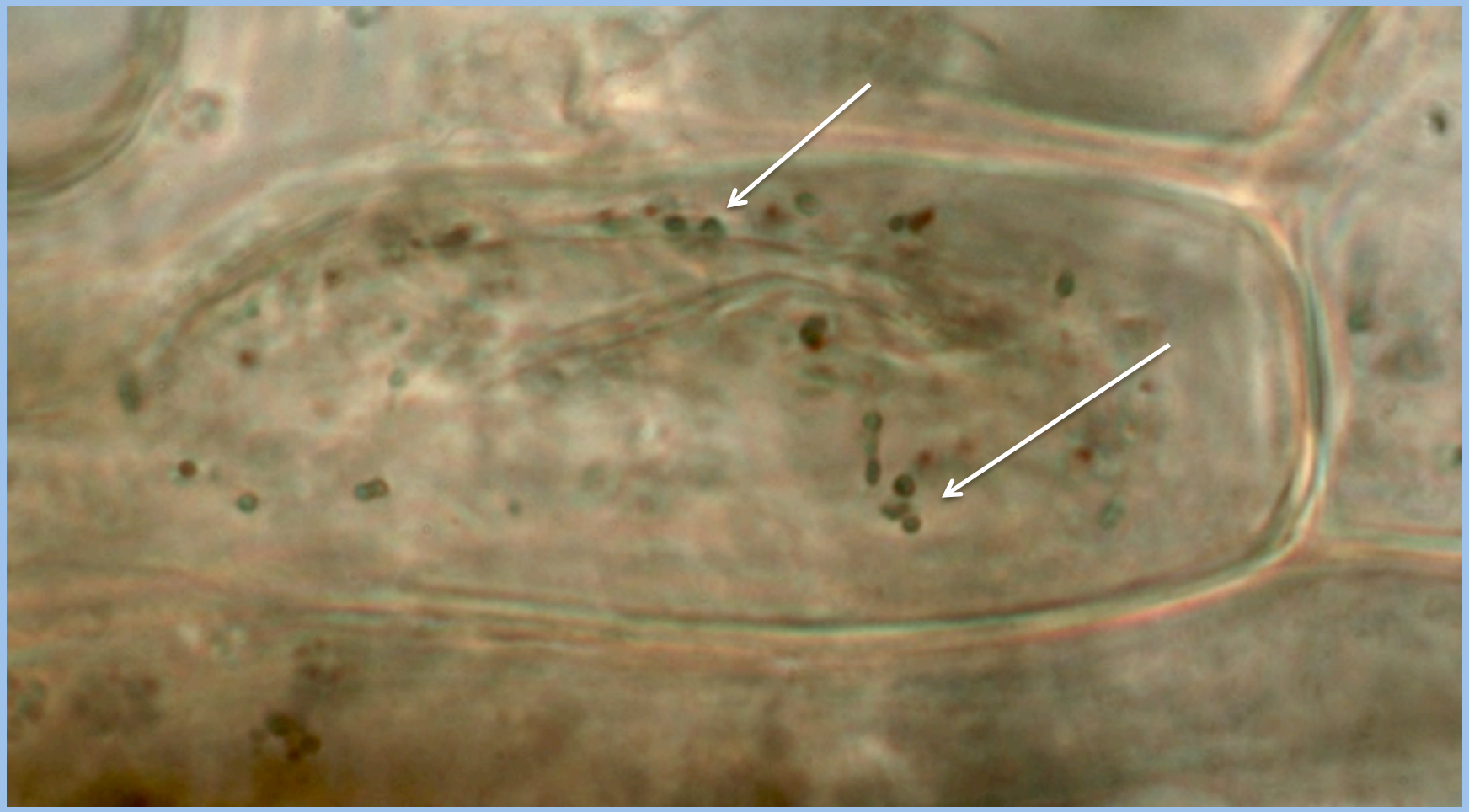
Close-up of *Micrococcus* tetrads (arrows) in periplasmic space of root meristematic cells (stained with DAB and aniline blue).



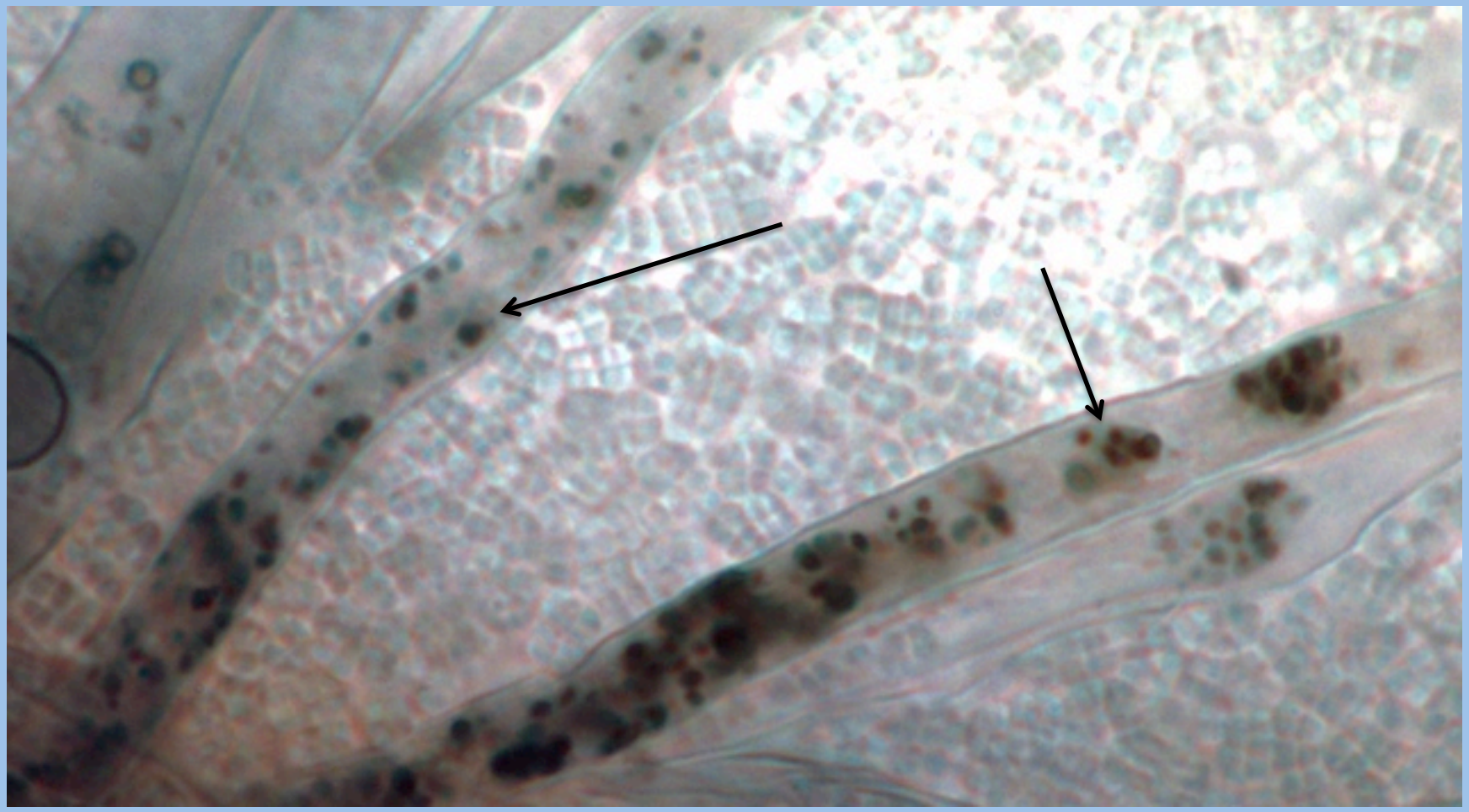
In older cells tetrads lose clustered form convert into smaller spherical cells (arrows). These are staining with aniline blue. These are likely wall-less protoplasts.



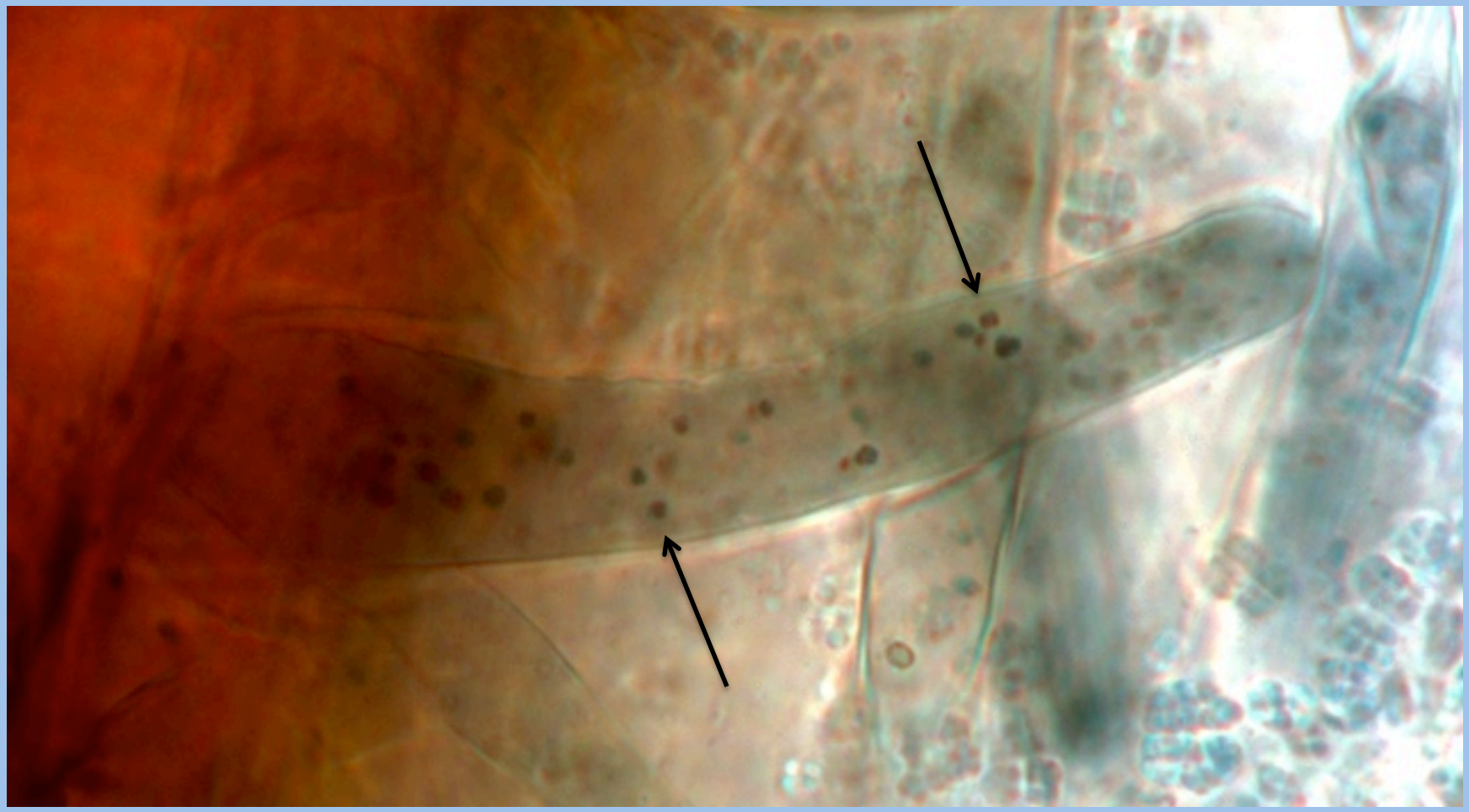
Spherical L-forms (arrows) in the periplasmic space of root parenchyma.



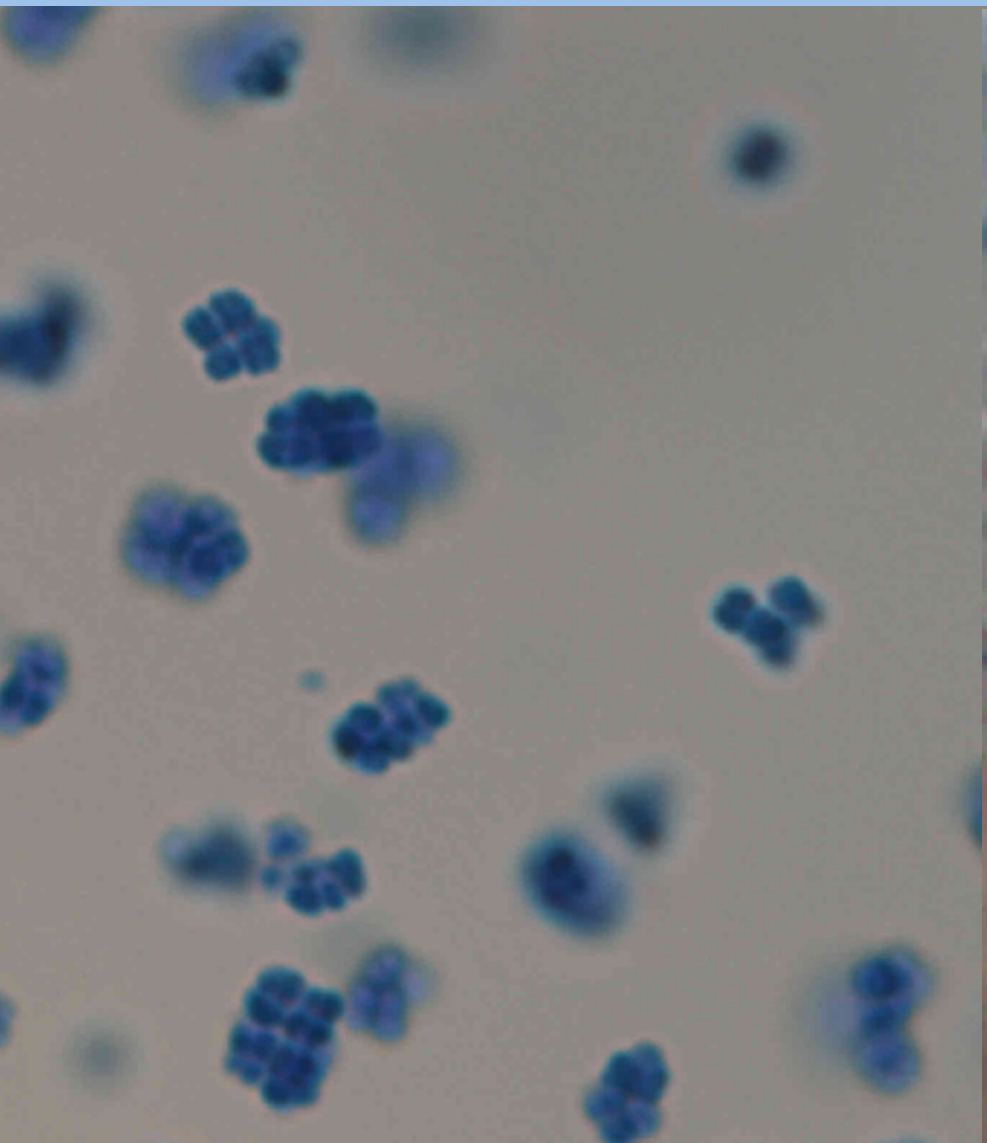
Spherical protoplasts (arrows) are present in root hairs as they form. Reddish brown color is due to reactive oxygen secreted from NADPH oxidases on the plant cell membrane. Blue is from aniline blue stain.

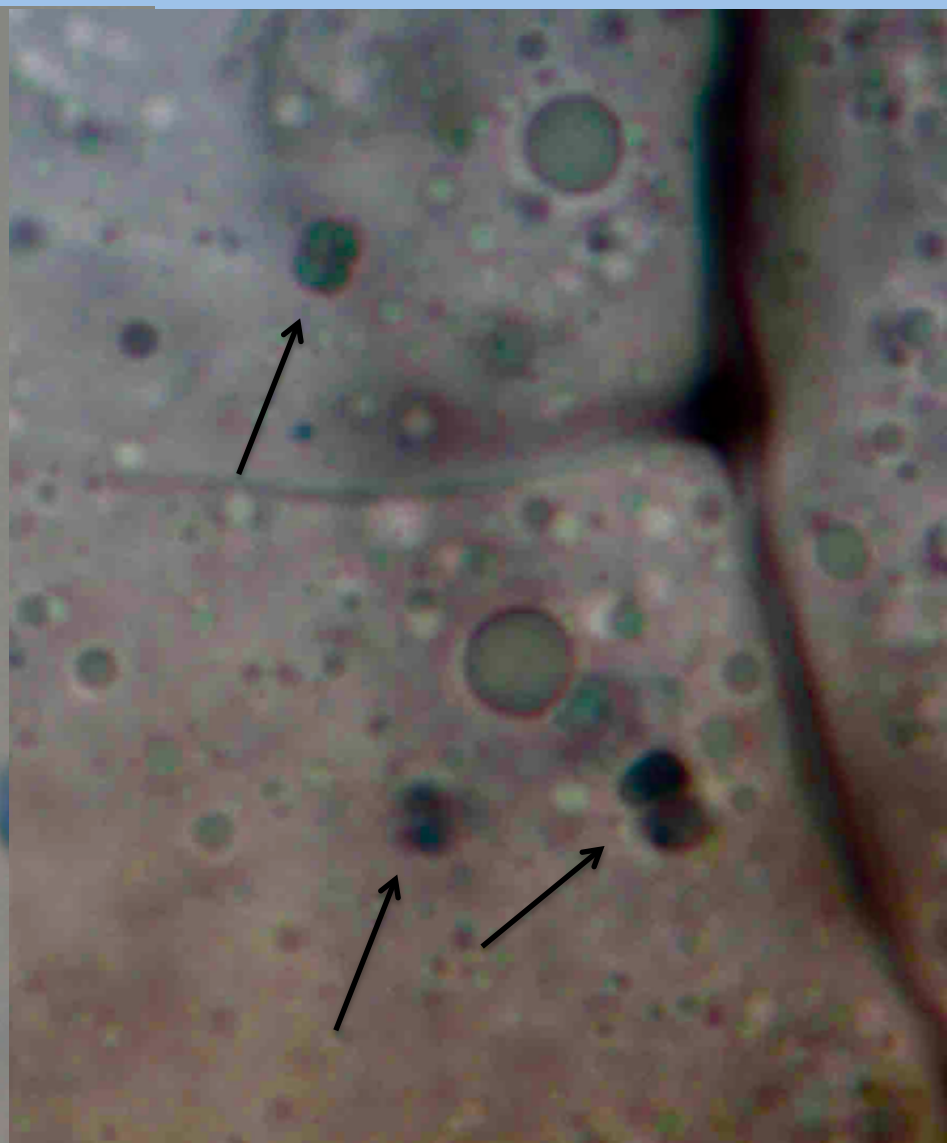


Spherical protoplasts (arrows) of bacterium (*M. luteus*) in developing root hair.

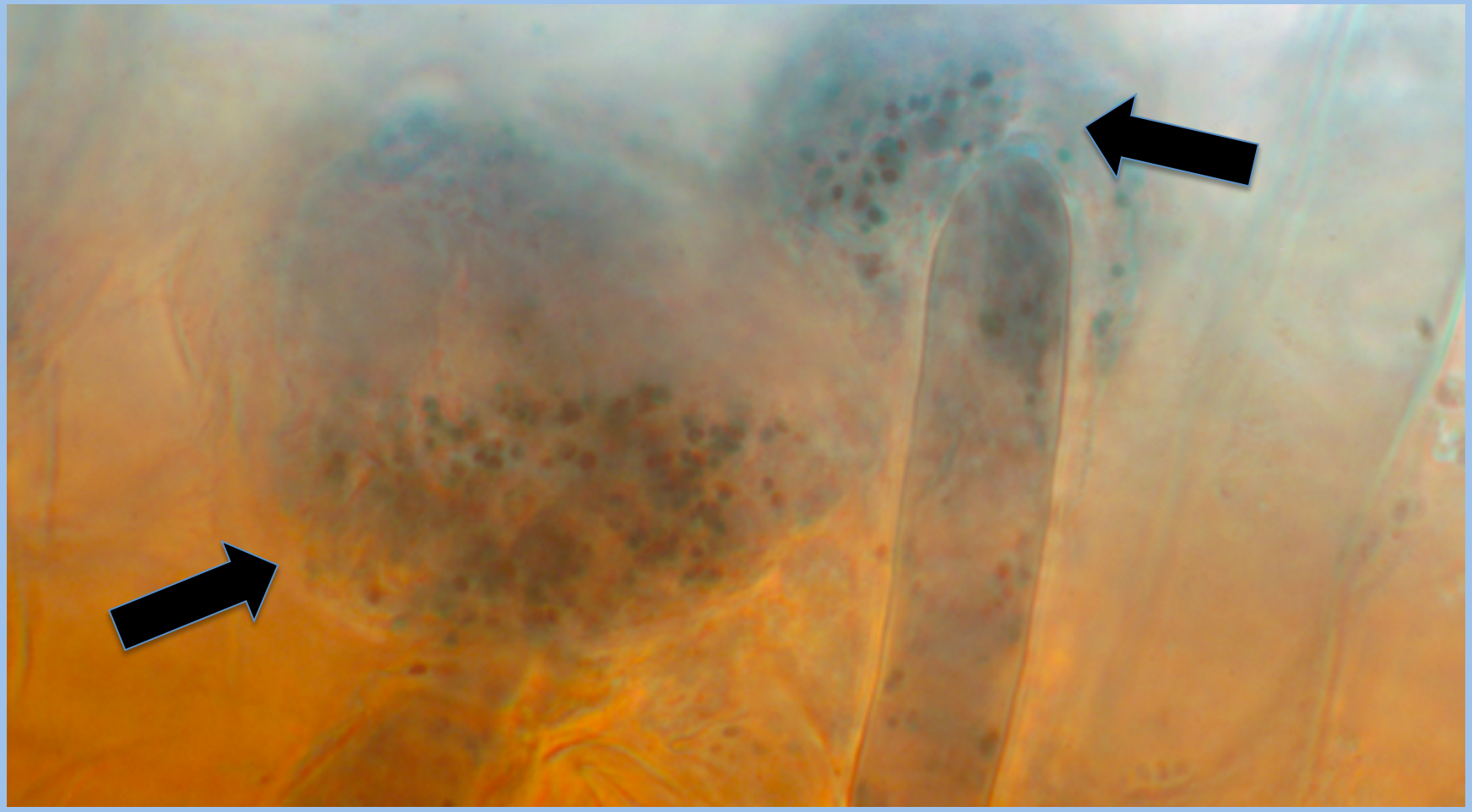


In some cases protoplasts reform tetrads (with walls) in root cells. The image to the left is of *Micrococcus luteus* from culture. The image to the right shows protoplasts and tetrads (arrows) reforming in root parenchyma cells of a dandelion seedling.

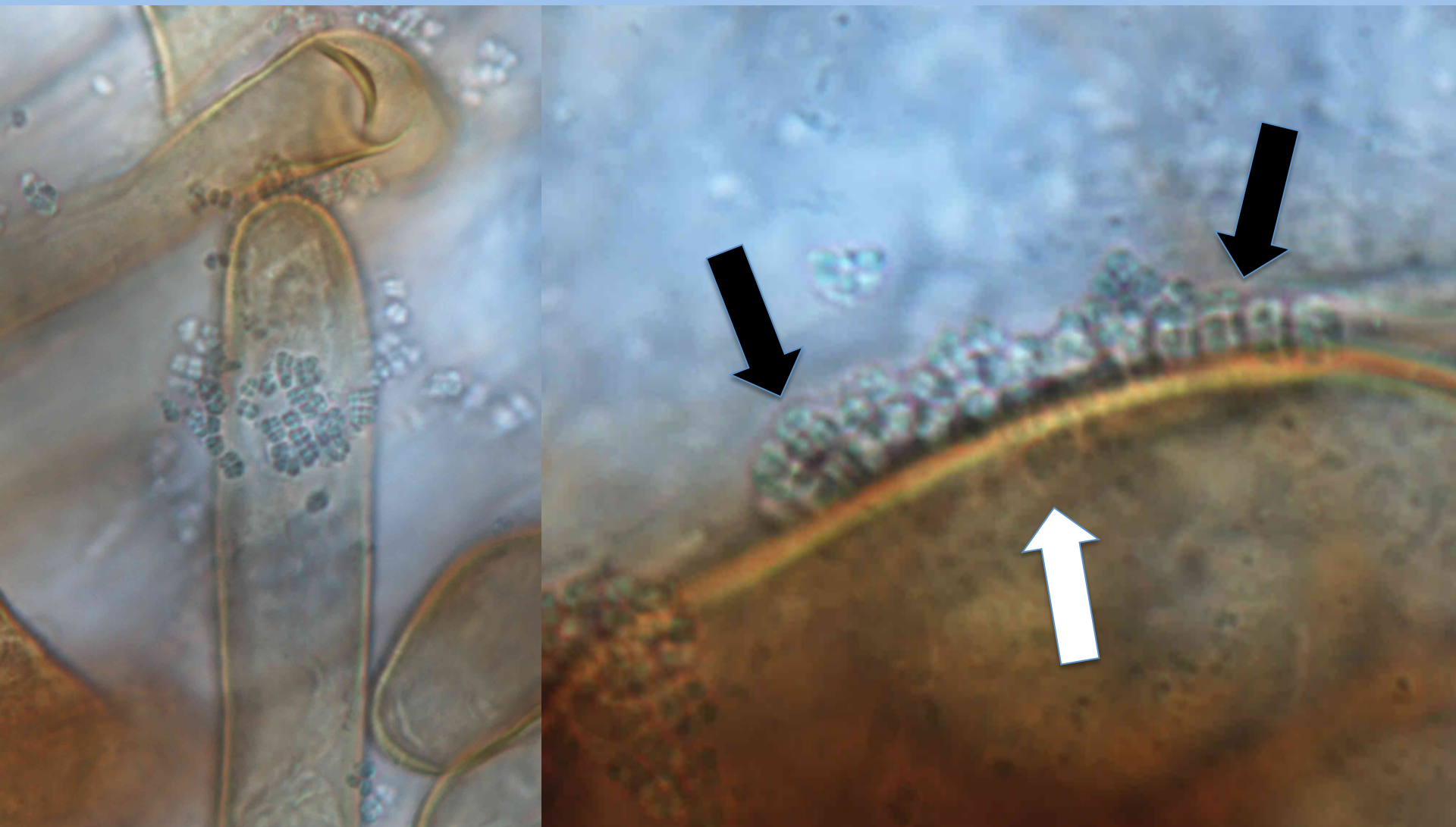




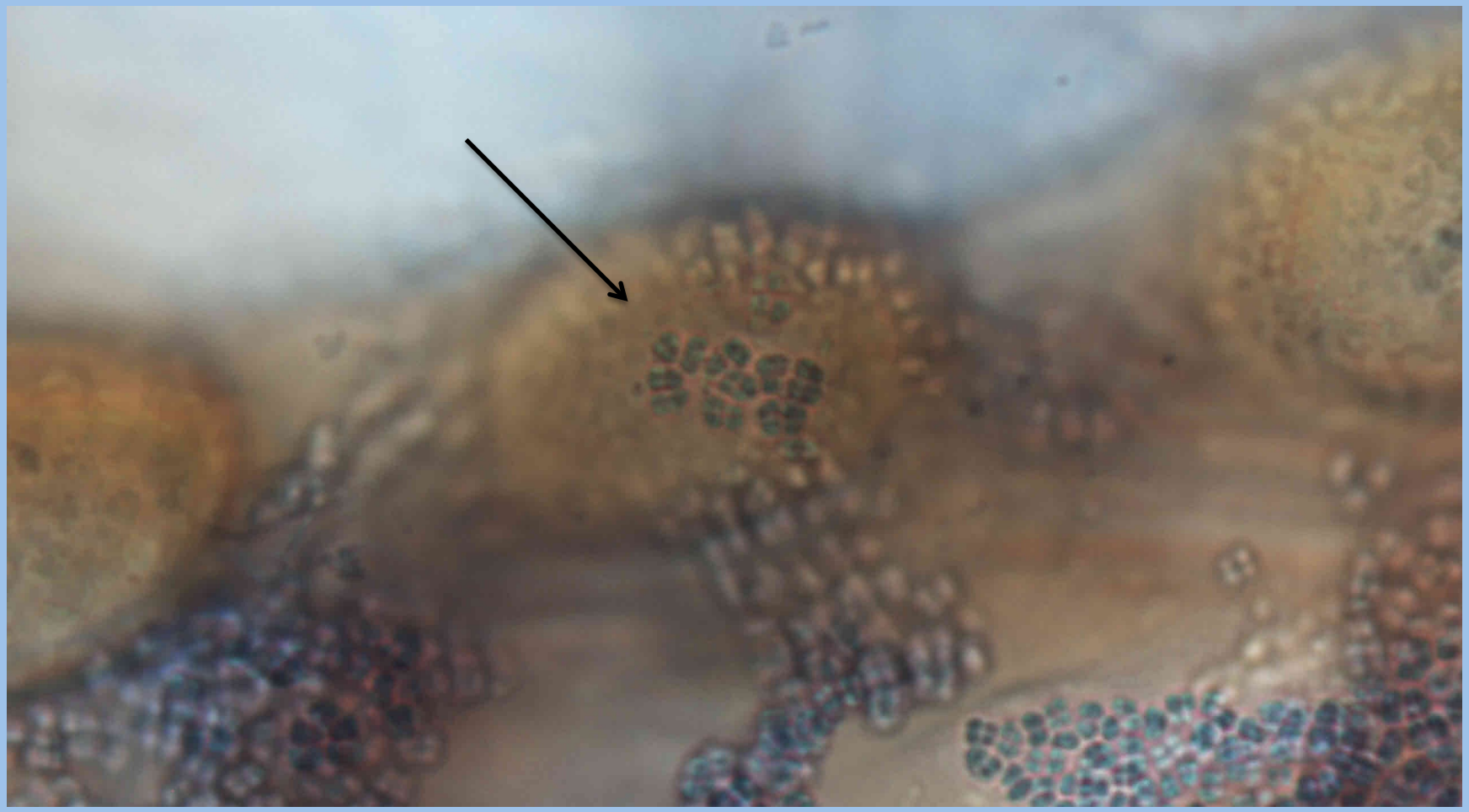
Two root hairs showing masses of bacteria (arrows) emerging from hairs.



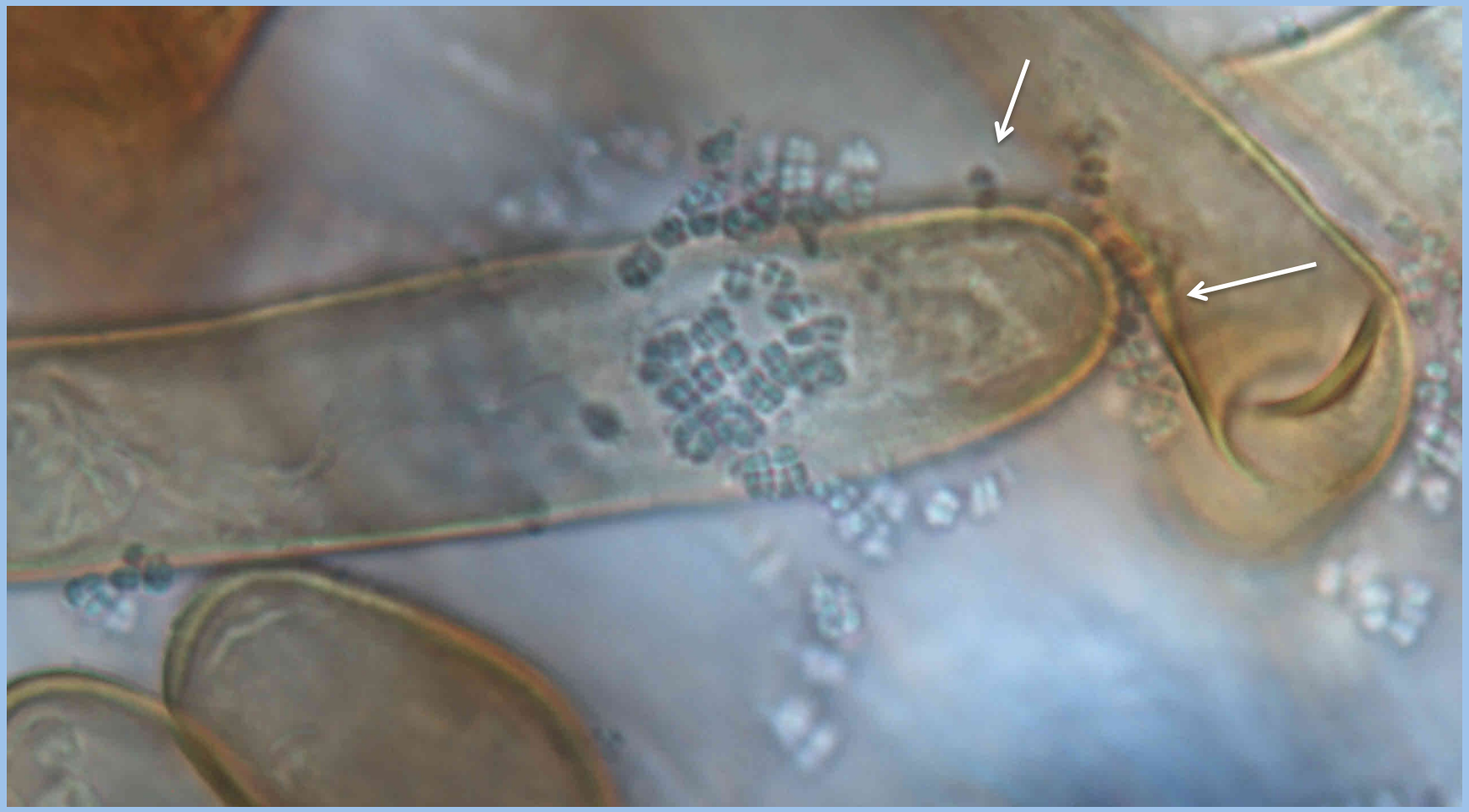
Micrococcus luteus bacteria exiting root hair tips. Spherical L-forms (white arrow) visible within hair beneath plant cell wall, tetrads of cells (black arrows) outside wall, and channels visible through plant cell wall. Host is *Rumex crispus* seedling.



Rumex root hair initial showing bacterial emergence to surface (arrow). Bacterial cells appear to be spilling off of the hairs and accumulating around the hairs. Note that the youngest (smallest and lightest staining) bacterial cells are on the surface of the hair initial.



Rumex root hair showing emergence of bacterial cells through channels at tip (arrow).



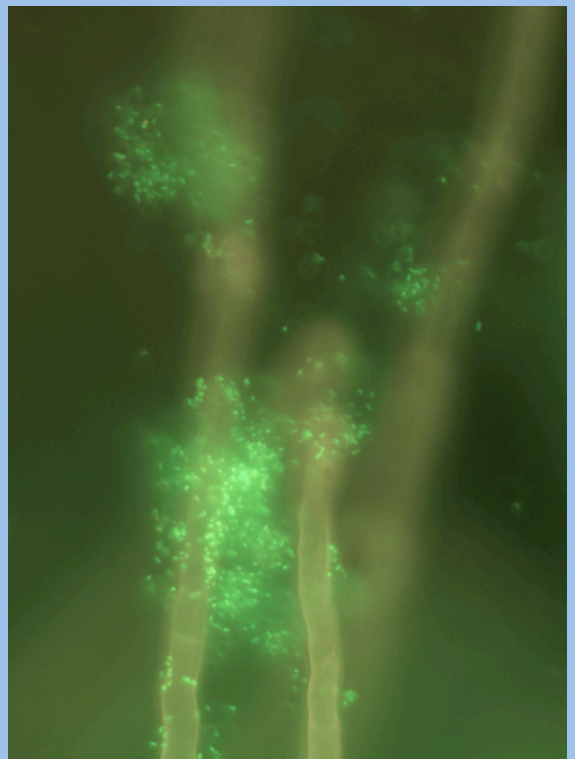
What is the function of root hairs?

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.

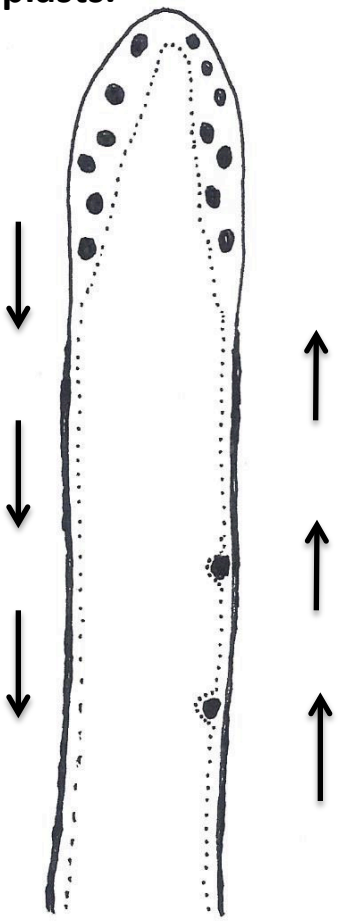
Bacteria emerging from tips of elongating root hairs. Stained with nuclear stain Syto 13.

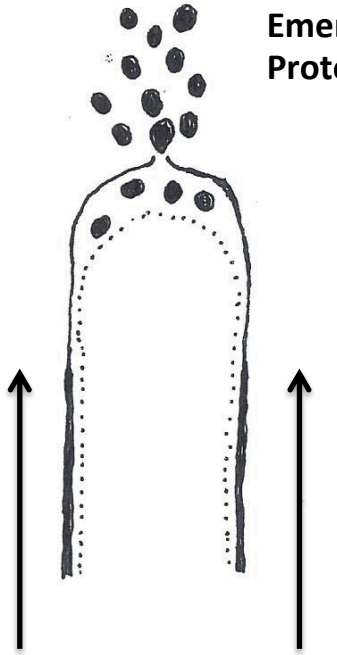


Bacteria emerging from root hair tip. Bacteria in hairs are present as wall-less L-forms. Bacteria reform their walls after exiting from the tip of the hair.

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.





Expansion Wave

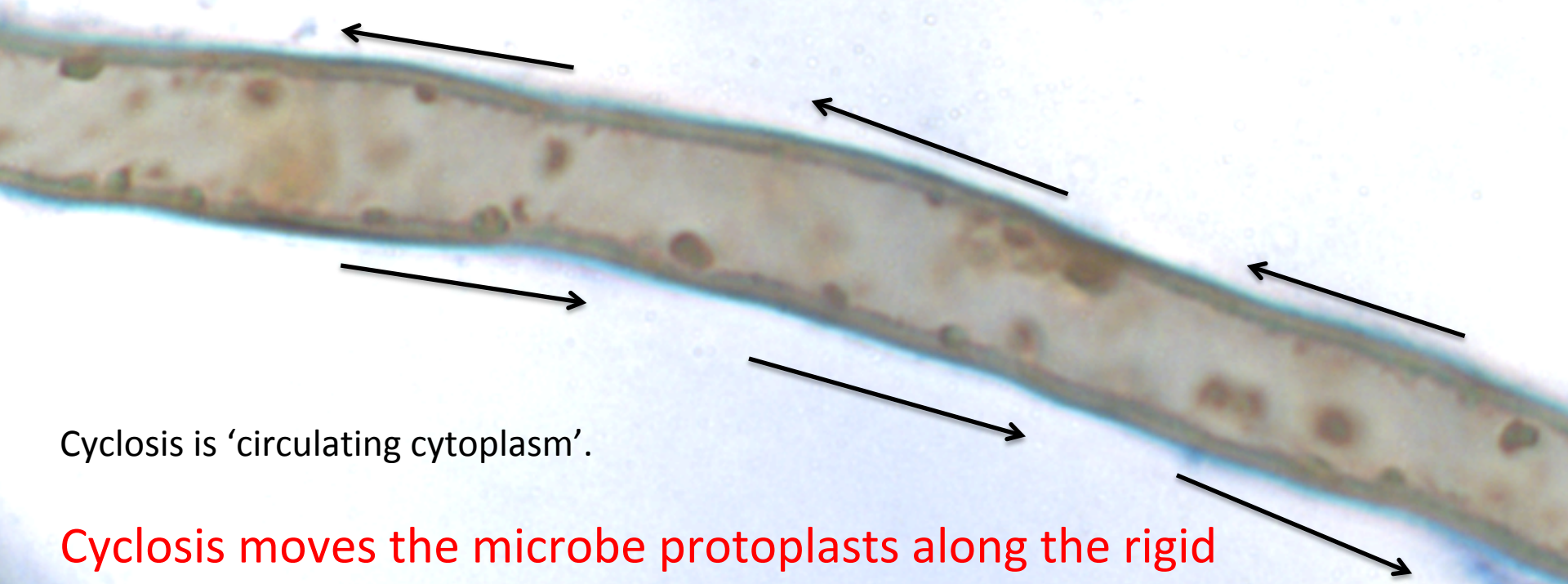
2. A wave of expansion in the hair protoplast begins in the base of the hair and progresses to the tip of the hair. This expansion wave forces microbe protoplasts through pores that form in the thin wall at the hair tip.

James White
Rutgers University
Feb. 23, 2019

Fimbristylis cymosa

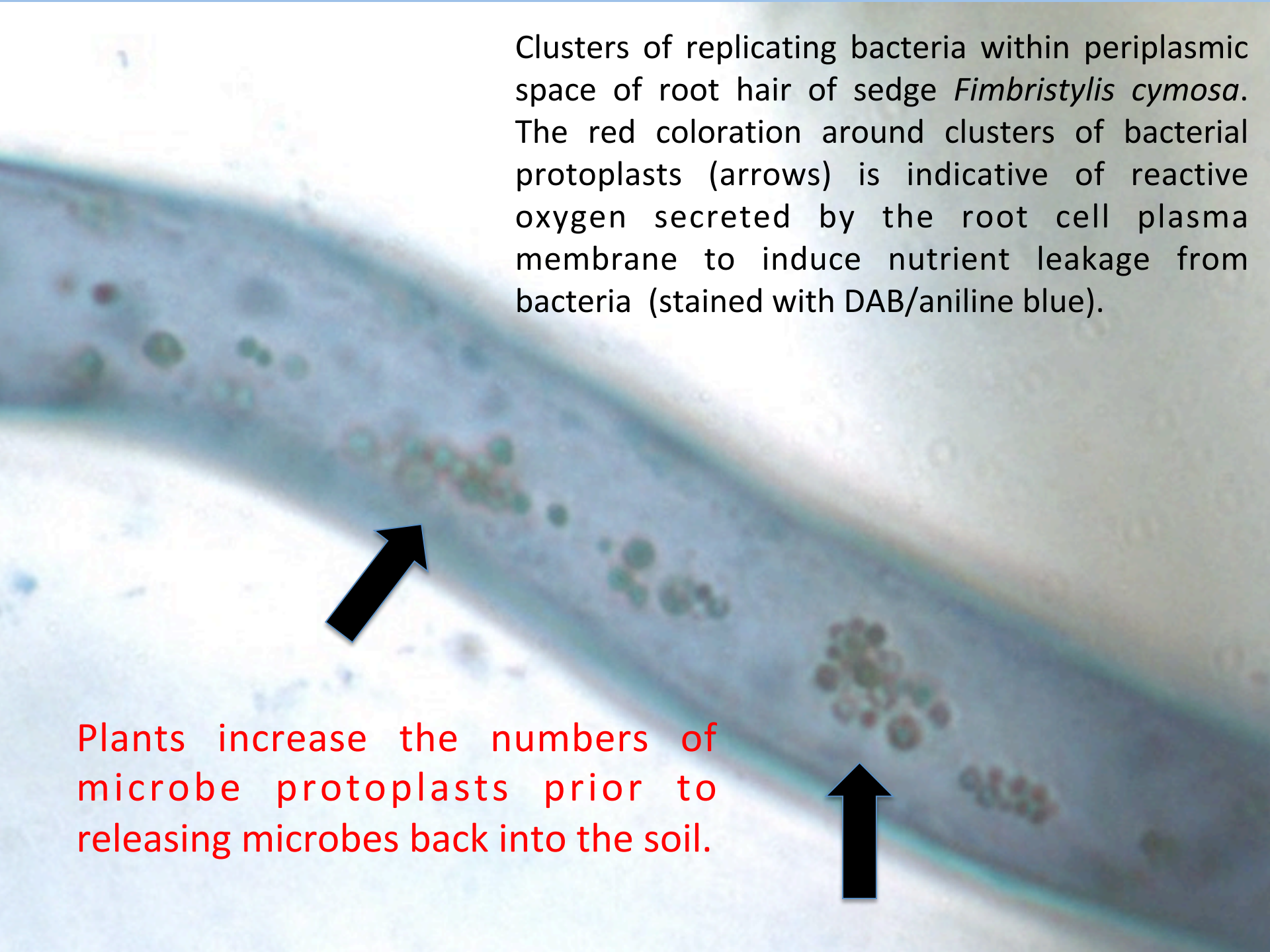
Plant grows in pits and crevices of limestone or in sand along high salt Caribbean shore environments.

Root hair of *Fimbristylis cymosa* showing bacterial protoplasts in periplasmic space. Bacteria are seen to be surrounded by red-staining reactive oxygen. Bacteria are transported to the tip of the hair and deposited by the action of unidirectional cyclosis in the root hair cell.



Cyclosis is ‘circulating cytoplasm’.

Cyclosis moves the microbe protoplasts along the rigid lateral walls of the hair. This reduces degradation of protoplasts and increases their recovery and replication.

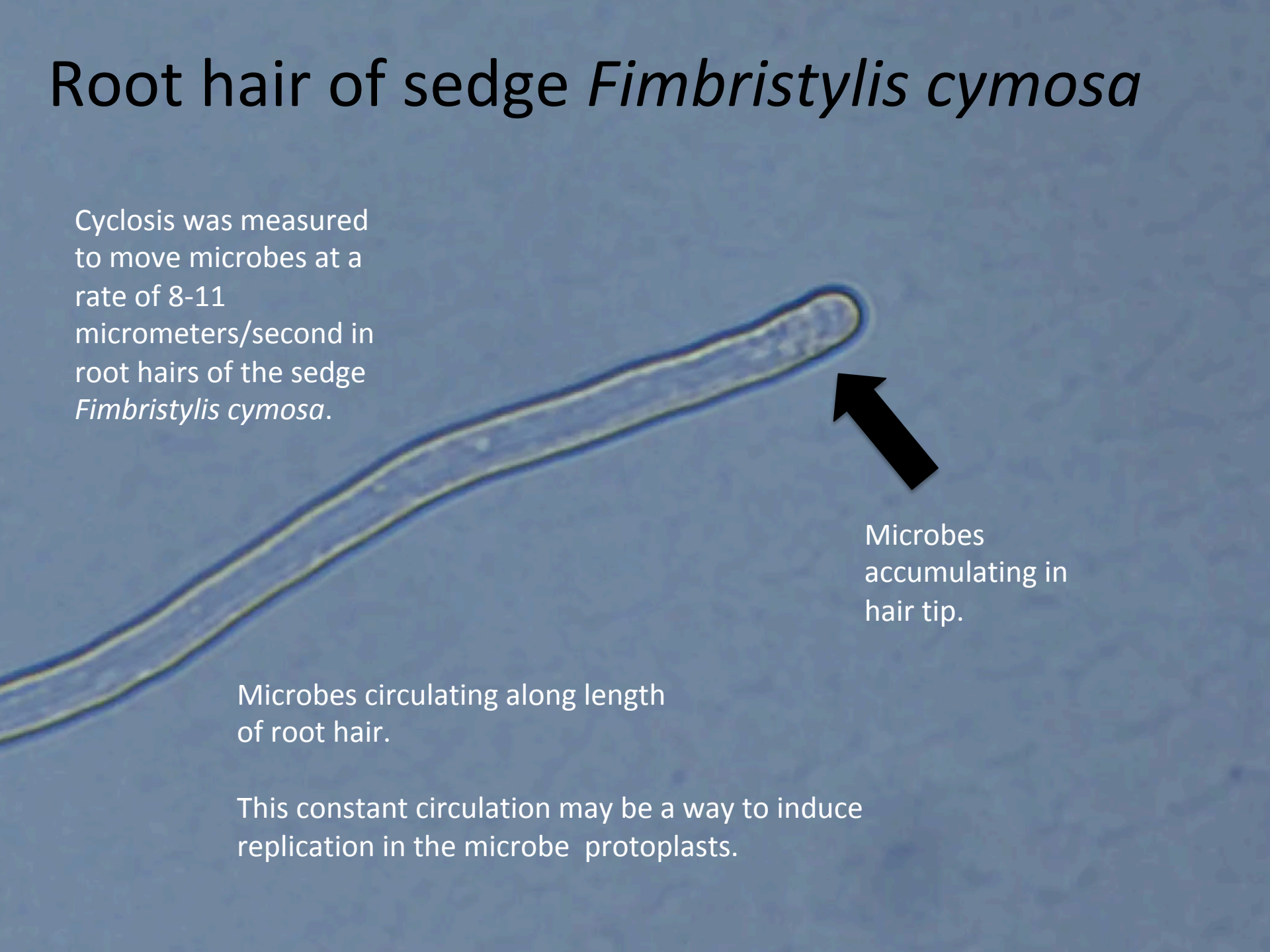


Clusters of replicating bacteria within periplasmic space of root hair of sedge *Fimbristylis cymosa*. The red coloration around clusters of bacterial protoplasts (arrows) is indicative of reactive oxygen secreted by the root cell plasma membrane to induce nutrient leakage from bacteria (stained with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

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

Root hair of sedge (*Fimbristylis cymosa*) 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.

Root hair of sedge *Fimbristylis cymosa*



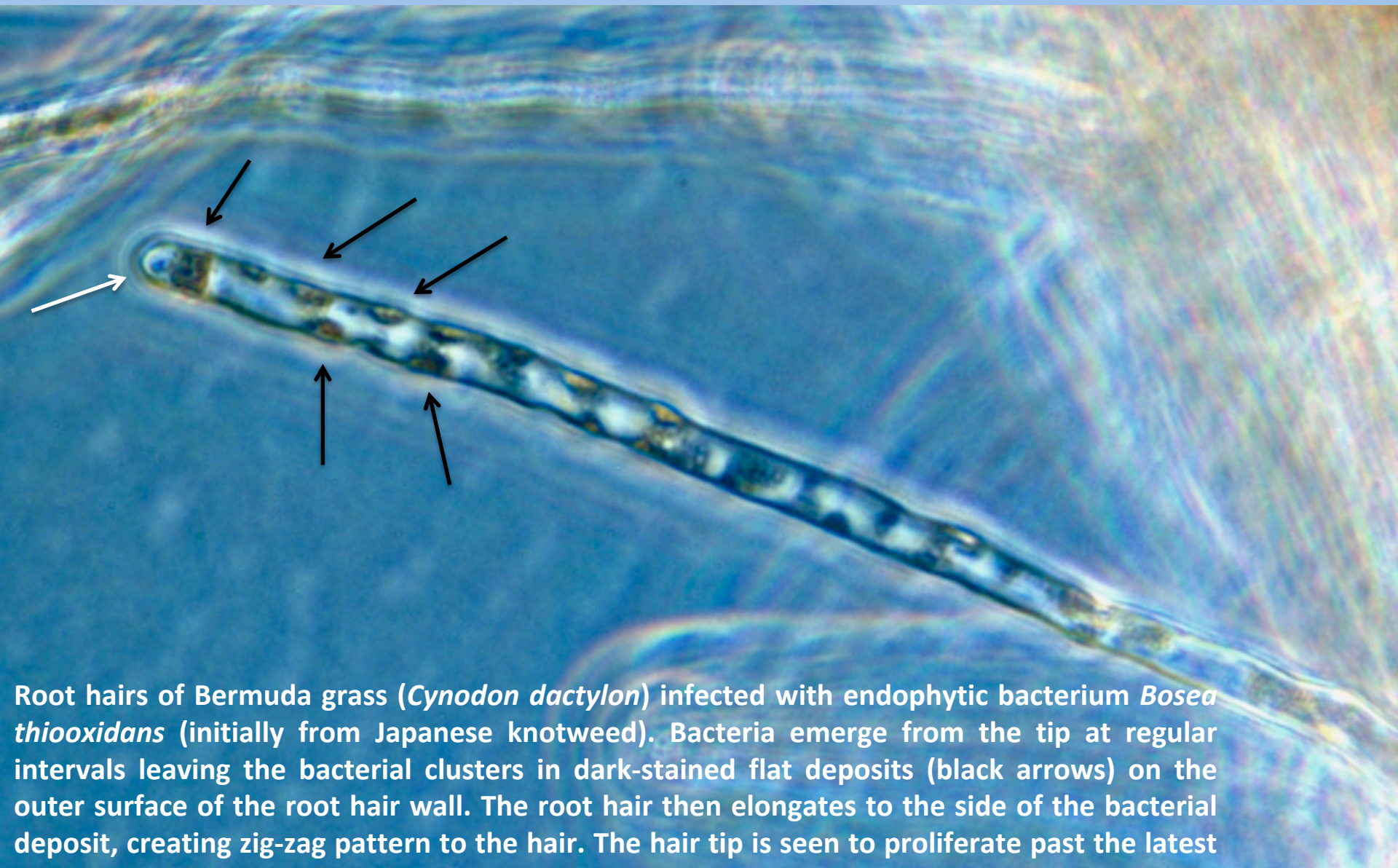
Cyclosis was measured to move microbes at a rate of 8-11 micrometers/second in root hairs of the sedge *Fimbristylis cymosa*.

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.

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

Mystery of the vanishing nutrients!!

As Carbon Dioxide Levels Rise, Major Crops Are Losing Nutrients

1) Wheat (C3 plant) showed declines in protein, magnesium, iron and zinc.
2) Soybeans and field peas (with rhizobia) showed declines in magnesium, iron and zinc.
3) Maize and sorghum (C4 plants) were less affected.

Myers, S. et al. 2014. Increasing CO_2 threatens human nutrition. Nature 510: 139-142.

Hypothesis

We hypothesize that elevated CO_2 inhibits the extraction of nutrients from microbes in the rhizophagy cycle through inhibition of superoxide formation in root cells.

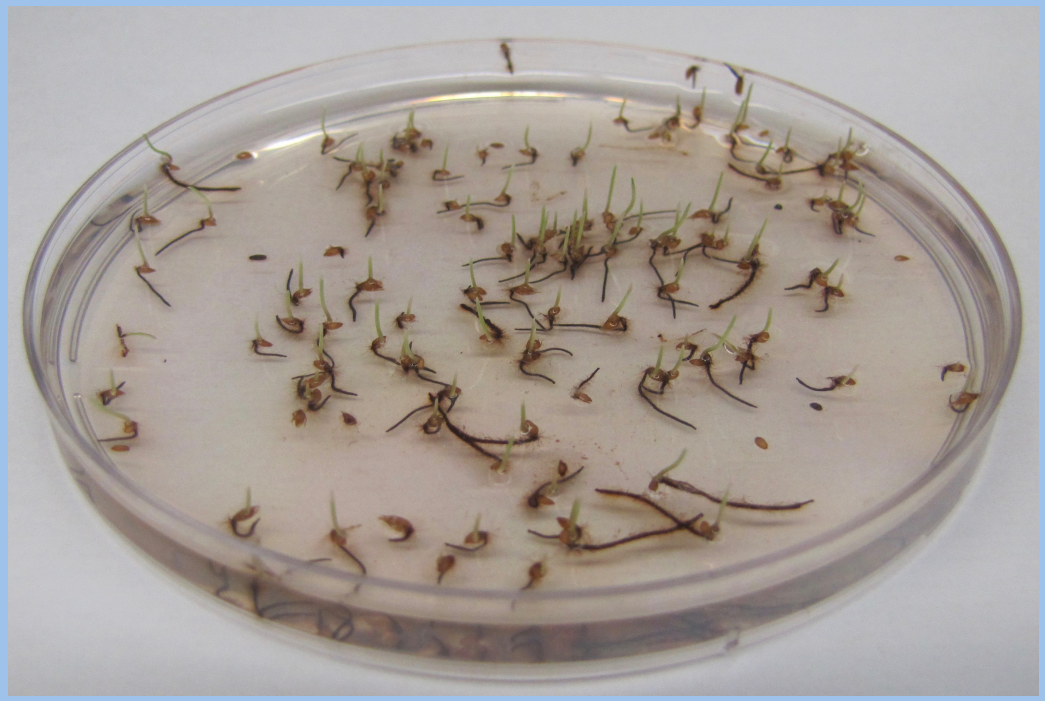
Carbon dioxide inhibits generation of superoxide that plants use to extract nutrients from microbes!

Kogan et al. 1997. Carbon dioxide--a universal inhibitor of the generation of active oxygen forms by cells (deciphering one enigma of evolution). Izvestiia Akademii nauk. Seriia biologicheskaia / Rossiĭskaia akademiia nauk. 1997 Mar-Apr. 204-217.

Bolevick S, et al. 2016. Protective role of carbon dioxide (CO2) in generation of reactive oxygen species. Molecular and Cellular Biochemistry 411: 317-330.

Rajan Verma

Experiments with seedlings in elevated CO_2 atmospheres



Experiments were conducted where seedlings of plants were grown under two levels (non-elevated and elevated) of atmospheric carbon dioxide. Dry ice was used to elevate carbon dioxide in chambers.

Experiments with tall fescue seedlings

Non-Elevated 0.04% CO_2

Roots from seedlings in the non-elevated level of CO_2 (0.04%) show superoxide presence (blue color due to NBT staining) in cells around the root tip meristem. Roots also show formation of long root hairs just behind the root tip meristem. The long root hairs form because of the abundant presence of microbe protoplasts in root hairs.

Elevated 0.06% CO_2

Tall fescue seedling roots in the elevated CO_2 treatment (0.06%) do not show superoxide formation (blue color) in cells around the root tip meristem. Although, some blue color is observable in the interior of the meristem. In this treatment root hair development appears to be suppressed with no root hairs forming near the root tip.

Non-elevated 0.04% CO_2

Superoxide is visible in the tips of the root hairs where microbe protoplast accumulate. It is the presence of these microbes in root hairs that stimulates hairs to elongate. This may be the result of nitric oxide signaling by microbes in hair tips.

Elevated 0.06% CO_2

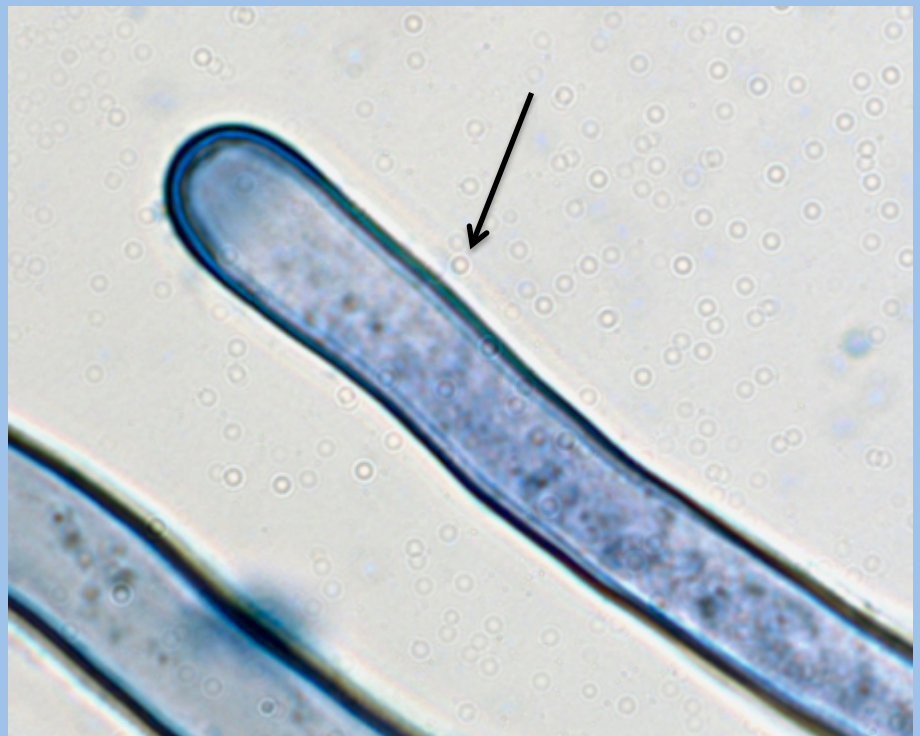
Tall fescue seedling roots in the elevated CO_2 treatment (0.06%) do not show superoxide formation (blue color) in root hairs. In this treatment root hair development appears to be suppressed.

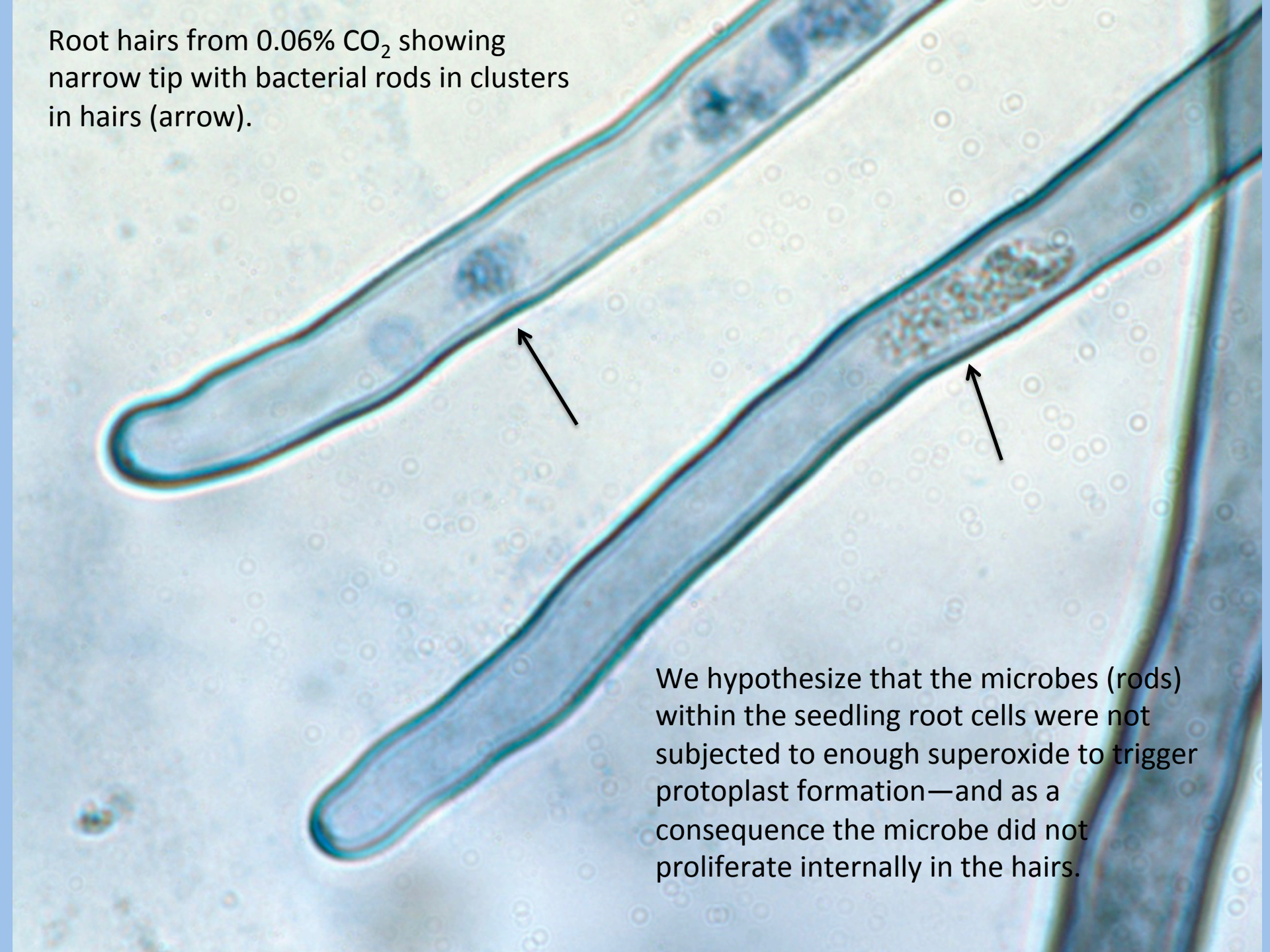
Wheat elevated CO_2 experiment in protein-agarose

- Winter wheat seeds surface disinfected (45 min 4% NaOCl) to reduce surface microbes.
- Microbes vectoring within seeds were not affected.
- Seeds placed onto 0.1% protein (denatured lipase) agarose (protein stimulates root hair elongation in absence of microbes).
- Seedlings germinated and grown for 3 days in lab ambient air (approx. 0.04% CO_2).
- Seedlings placed in gas chambers with 0.04% CO_2 (current atmosphere concentration) or 0.06% CO_2 (elevated level) and incubated 4 days at room temperature.
- Seedlings removed from chambers and stained for 15 hours by flooding plates with diaminobenzidine tetrachloride stain to visualize H_2O_2.
- Seedlings from both treatments examined for microscopic evidence of rhizophagy cycle activity in roots.

Root hair from 0.06% CO_2 showing narrow tip without internal bacteria (arrow).

Root hairs from 0.04% CO_2 showing swollen tips with masses of bacteria evident internally at tip (arrows; DAB stained).

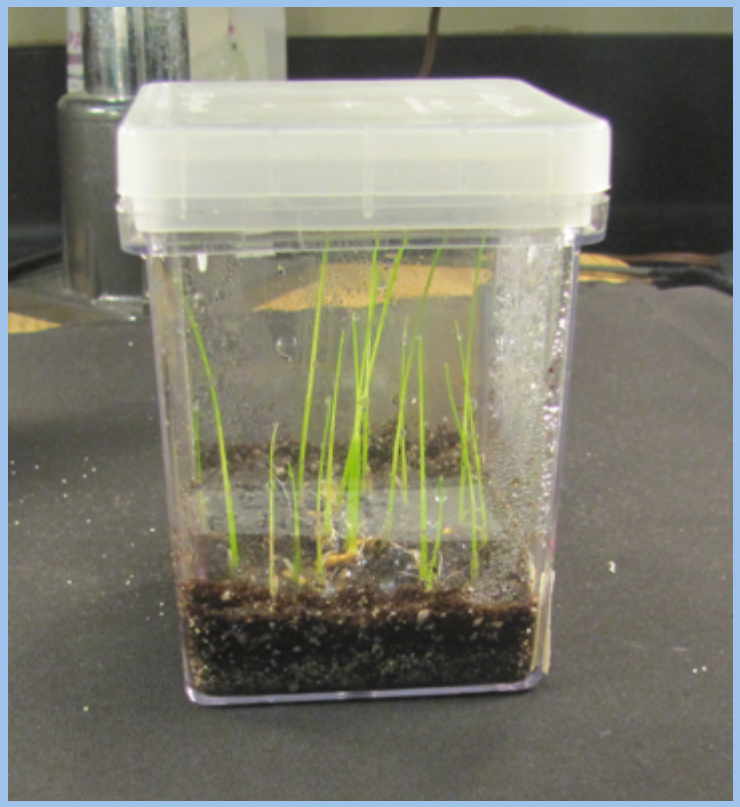



Root hairs from 0.06% CO_2 showing narrow tip with bacterial rods in clusters in hairs (arrow).
We hypothesize that the microbes (rods) within the seedling root cells were not subjected to enough superoxide to trigger protoplast formation—and as a consequence the microbe did not proliferate internally in the hairs.

Examination of effects of elevated CO_2 on rhizophagy cycle and root hair formation in winter wheat in potting mix

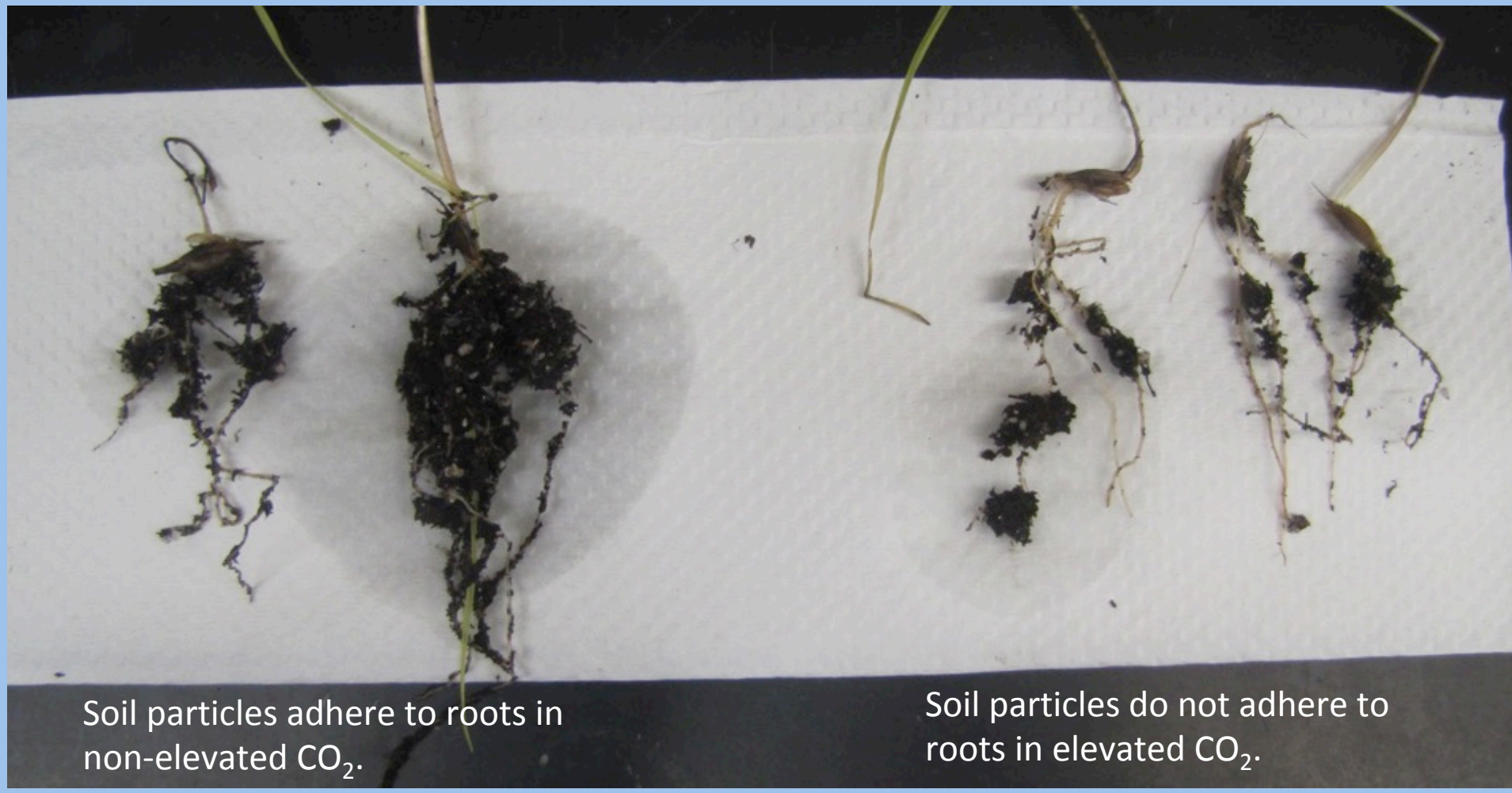
- Wheat seeds were germinated in potting mix and grown in chambers with air at 0.04% CO_2 (non-elevated) or air with elevated CO_2 (approx. 0.06%).
- Incubated for 21 days in the laboratory.
- Chambers were opened and plants pulled from potting mix to examine roots.
- Seedling roots were then examined to determine differences in root development between the two treatments.

Elevated Atmospheric CO_2 Experiments



Plants in non-sterile potting mix

Plants placed in gas chambers

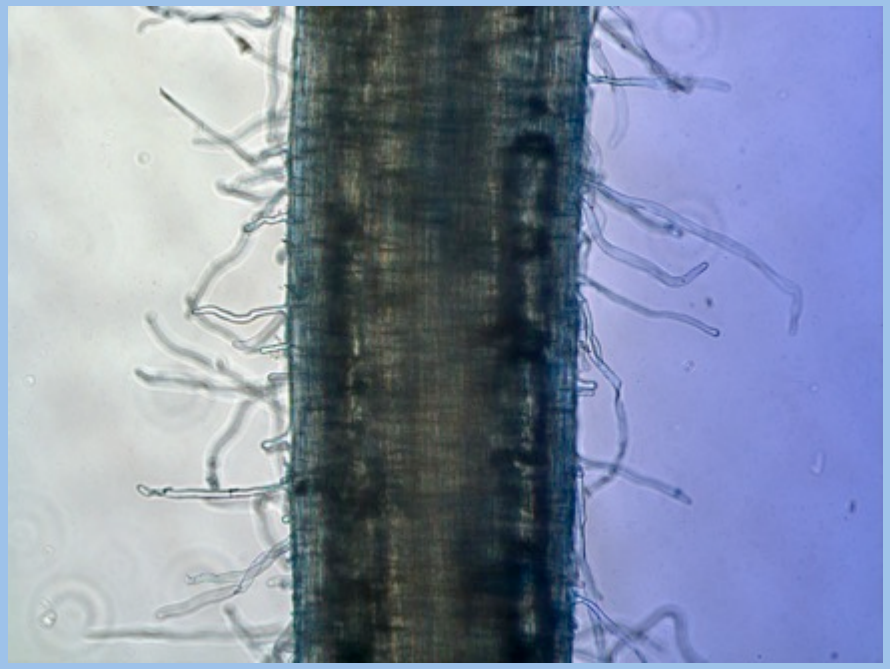


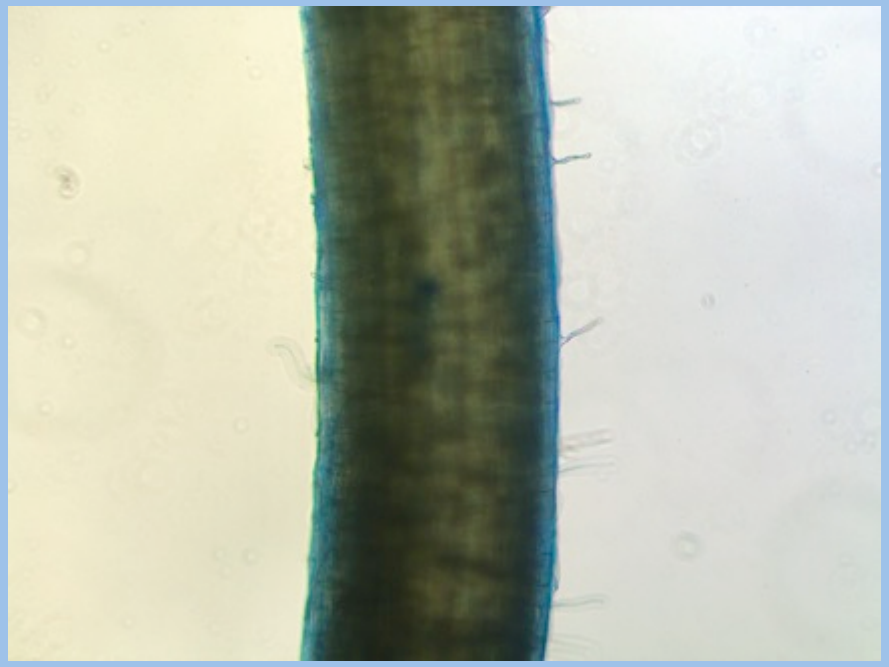
Non-Elevated CO_2 (0.04%)

Elevated CO_2 (0.06%)

Reduced soil adherence to roots in elevated CO_2 may be the result of reduced secretion of root exudates, reduced microbial activity in soil, and reduced root hair elongation.

Elevated CO2 suppresses root hair formation in winter wheat. Where more microbes are present in root cells, root hairs are longer; absence of microbes in root cells results in failure of hairs to form. Root hair elongation may be the result of nitric oxide signaling by the microbes accumulating in the tip of the root hair.





Non-Elevated CO_2 (0.04%)

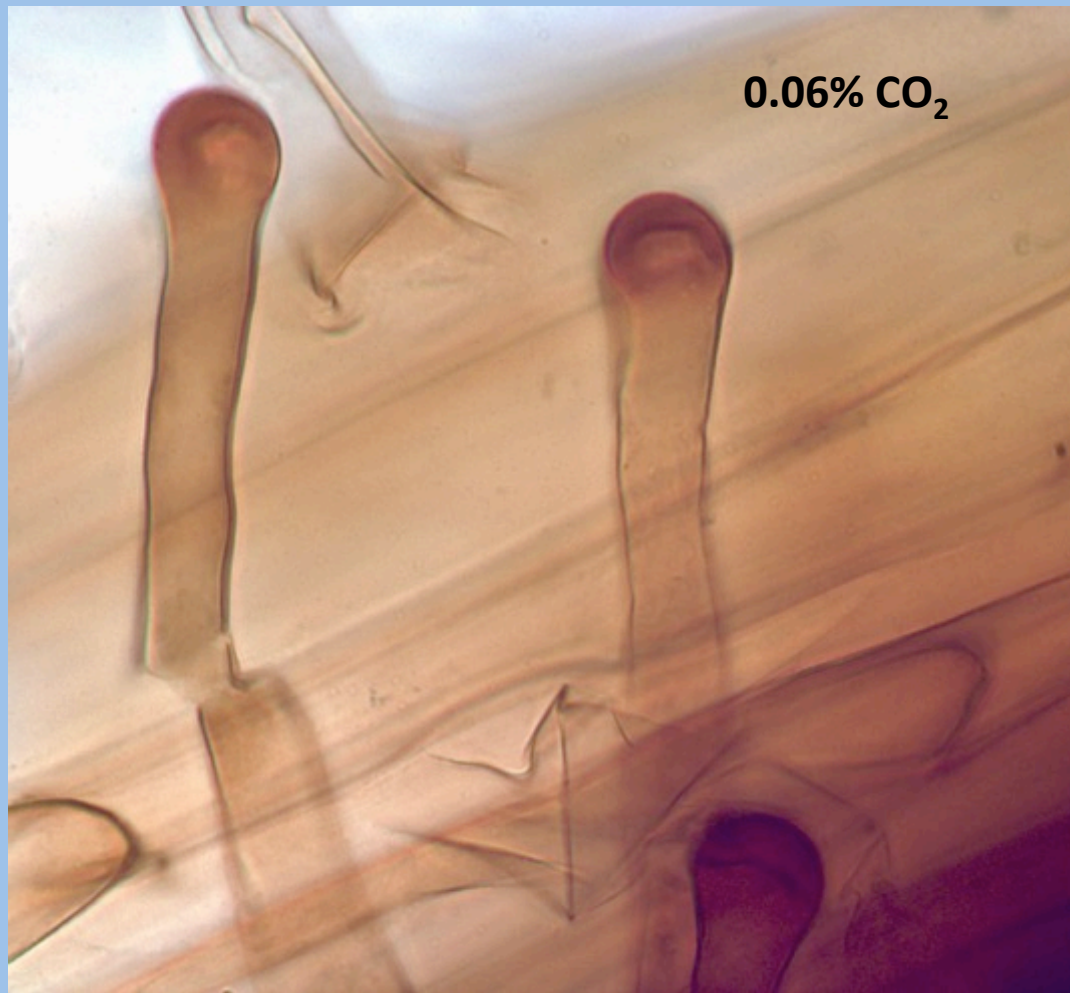
Elevated CO_2 (0.06%)

Nkolika Obi

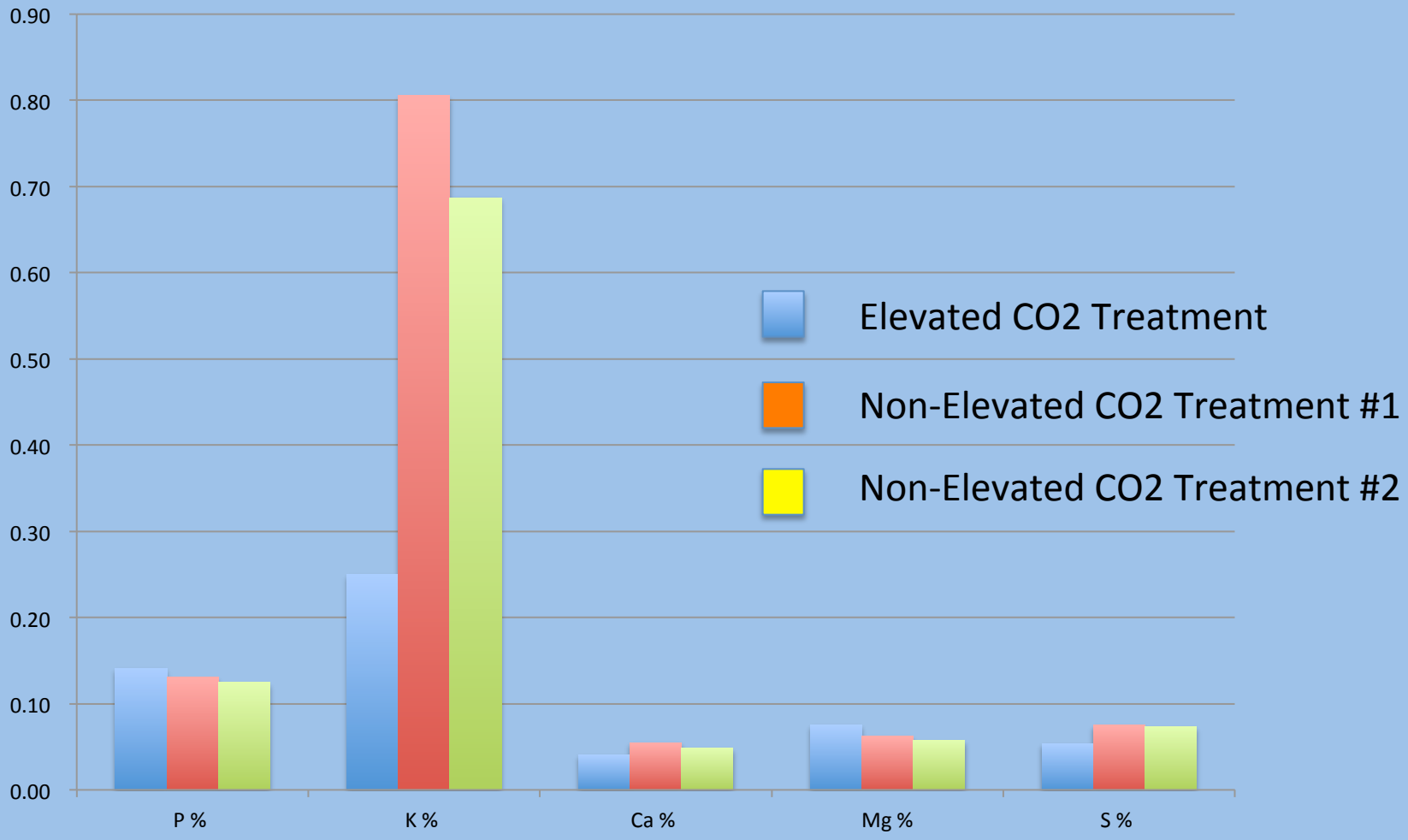
Tomato Seedlings: Elevated CO_2 Experiment

- Tomato seeds surface disinfected (5 min 4% NaOCl) to reduce surface microbes.
- Seeds placed into potting mix.
- Seeds placed in gas chambers with 0.04% CO_2 (current atmosphere concentration) or 0.06% CO_2 and incubated 21 days at room temperature.
- Seedlings removed from chambers and stained for 15 hours by flooding plates with diaminobenzidine tetrachloride stain to visualize H_2O_2.
- Seedlings from both treatments examined for microscopic evidence of rhizophagy cycle activity in roots.
- Seedling shoots analyzed for nutrient content at Penn State Plant Analysis Lab.

Microbe protoplasts (arrows) may be seen within root hairs of the 0.04% CO_2 air; but were not visible in hairs of the elevated CO_2 treatment (DAB stained).



Tomato seedling nutrient absorption experiment (macronutrients)



Conclusions

- Elevated CO_2 inhibits superoxide formation in plant root cells.
- Without superoxide, microbes do not convert to protoplasts within root cells.
- Without protoplast phases in root cells, root hairs do not elongate.
- In elevated CO_2 , the rhizophagy cycle in roots is suppressed and nutrient absorption into roots is reduced.

What happens to plants without the rhizophagy cycle?

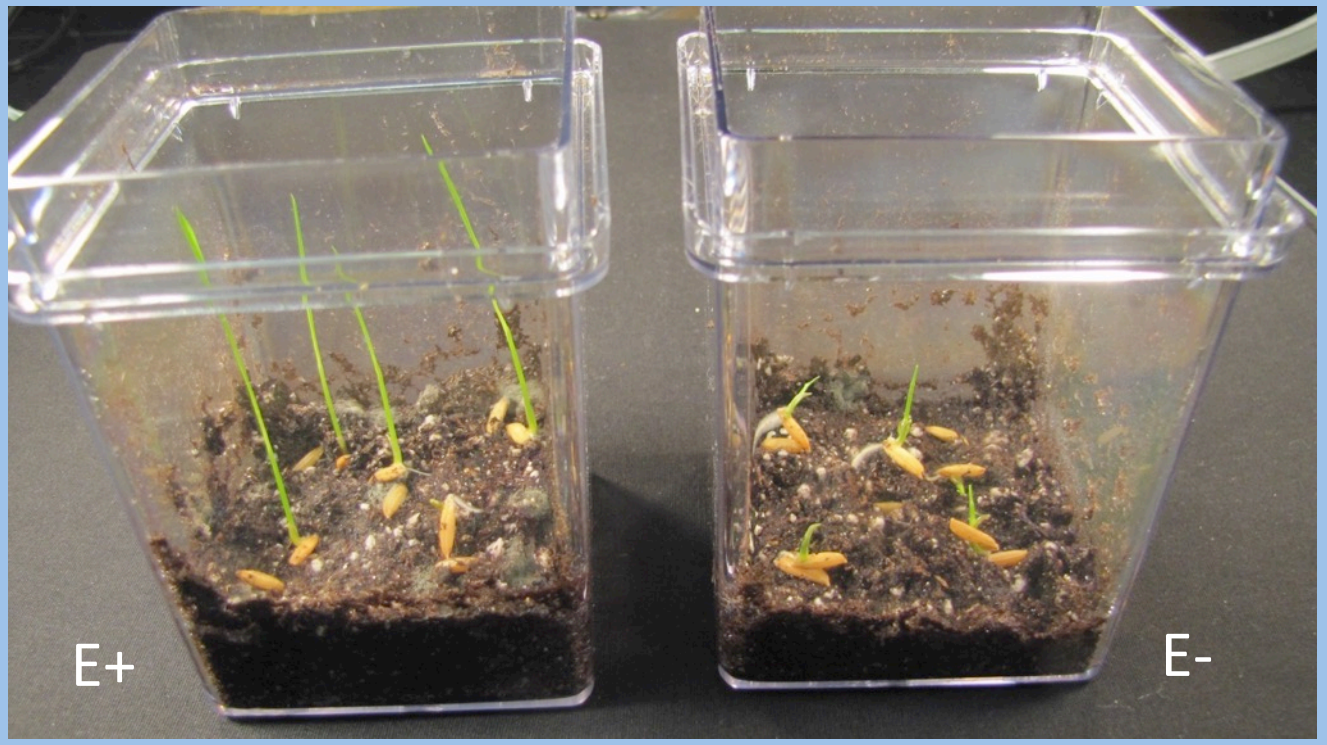
Satish K. Verma

Kate Kingsley

Kurt Kowalski

Rice:

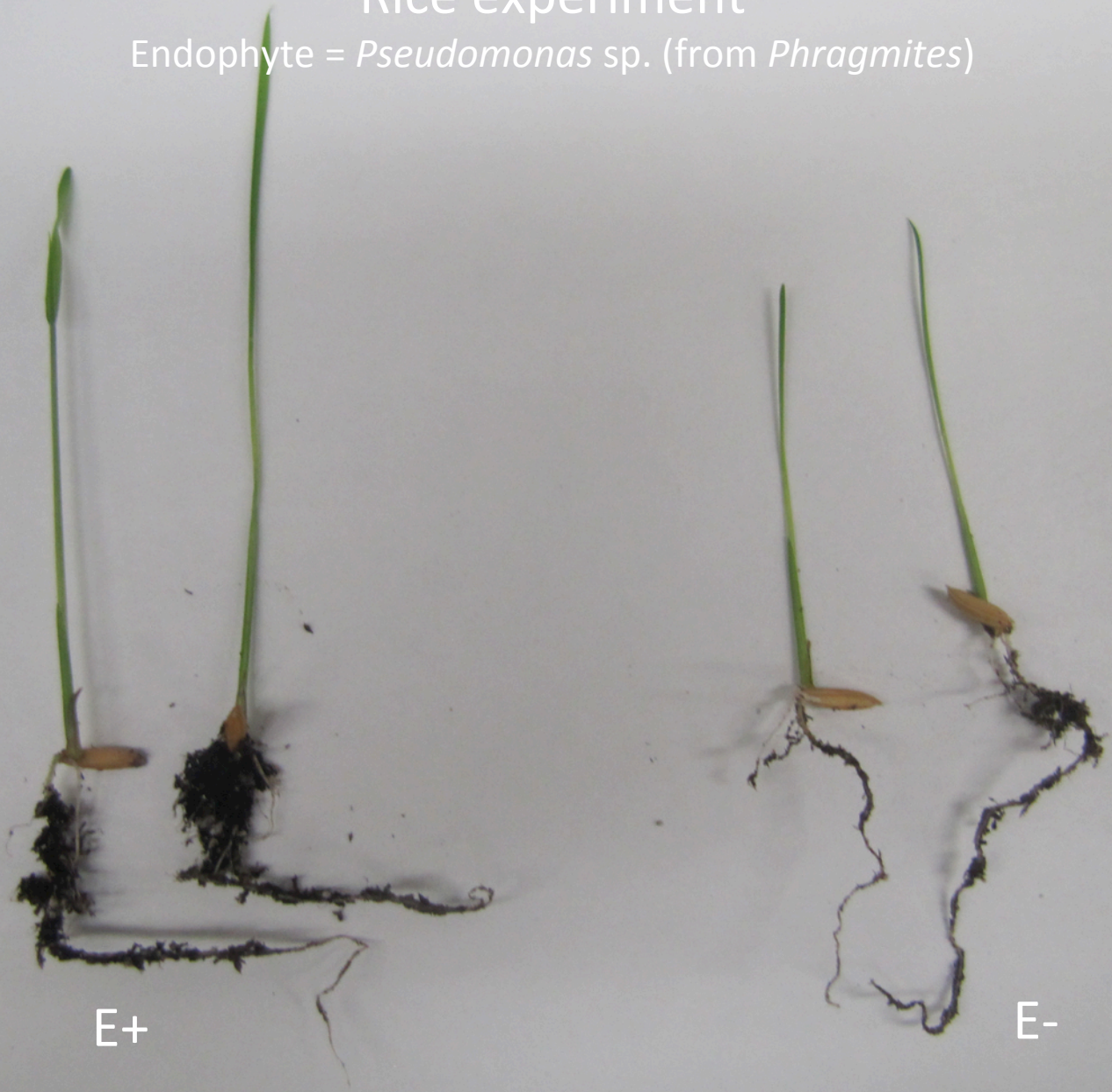
Growth
Promotion!



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

Rice experiment

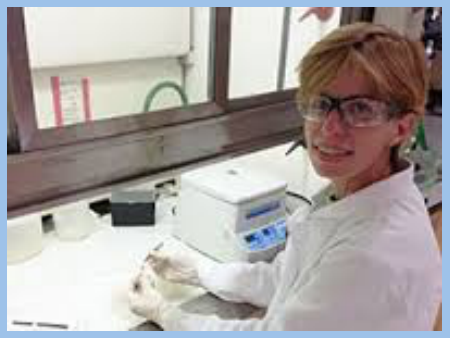
Endophyte = *Pseudomonas* sp. (from *Phragmites*)

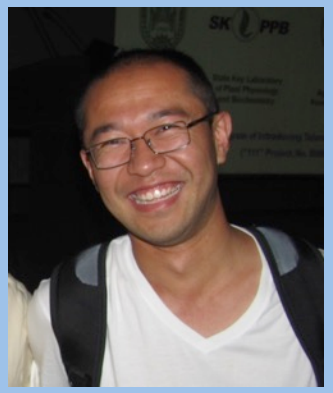


Rhizophagy Cycle Implications for Sustainable Plant Cultivation

- Preserve native microbes on seeds (i.e., no antimicrobials on seeds, and no removal of seed tissues (de-husking)).
- Use organic amendments in soil to build up the soil microbial community.
- No-till is a good practice to keep the topsoil microbial community intact.
- Applications of inorganic fertilizers to plants and soil may reduce benefit of nutrient acquisition through the rhizophagy cycle (e.g., nitrate applications to soil inhibits nitrogen fixation in soil).

Acknowledgments





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Qiang Chen
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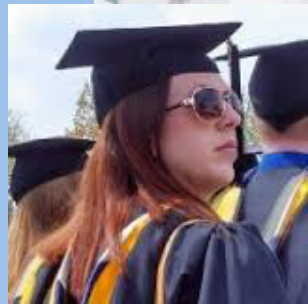

For Funding:

John and Christina Craighead Foundation;
New Jersey Agric. Exp. Sta.;
USDA NIFA Multistate 3147;
Rutgers Center for Turfgrass Science;
USGS-Rutgers U (CESU Study Agreement)