



Biological Products Industry Alliance (BPIA) Conference

How do biostimulant microbes and endophytes stimulate plant growth?

James F. White

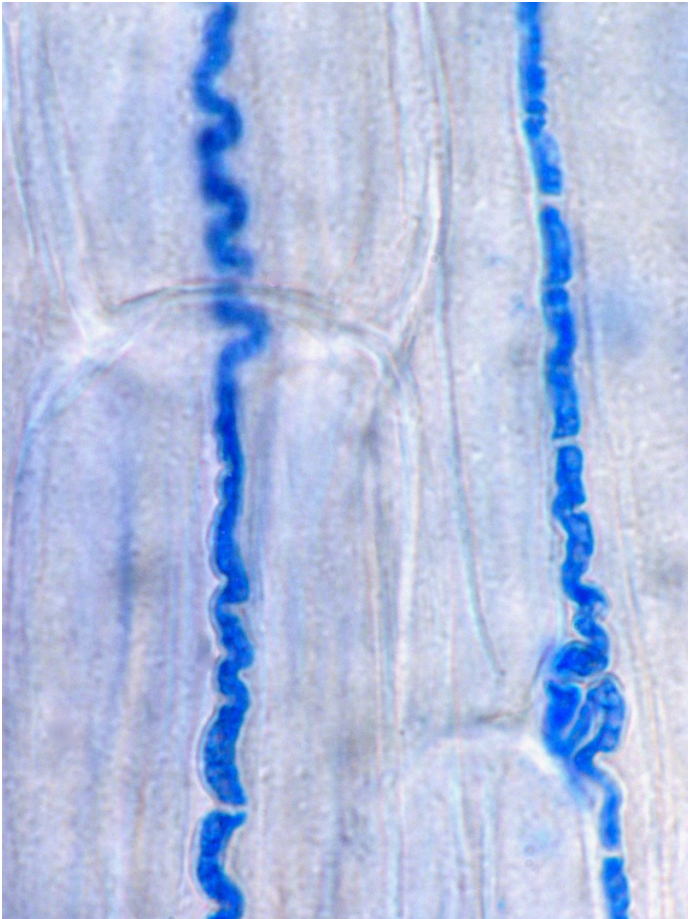
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2/22/2023

Seed plants absorb soil bacteria into growing cells and tissues. These microbes are referred to as endophytes.

What are endophytes?

(Botany): Endophytic/endosymbiotic non-pathogenic microbes (fungi, bacteria or algae) present asymptotically 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.

‘Cadushy’ cactus: *Subpilocereus repandus* in Bonaire



Cadushy fruits



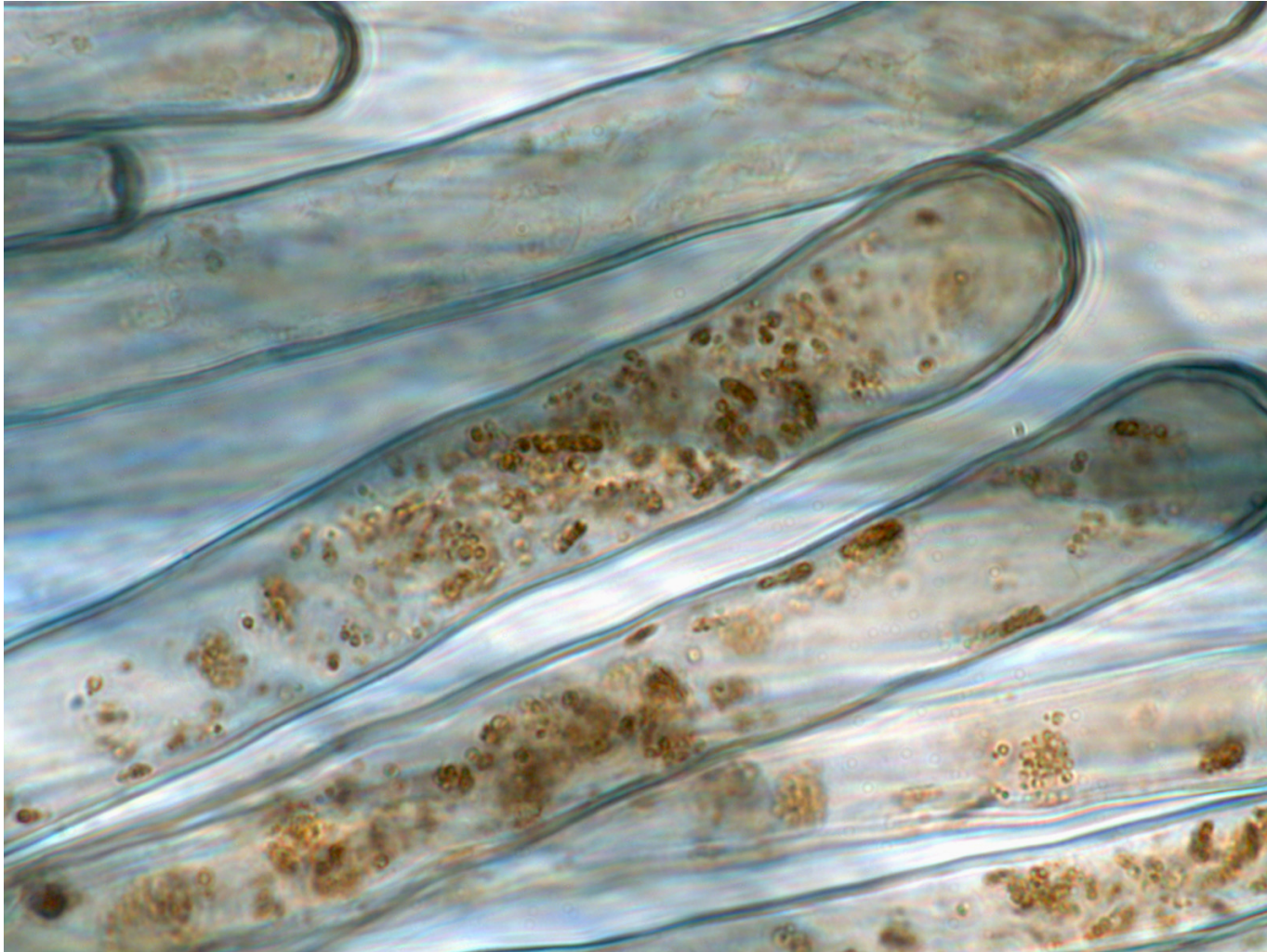
Seeds



Cadushy seedling



Bacteria in root hairs (Stained in DAB).



All plants naturally host a community of endophytes!

But you need a microscope to see them.



*Endophytes
are
everywhere!*

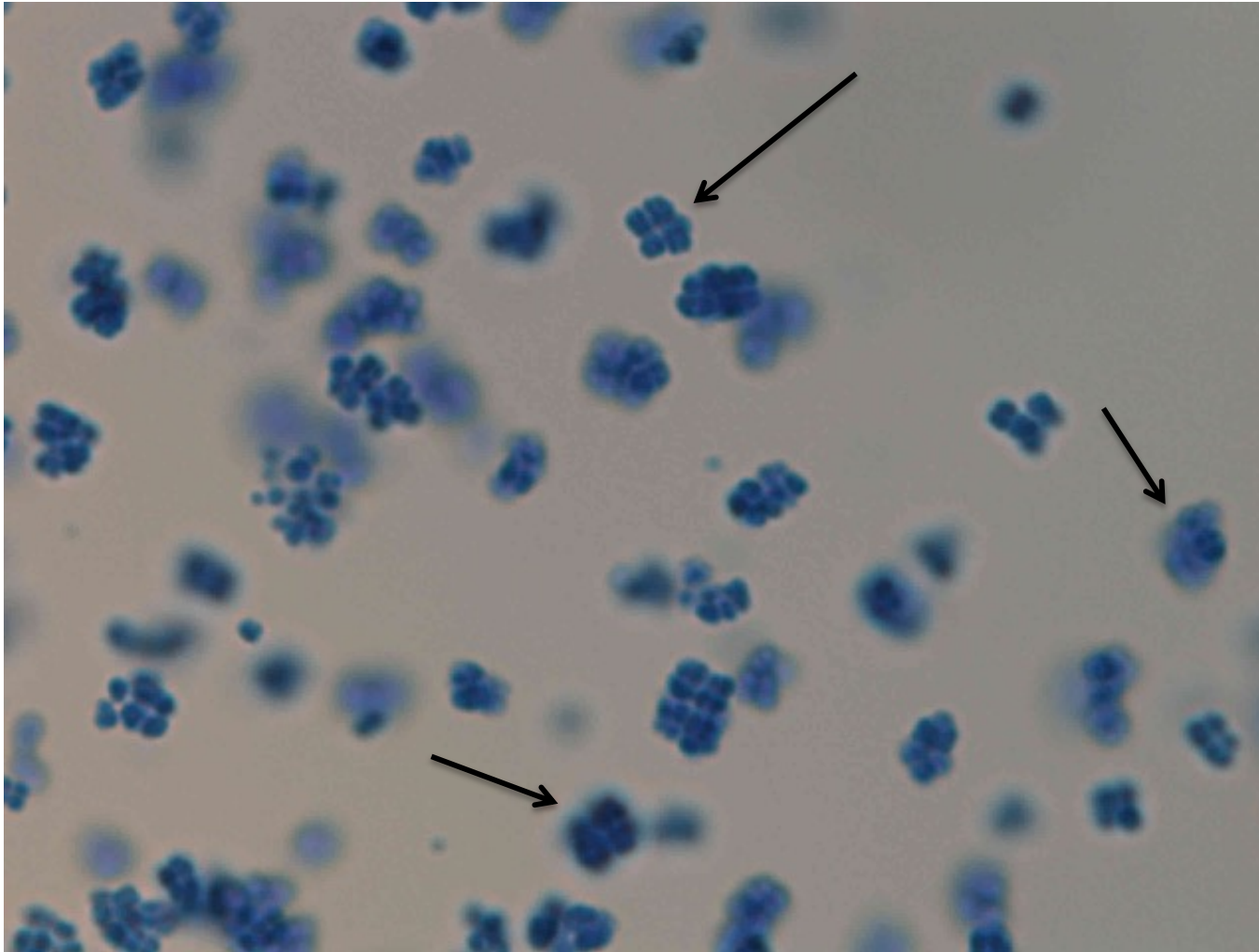
What about crop plants?

All crops harbor endophytes!

Bacterial endophytes colonize seeds.

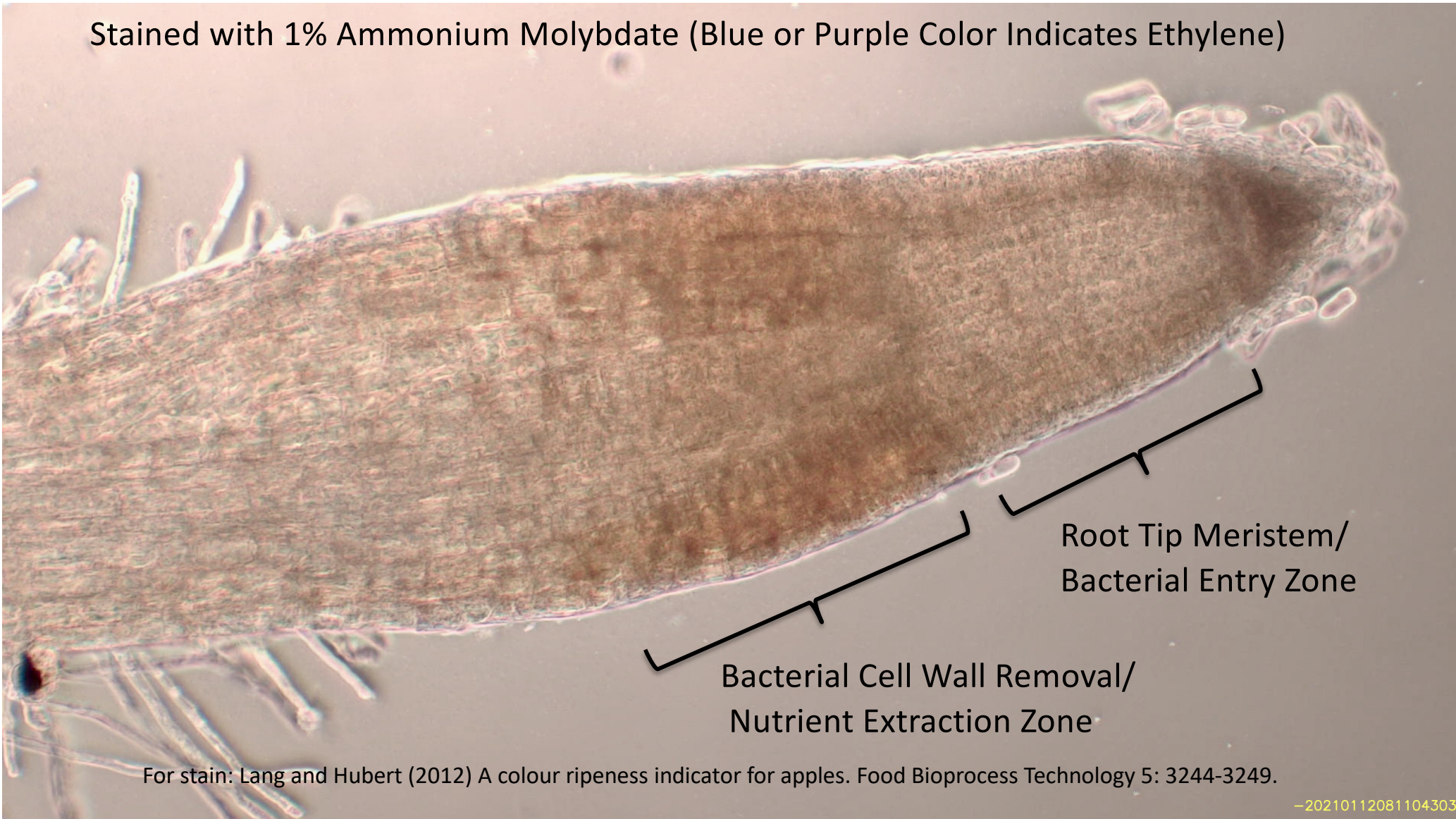


Micrococcus luteus is a bacterial endophyte from tomato seedlings. This photo shows tetrads (clusters of 4 cells) (arrows) of this bacterium.



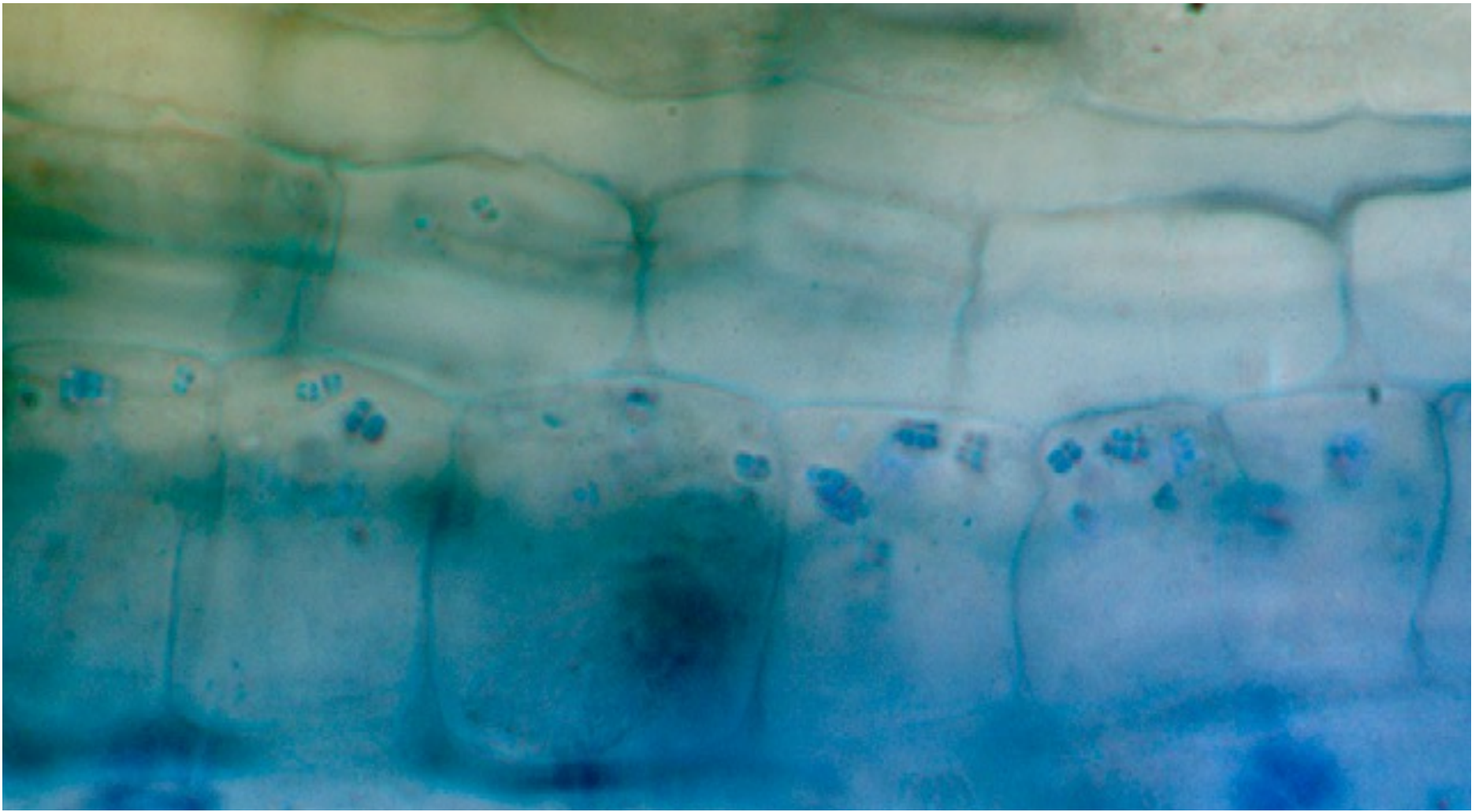
Tomato seedling root tip showing high ethylene areas to either side of the root tip meristem.

Stained with 1% Ammonium Molybdate (Blue or Purple Color Indicates Ethylene)



For stain: Lang and Hubert (2012) A colour ripeness indicator for apples. Food Bioprocess Technology 5: 3244-3249.

Close-up of *Micrococcus* tetrads in periplasmic space of root meristematic cells.



Plant microbes are:

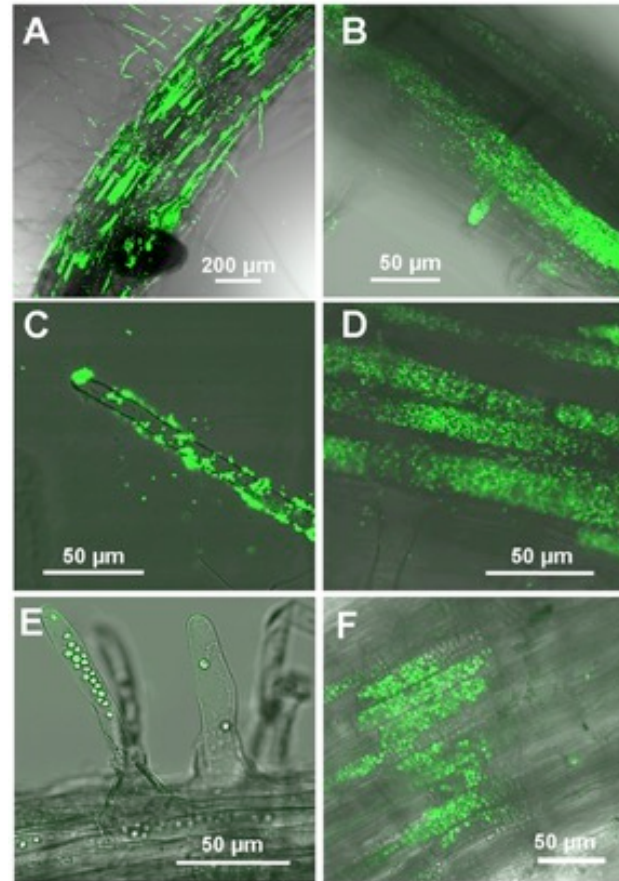
1) Soil microbes

2) Seed vectored

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?



Chany Paungfoo-Lonhienne



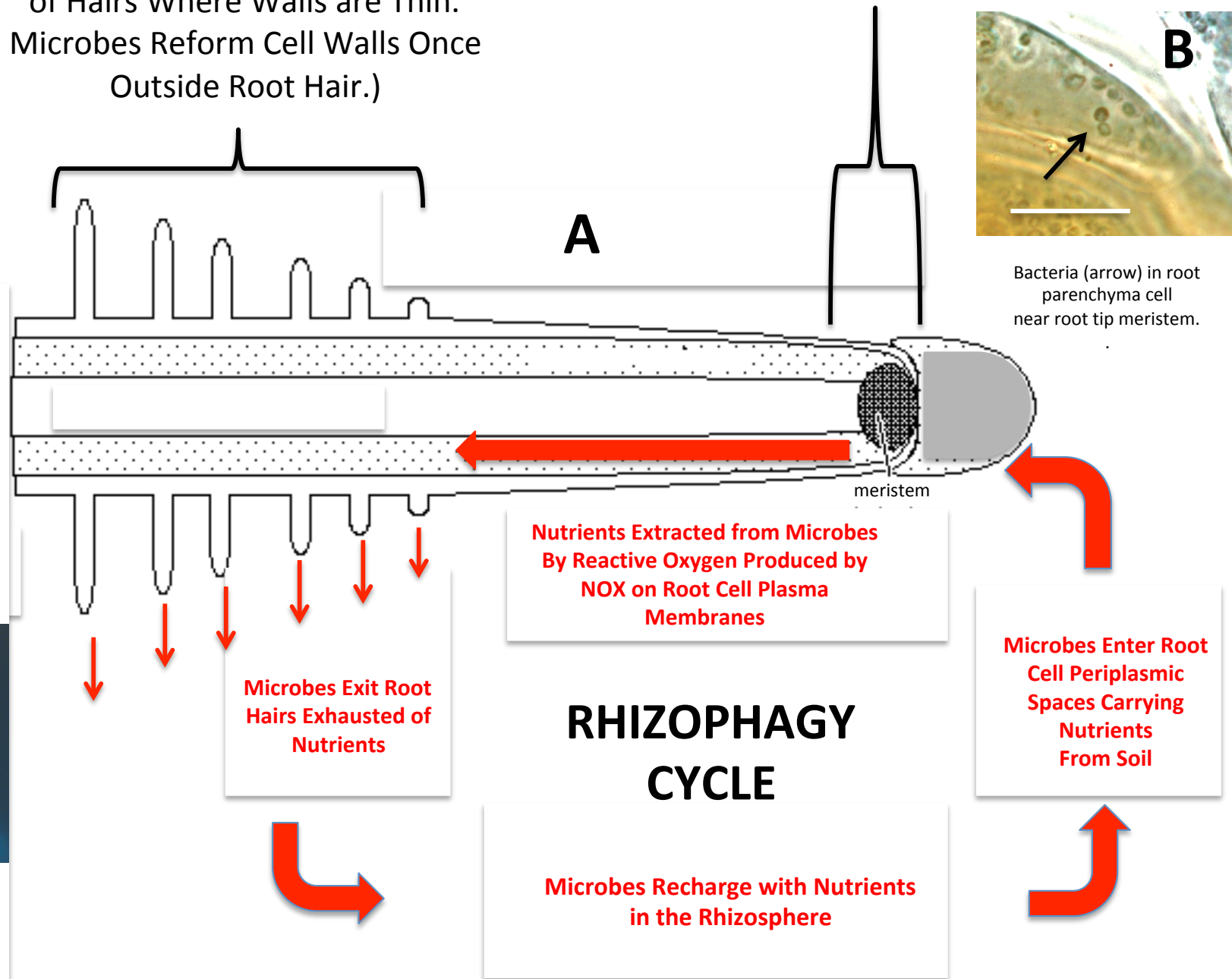
Suzanne Schmidt

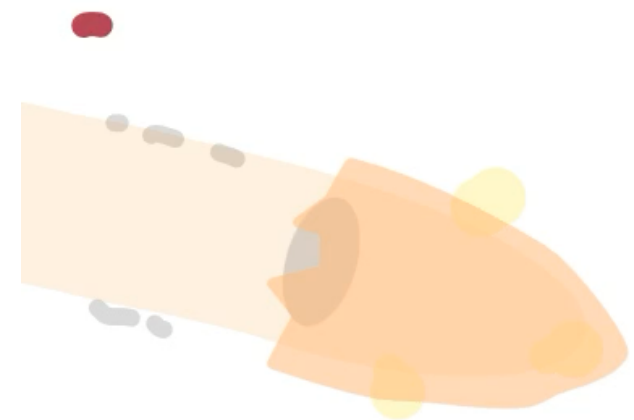
Paungfoo-Lonhienne C et al. 2010.
Turning theTable: Plants Consume Microbes as a Source of Nutrients.
PLoS ONE 5(7): e11915, doi:10.1371/journal.pone.0011915

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.)





Grass roots show numerous roots tip meristems. These root tip meristems are the sites of internalization of microbes and extraction of nutrients from microbes in the rhizophagy cycle.

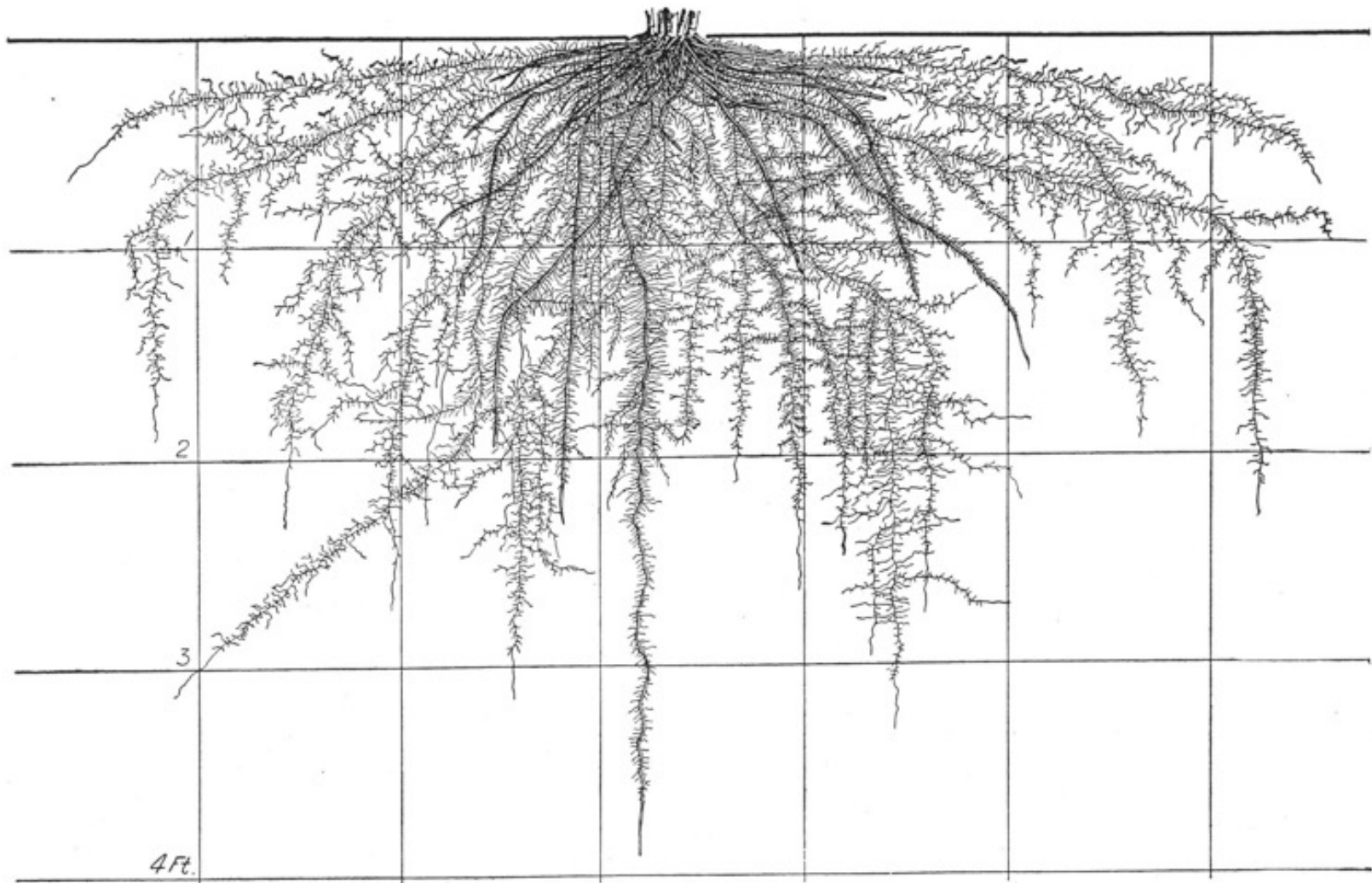
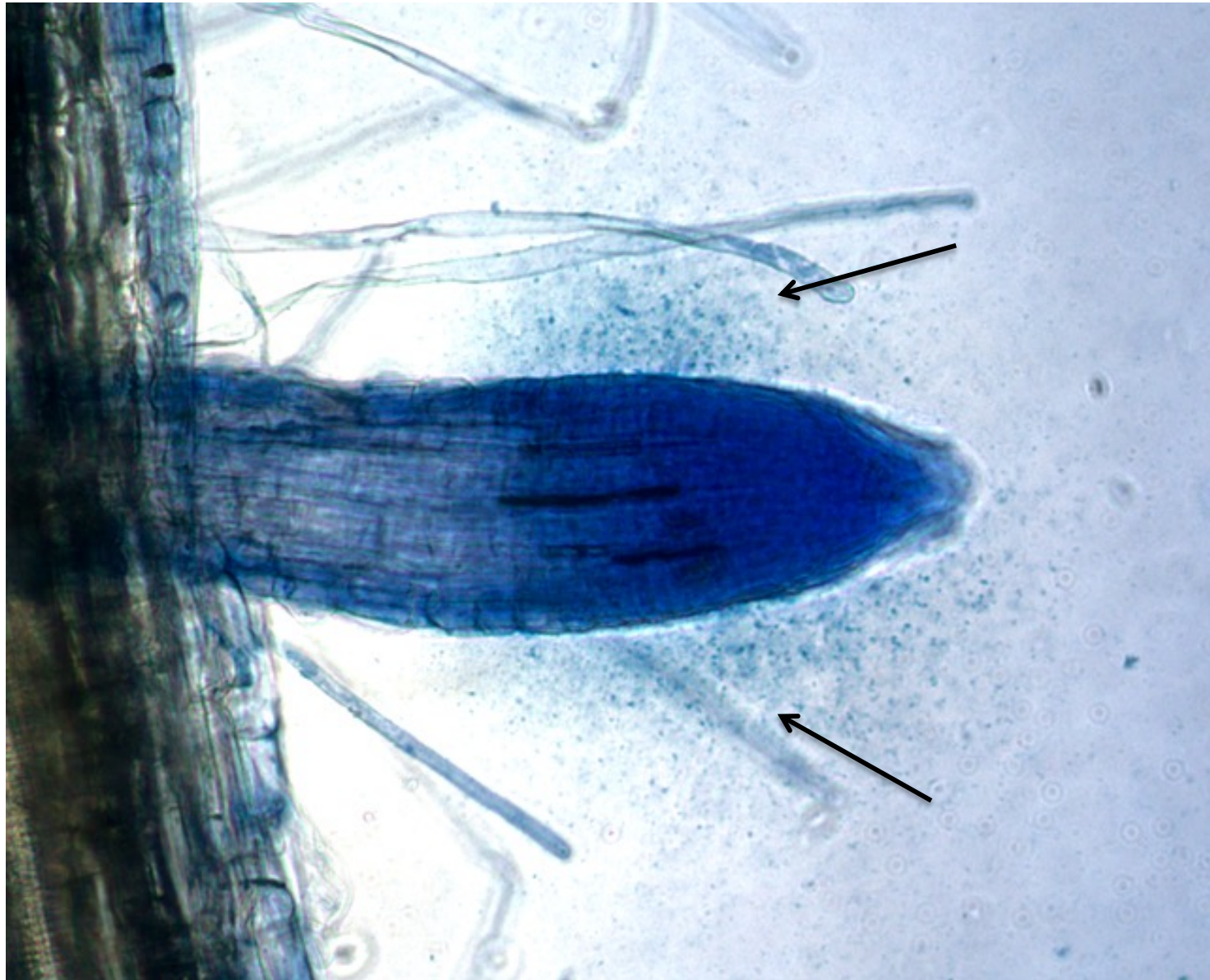
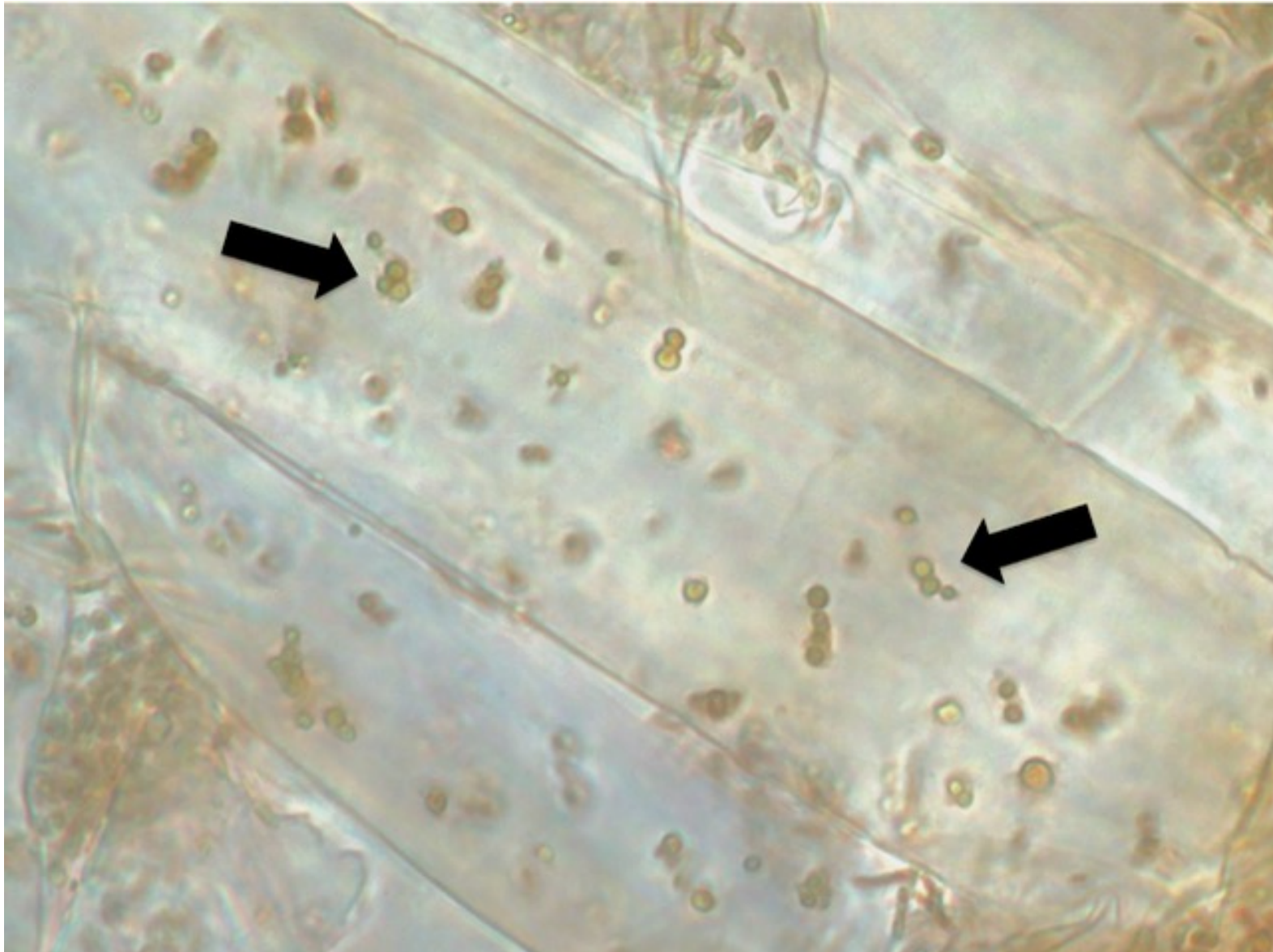


Illustration of a root system of corn (Illustration by Botanist John E. Weaver, 1927)

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.



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*.





Celeste Zhang

Confocal Microscopy:

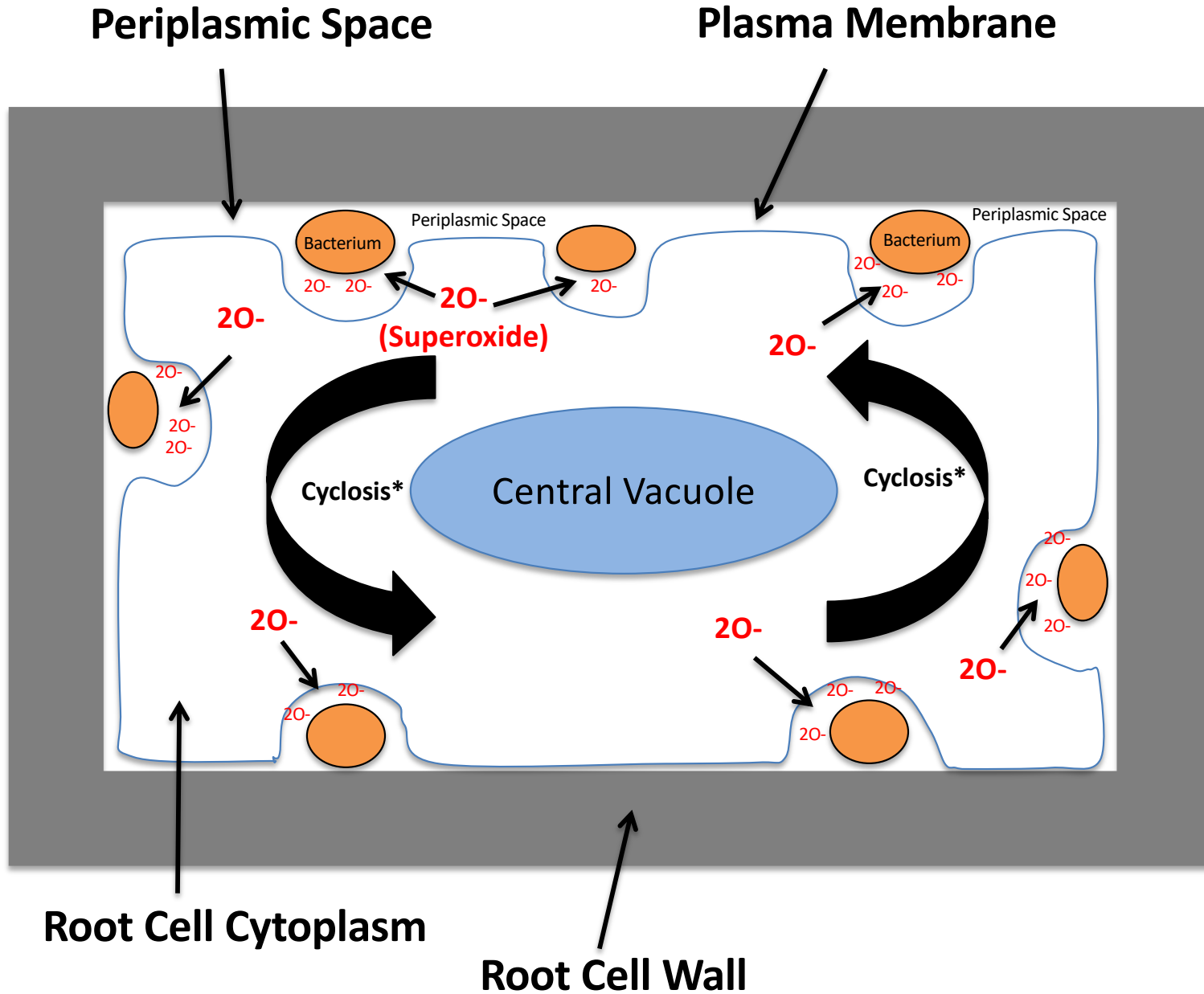
Pseudomonas sp. tagged with M-Cherry and inoculated into clover plants. Bacteria fluoresce red in the root cap cells.

Blue = calcofluor white (plant cell walls)

Green = syto13 (nucleic acid)

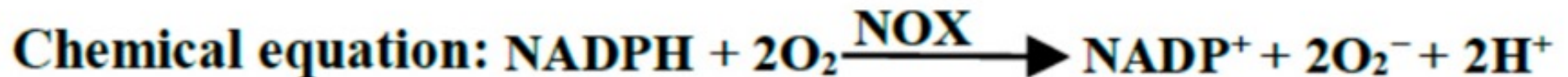
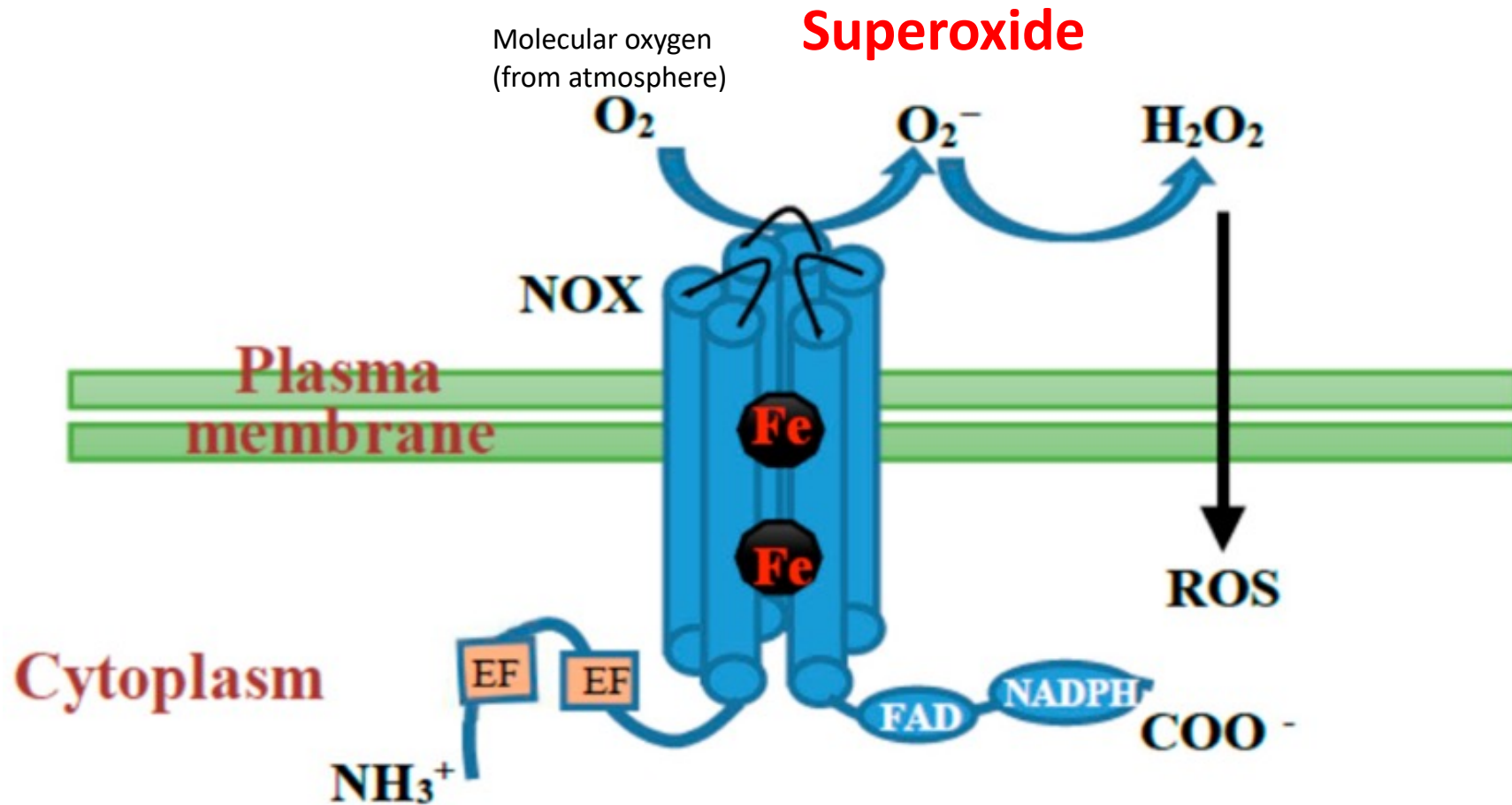
Red = mCherry tagged bacteria

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



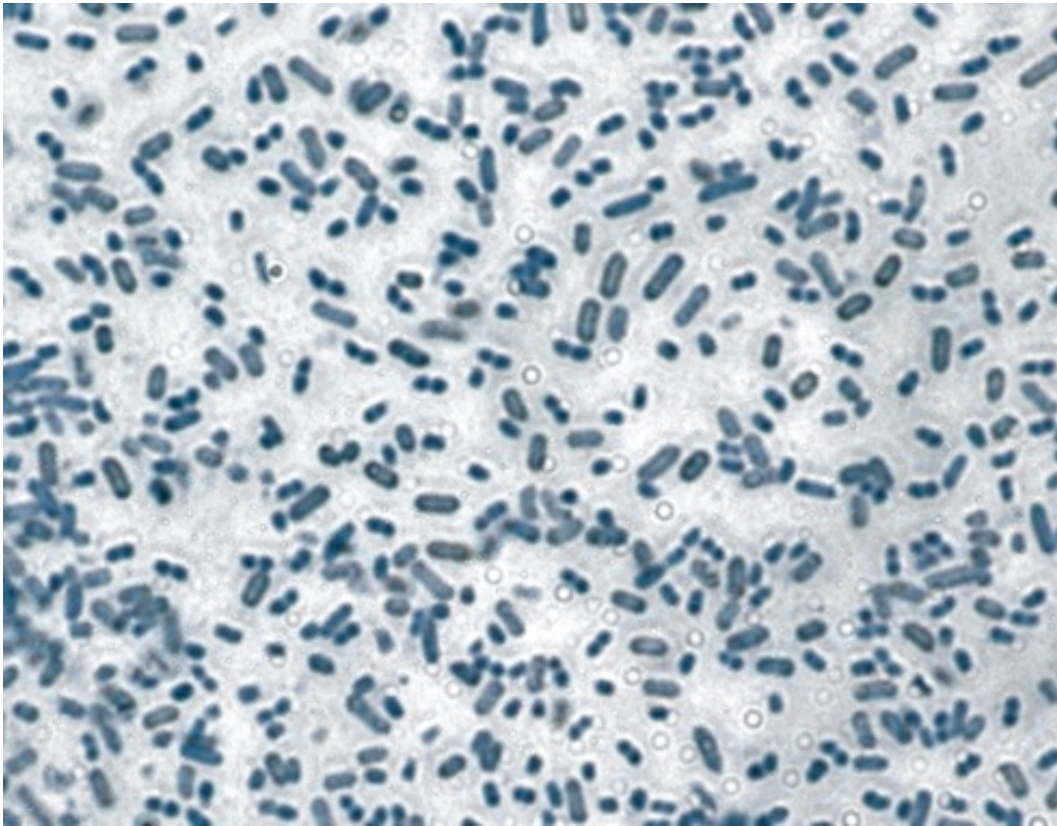
*Cyclosis = Cytoplasmic Movement

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



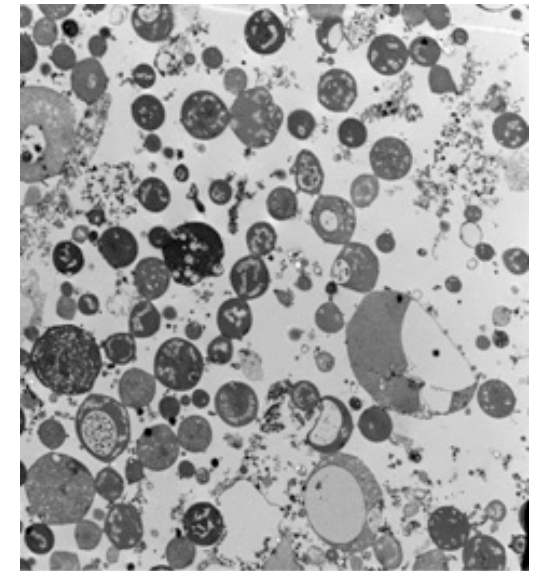
Bacterium *Bacillus subtilis*

Bacteria with cell walls (rods)



Spherical bacterial protoplasts
(no cell walls)

Reactive oxygen
(superoxide)



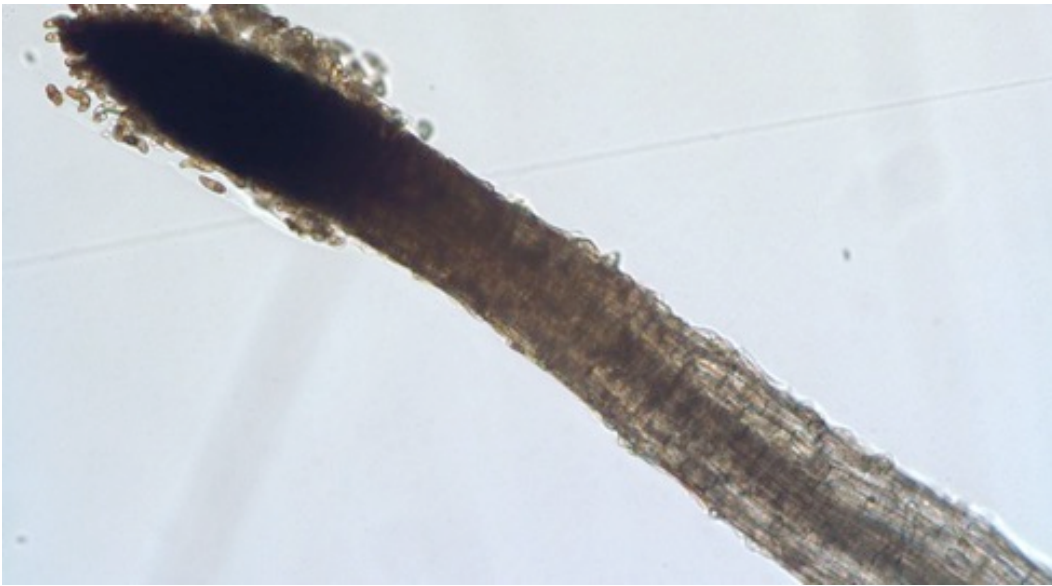
Bacterial protoplasts
are called L-forms.

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

Rhizophagy cycle microbes modulate development of seedlings

- Microbes trigger root hair elongation
- Microbes trigger the gravitropic response in roots
- 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

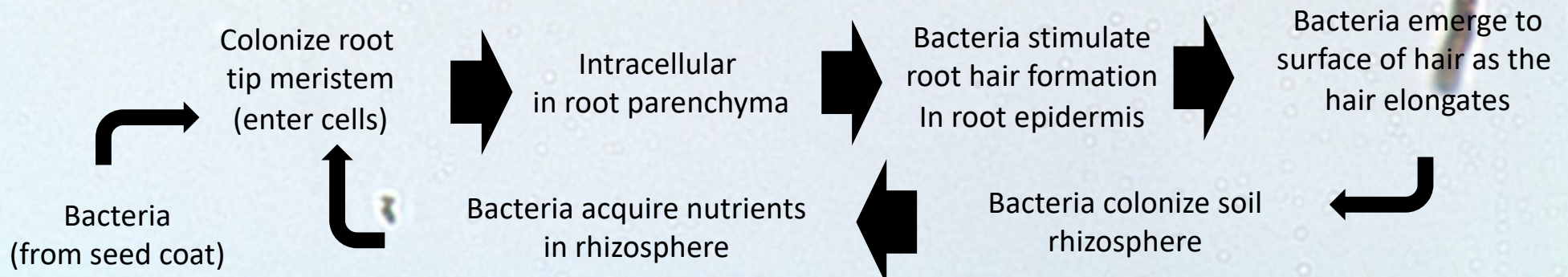


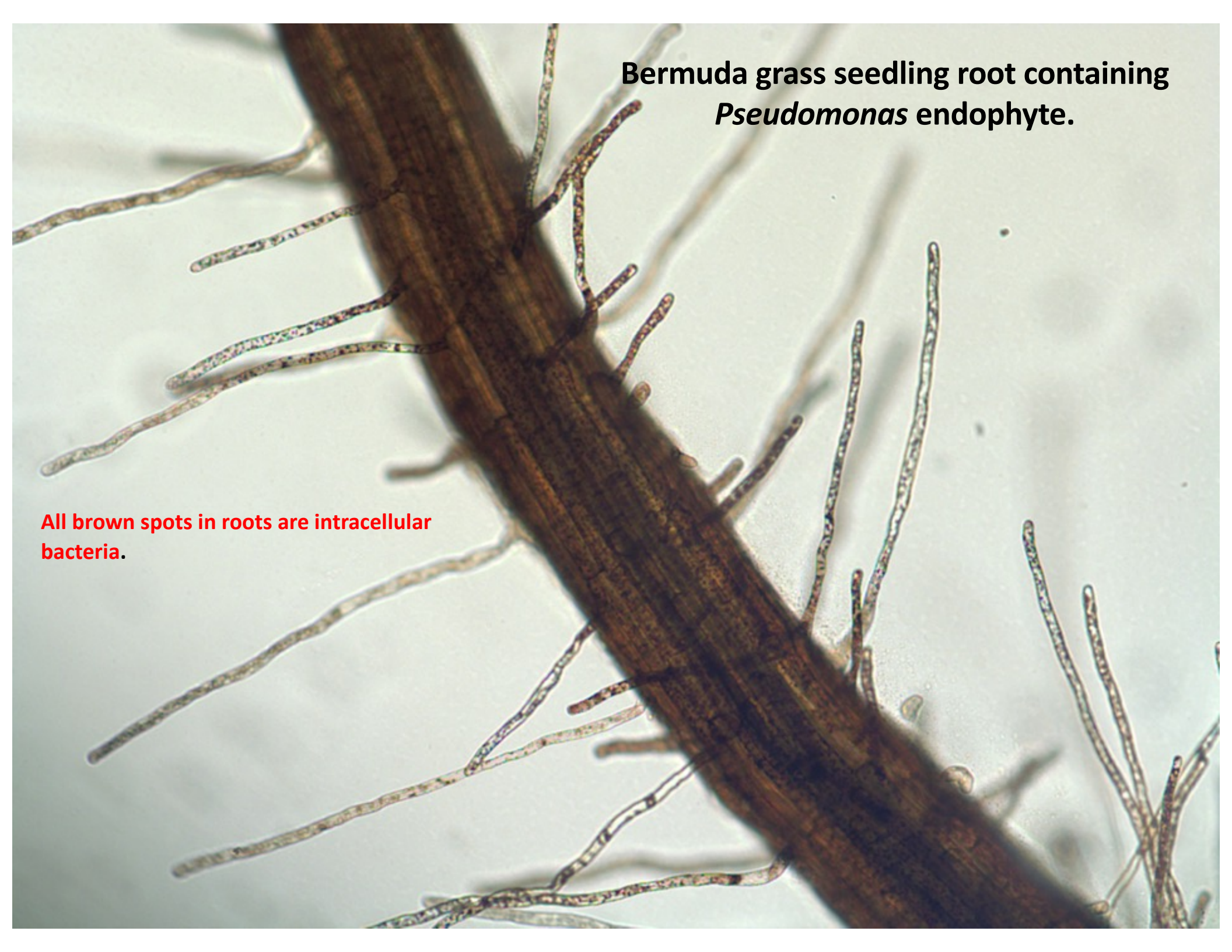
Bermuda grass root containing *Pseudomonas* (bacterium)

Route of endophyte colonization of root at root tip and reentry to rhizosphere from root hairs



RHIZOPHAGY CYCLE

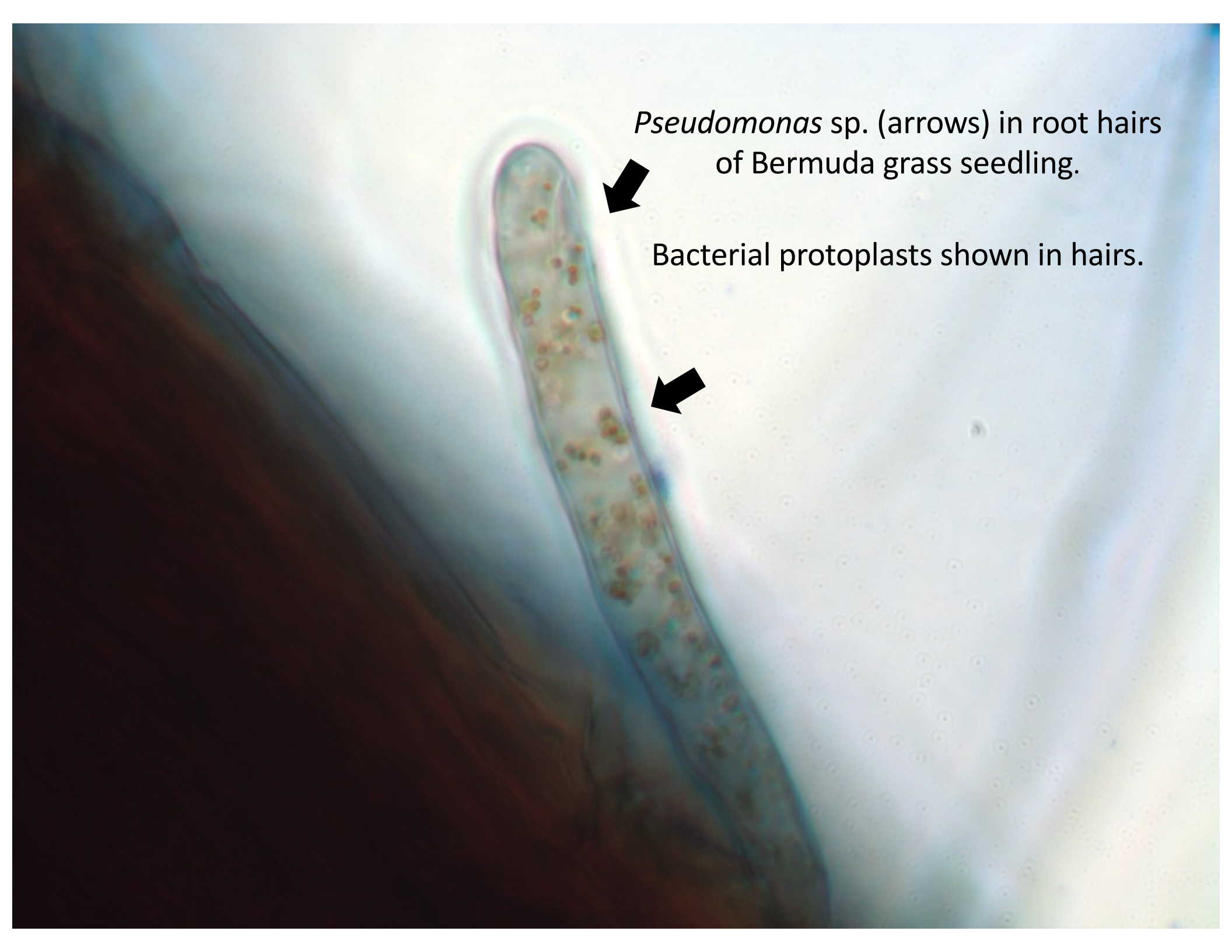




A light micrograph showing a thick, dark brown root of a Bermuda grass seedling. Numerous thinner, lighter-colored roots branch out from the main root. The thinner roots are covered with small, brown, oval-shaped spots, which are intracellular bacteria. The background is a light, uniform color.

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.

This is a light micrograph showing a root hair of a Bermuda grass seedling. The root hair is a long, thin, translucent structure. Inside, numerous small, circular, brownish-orange protoplasts are visible. Two black arrows point to these protoplasts. The background is a light, slightly textured surface.

Bacterial protoplasts shown in hairs.

Streptomycin treatment of tomato seedlings

Experiment: All seeds surface disinfected for 20 mins in 4% sodium hypochlorite—then washed.

½ seeds treated with streptomycin (100 mg/L) for 24 hours to inhibit growth of endophytic bacteria.

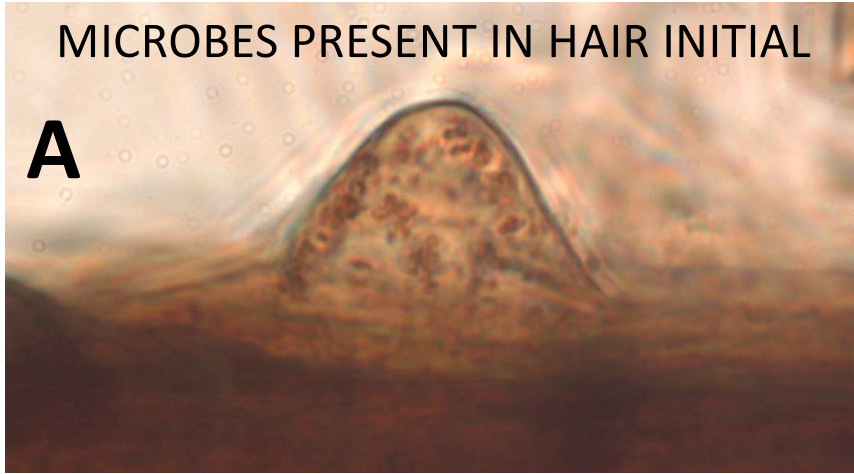
Results: Where bacteria are present in seedlings, tomato seedlings (3-days-old) show root hair formation (arrow); and where antibiotic limits bacterial growth no hairs form.



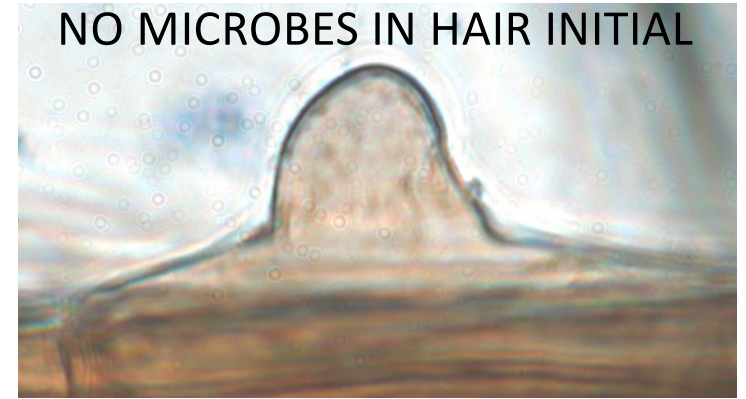
Mode of action: Streptomycin binds to the small 16S rRNA bacterial ribosome and inhibits protein synthesis.

MICROBES PRESENT IN HAIR INITIAL

A

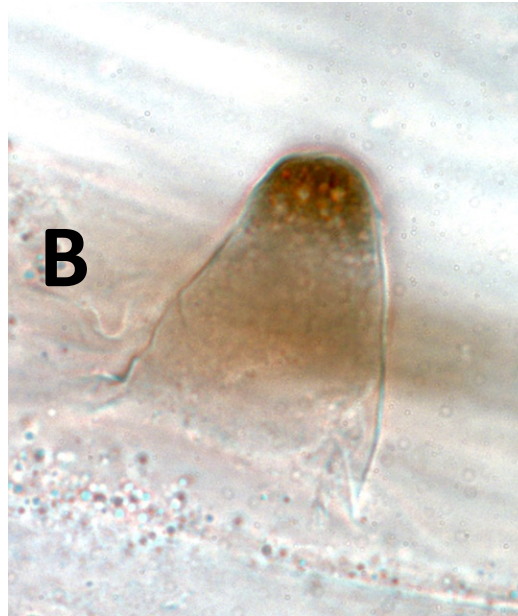


NO MICROBES IN HAIR INITIAL



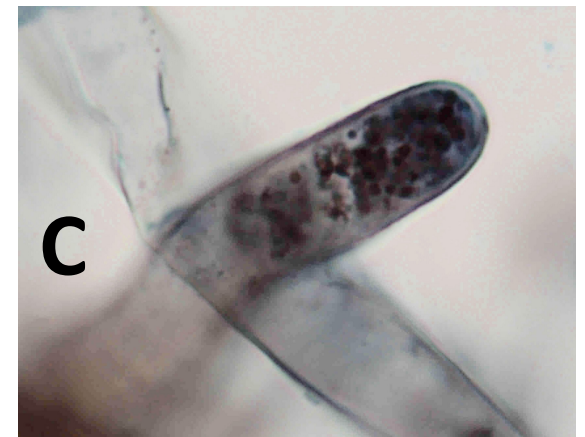
Tomato Root Hair Initial Without Internal Microbes Do Not Elongate.

B



Root hair growth is linked to presence of microbes in hair initials.

C

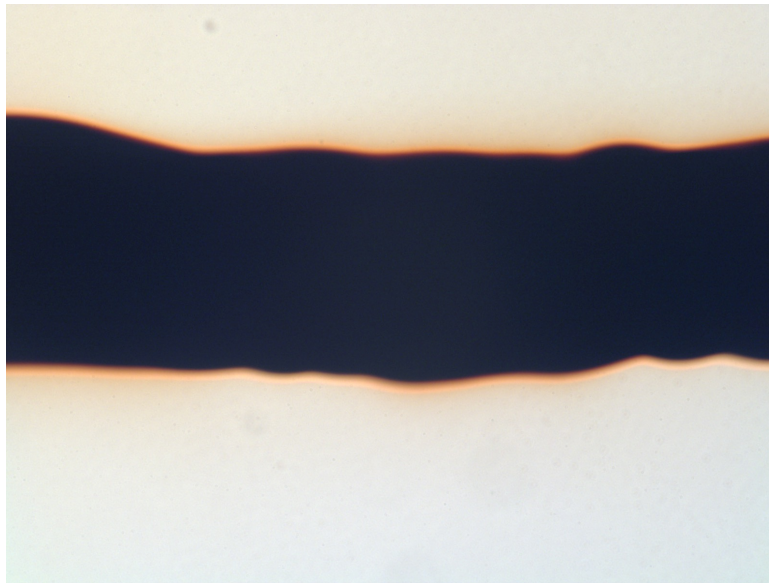


Why is root hair growth linked to presence of intracellular bacteria?



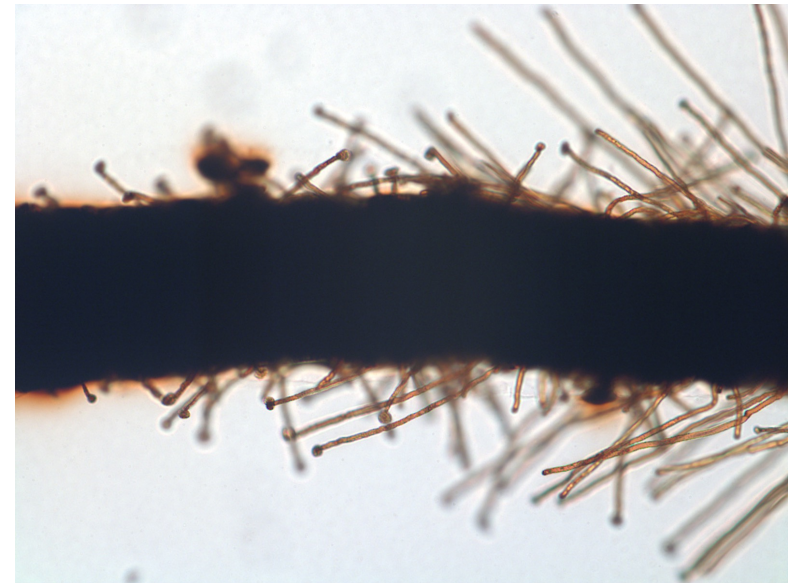
Xiaoqian (Ivy) Chang

Experiments to test the 'Microbial Stimulated Cell Growth Hypothesis'



**No microbes in
seedlings**

(Seeds disinfected rigorously.)



Bacterium present

(*Pseudomonads fluorescens* inoculated
onto disinfected seeds.)

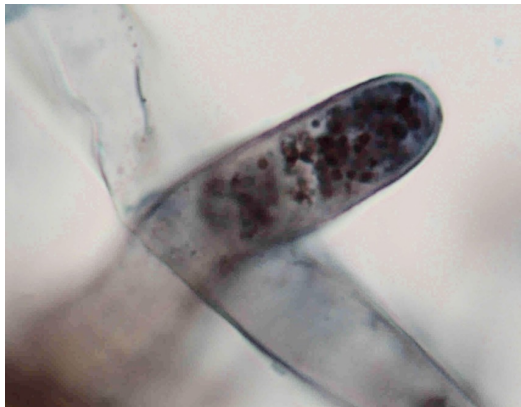
What stimulates the plant root hairs to elongate?

Microbe Produced Hormones Hypothesis

Microbial Ethylene and Nitric Oxide Stimulate Root Cell Growth

Microbes in root hair tip
produce ethylene and nitric oxide

Ethylene and nitric oxide
act as hormones,
causing root hair to elongate



Ethylene
Nitric oxide

Root Hair Elongates

Sedge (*Fimbristylis cymosa*)

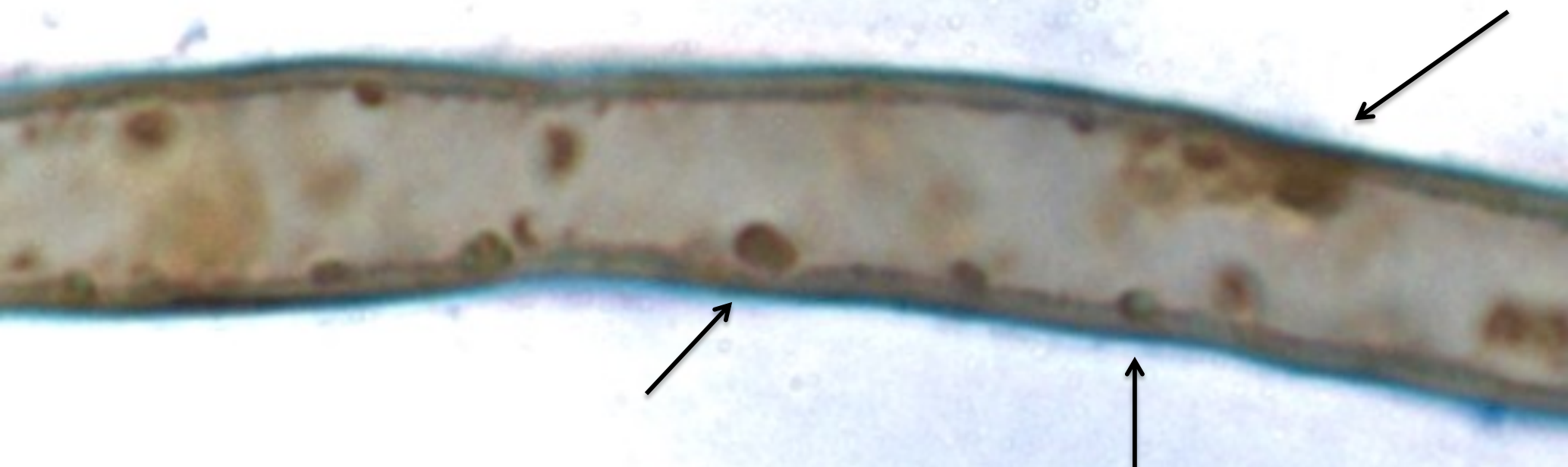
Plant grows in pits and crevices of limestone or in sand along high salt Caribbean shore environments.

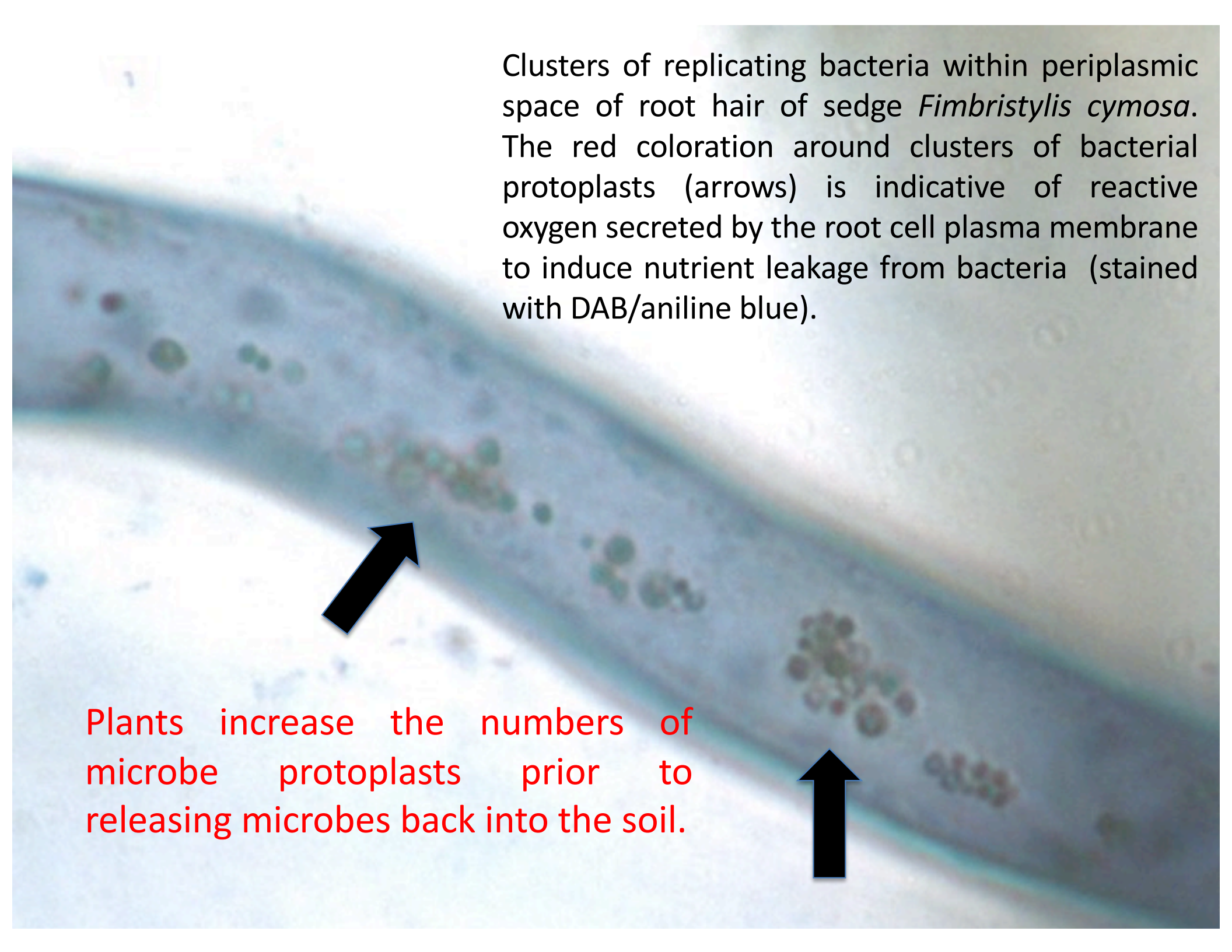


Root hair showing microbes circulating along interior of hair

Constant cyclosis of microbes enables efficient nutrient exchange between microbe and root cells and reduces exposure to superoxide (permitting microbe replication and N fixation).

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




A light micrograph of a sedge root hair, *Fimbristylis cymosa*, showing clusters of replicating bacteria within the periplasmic space. The root hair is a long, thin, curved structure. Inside, there are several clusters of small, dark, circular bacterial protoplasts. Some of these clusters are surrounded by a reddish-brown stain, which is indicative of reactive oxygen secreted by the root cell plasma membrane. Two black arrows point to specific clusters of bacterial protoplasts. The background is a light, slightly textured surface.

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.

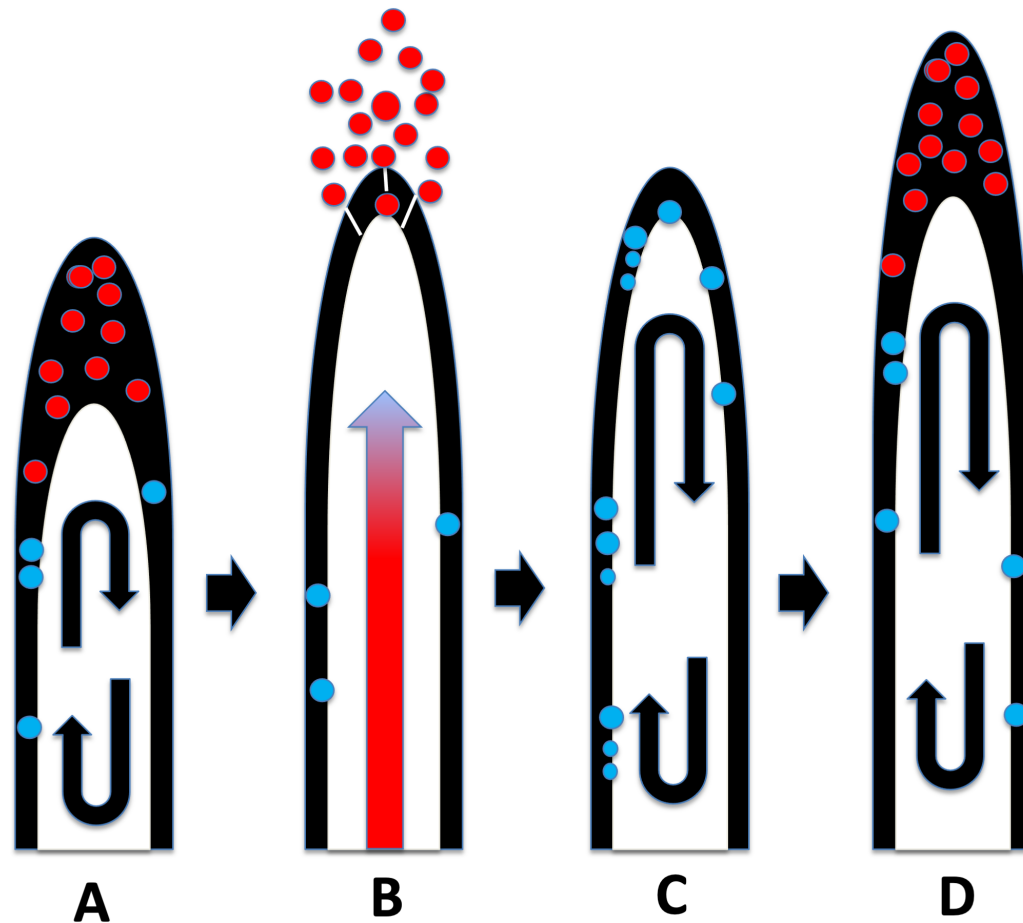
Qiang Chen



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.

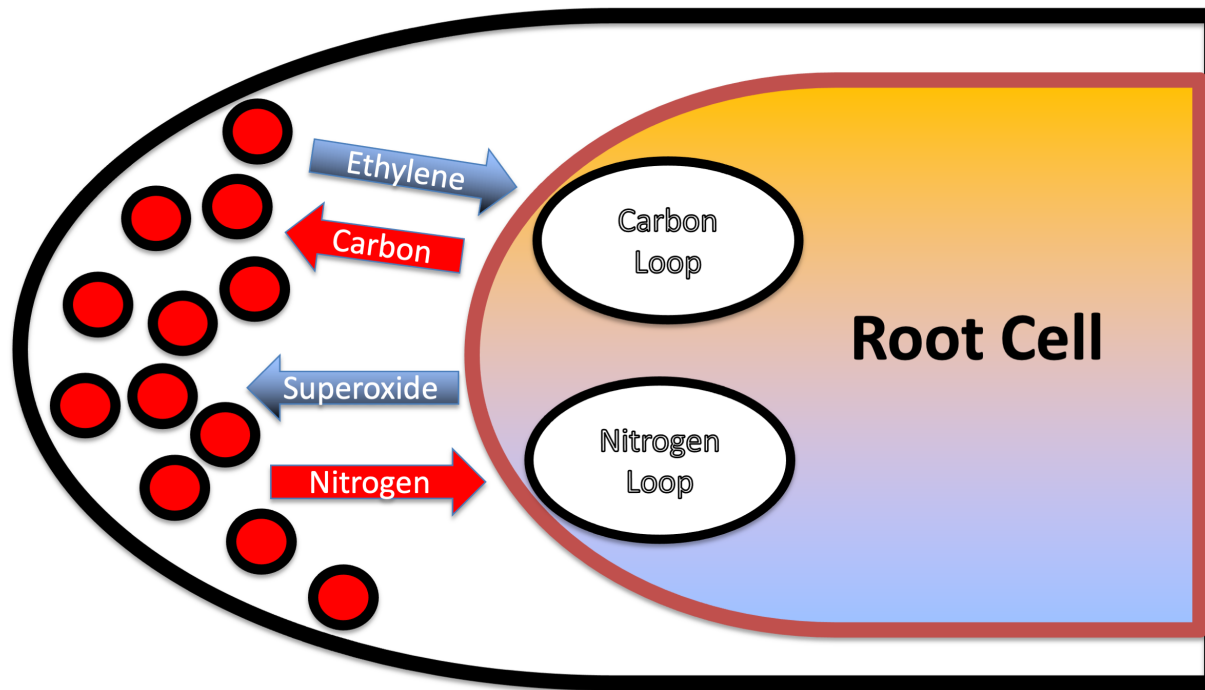


Sequence of periodic build-up and ejections of bacteria from root hairs.



Red bacteria are active in antioxidant nitrogen secretion while blue bacteria are active in nitrogen fixation.

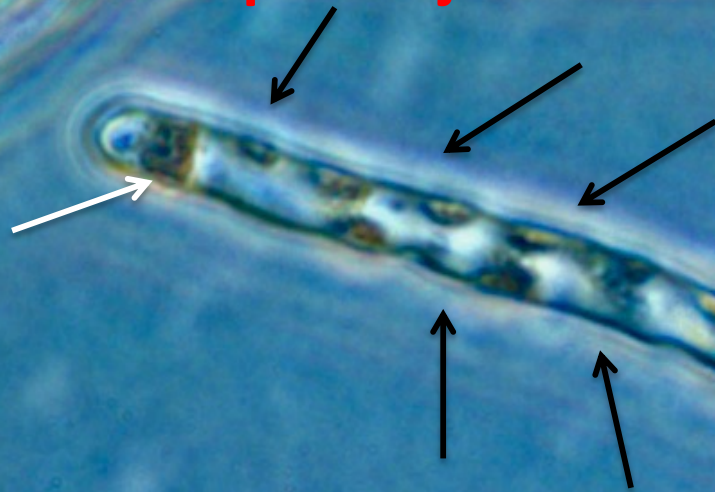
Nitrogen-transfer symbiosis in plant hairs



Nitrogenous antioxidants like nitric oxide are secreted by the bacteria to neutralize superoxide. Nitric oxide combines with superoxide to form nitrate. Nitrate is absorbed directly into the plant.

Microbe ejection appears to be periodic rather than continuous. Microbes may be ejected in clusters rather than 1 at a time. This may be the result of ethylene-triggered growth spurts. A growth spurt occurs after a critical mass of bacteria in hair tips secrete enough ethylene to cause hair elongation.

Incomplete ejection of microbes in hairs suggest periodic ejection.



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



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.

1

2

3



Nutrient Absorption Function of the Rhizophagy Cycle:

Plant nutrient sources



Ivy Chang

- 1) Nutrients that are dissolved in soil water**
- 2) Nutrients that must be oxidatively extracted from soil microbes within root cells**
- 3) Nutrients obtained from mycorrhizae**

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

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 biologicheskaja / Rossijskaja akademija 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.

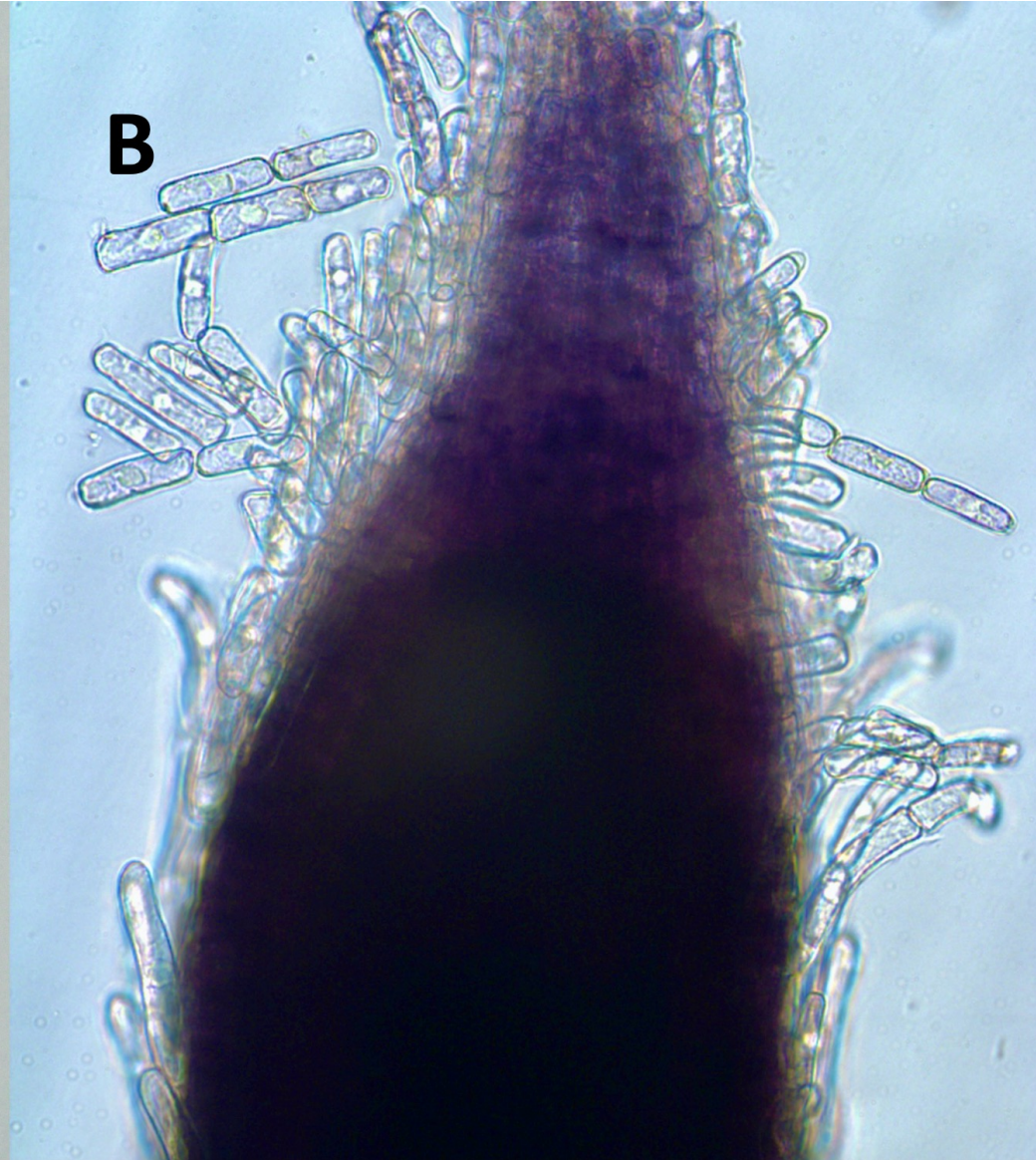
Winter wheat root tips showing: **A.** Repression of superoxide in epidermis layer after elevated CO_2 treatment. **B.** Root tip from non-elevated CO_2 treatment.

A



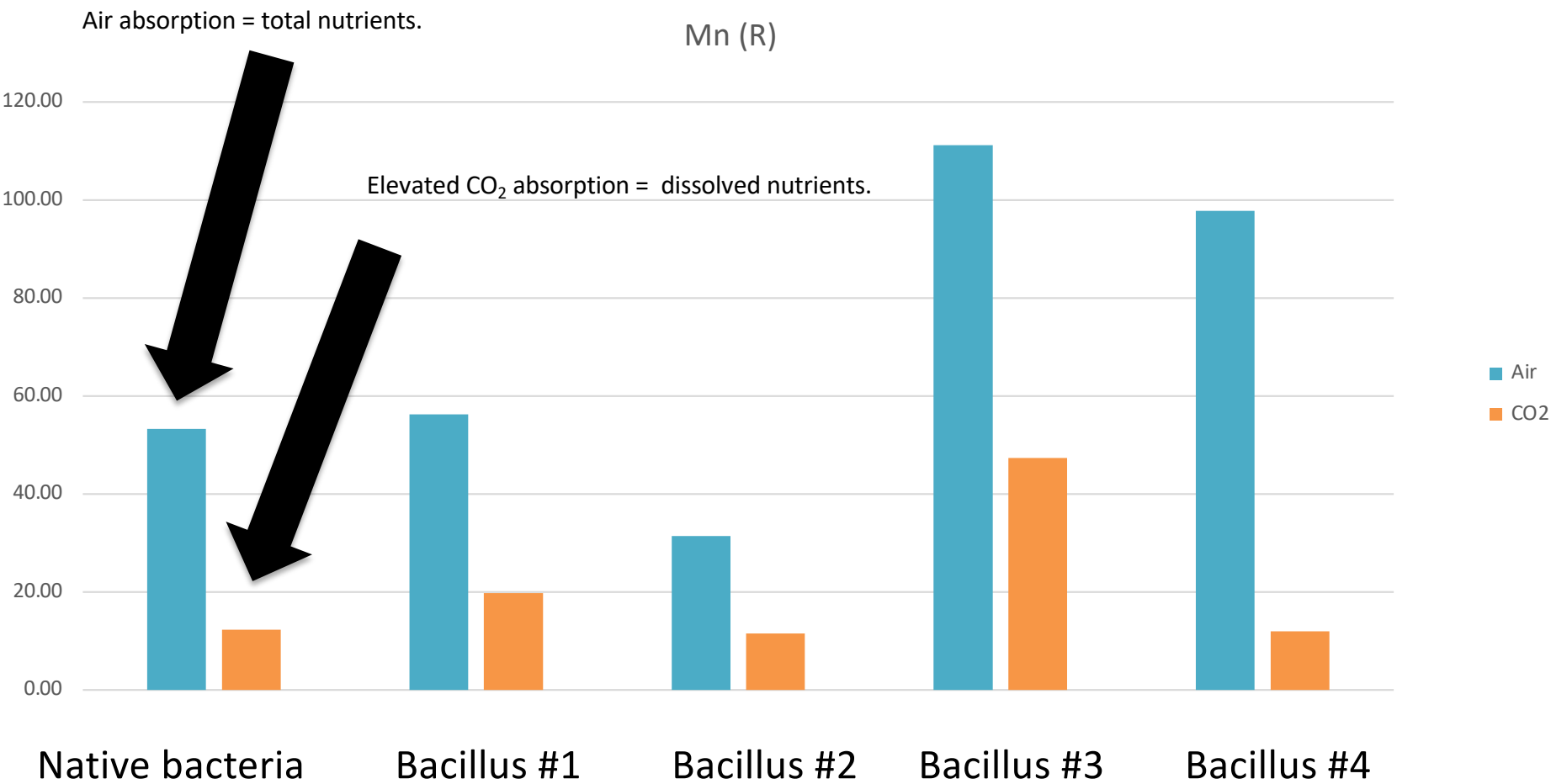
Elevated CO_2

B



Non-Elevated CO_2

MANGANESE (Mn) in Soybean Roots



Using *Bacillus* spp., the rhizophagy cycle provides 136% to 717% more manganese than absorption of soil dissolved manganese.

Rhizophagy Nutrients:

Micronutrients tend to be favored in oxidative extraction from bacteria in the rhizophagy cycle.

Sequence of oxidative extraction for nutrients:

Mn > Fe > Ca > Mg > S > Cu > N > Zn > P > K

Nitrogen Fixation by Endophytes

The first land plants (Bryophyta) internalized bacteria into their cells (hairs) to obtain nitrogen from them!

In plant hairs plants cultivate and extract nitrogen from nitrogen-fixing bacteria.



Lena Struwe

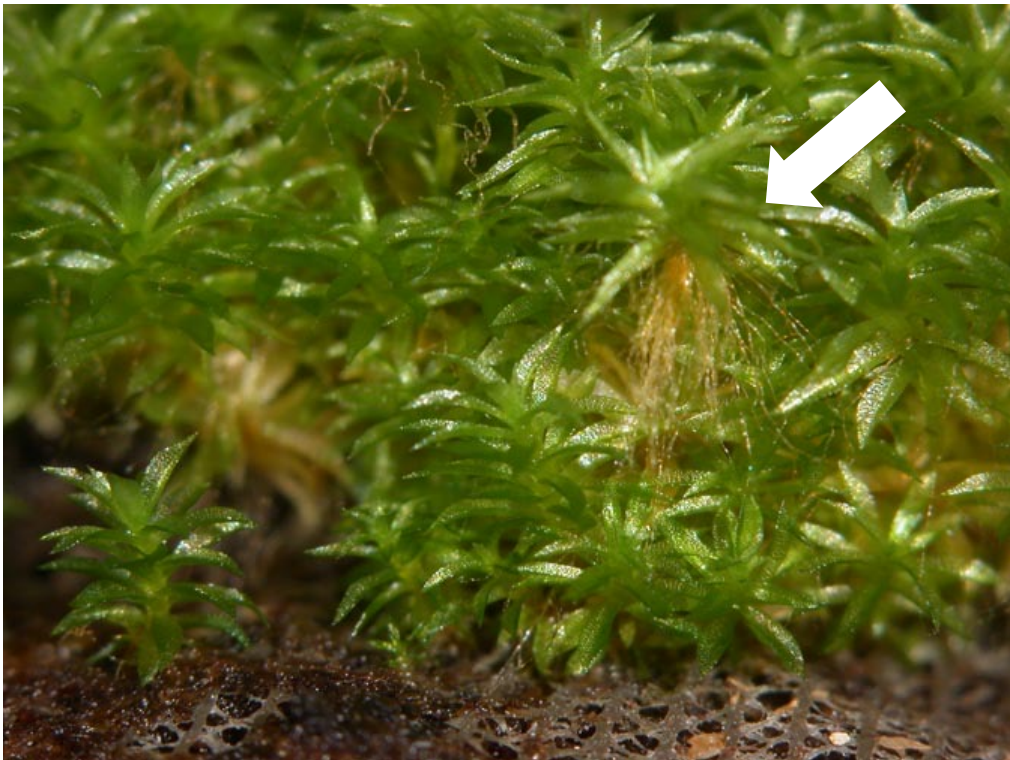


Nicole Vaccaro

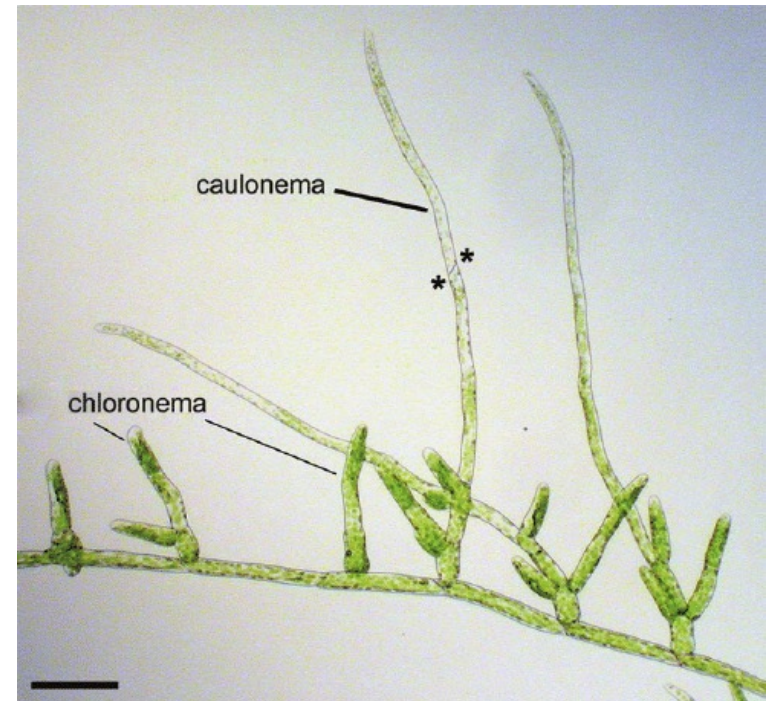


Blair Young

Moss (*Physcomitrella patens*) gametophytes have chloroplasts and do photosynthesis, but they also have non-photosynthetic tissues where nitrogen-transfer endosymbiosis occurs. Achlorophyllous filaments termed 'caulonemata' contain bacteria that transfer nitrogen to the moss gametophyte.



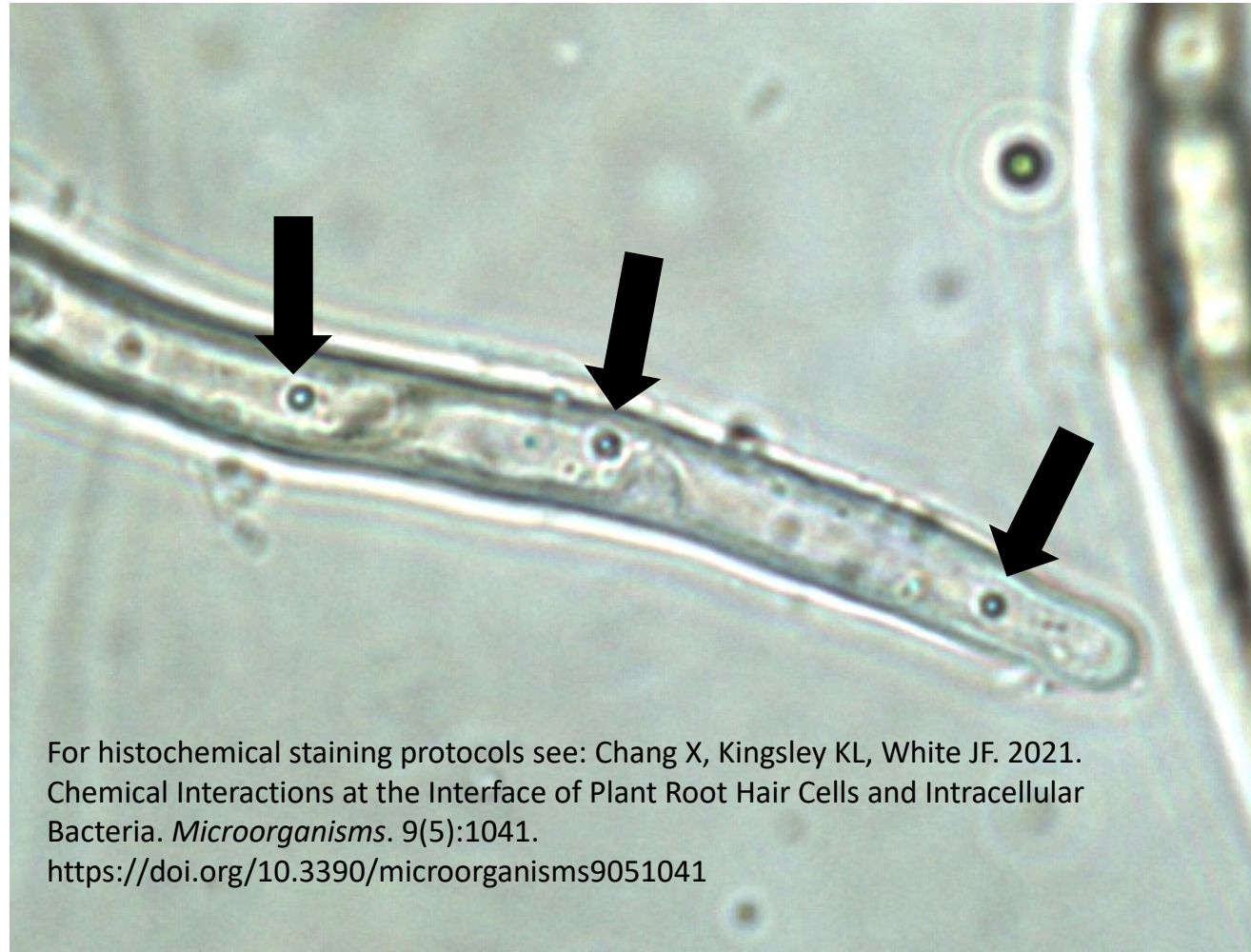
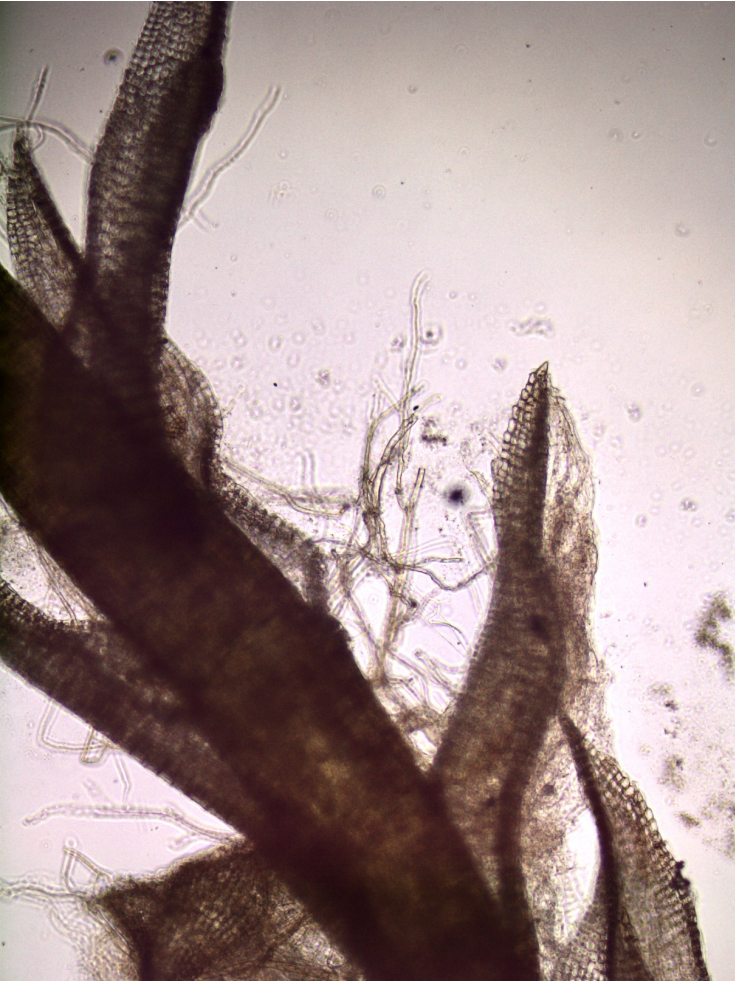
The brown filaments (arrow) in this image are caulonemata.



Chloronemata are photosynthetic filaments; while caulonemata function to fix atmospheric nitrogen and transfer it to the photosynthesizing gametophyte.

Caulonemata of moss stained for ethylene (blue color) around intracellular bacteria (arrows). Stain is ammonium molybdate.

Moss filaments are the earliest versions of plant hairs (trichomes) and they function to extract nitrogen from bacteria.



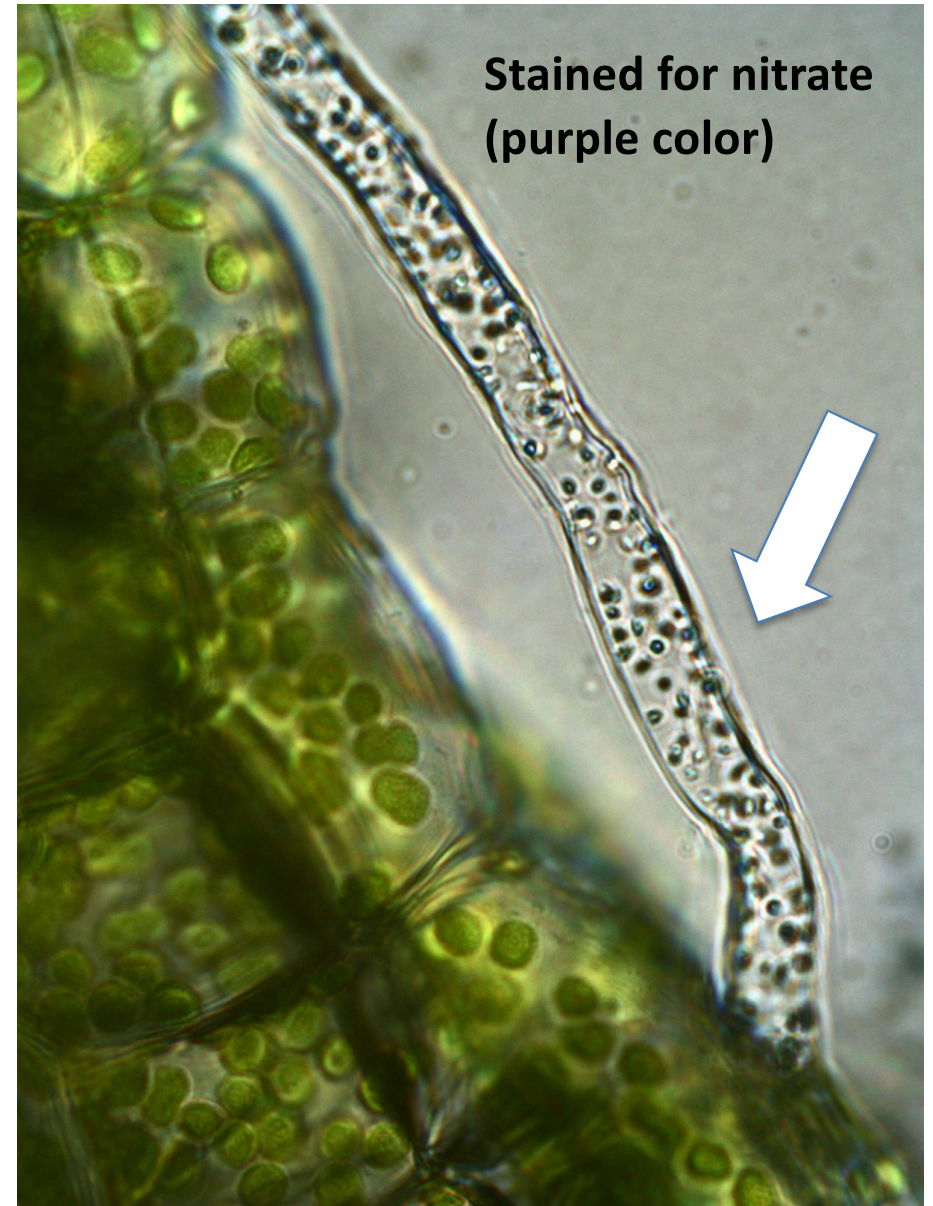
For histochemical staining protocols see: Chang X, Kingsley KL, White JF. 2021. Chemical Interactions at the Interface of Plant Root Hair Cells and Intracellular Bacteria. *Microorganisms*. 9(5):1041. <https://doi.org/10.3390/microorganisms9051041>

The very first land plants used endophytic microbes for nutrients from the start. These endophytes are about delivering nitrogen to plants.



Liverwort (*Riccia* sp.)

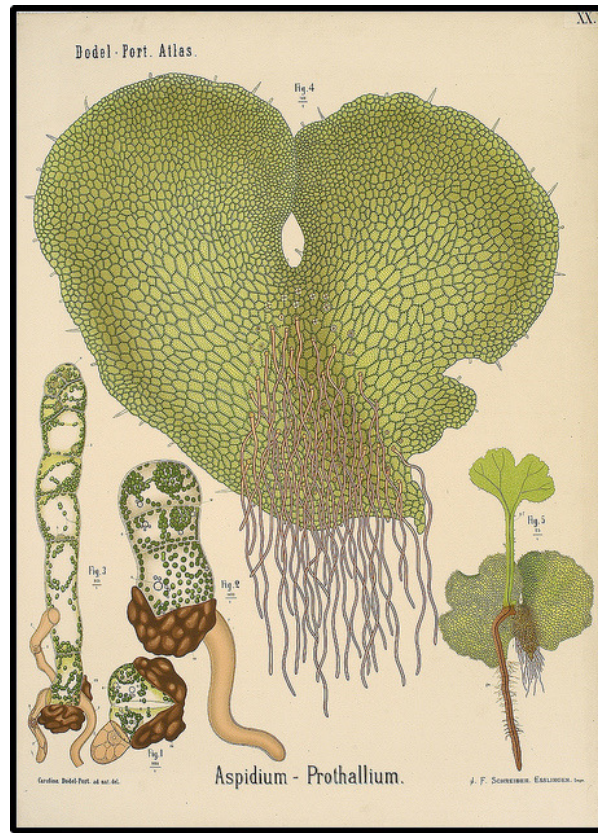
Plant lacks leaves and roots-but has non-photosynthetic filaments that contain bacteria (white arrow).





Boston Fern: *Nephrolepis exaltata*

Ferns are seedless plants that also produce filaments that contain bacteria and show formation of nitrogen around bacterial clusters (arrows) as evidenced by purple color when stained with bromothymol blue stain.



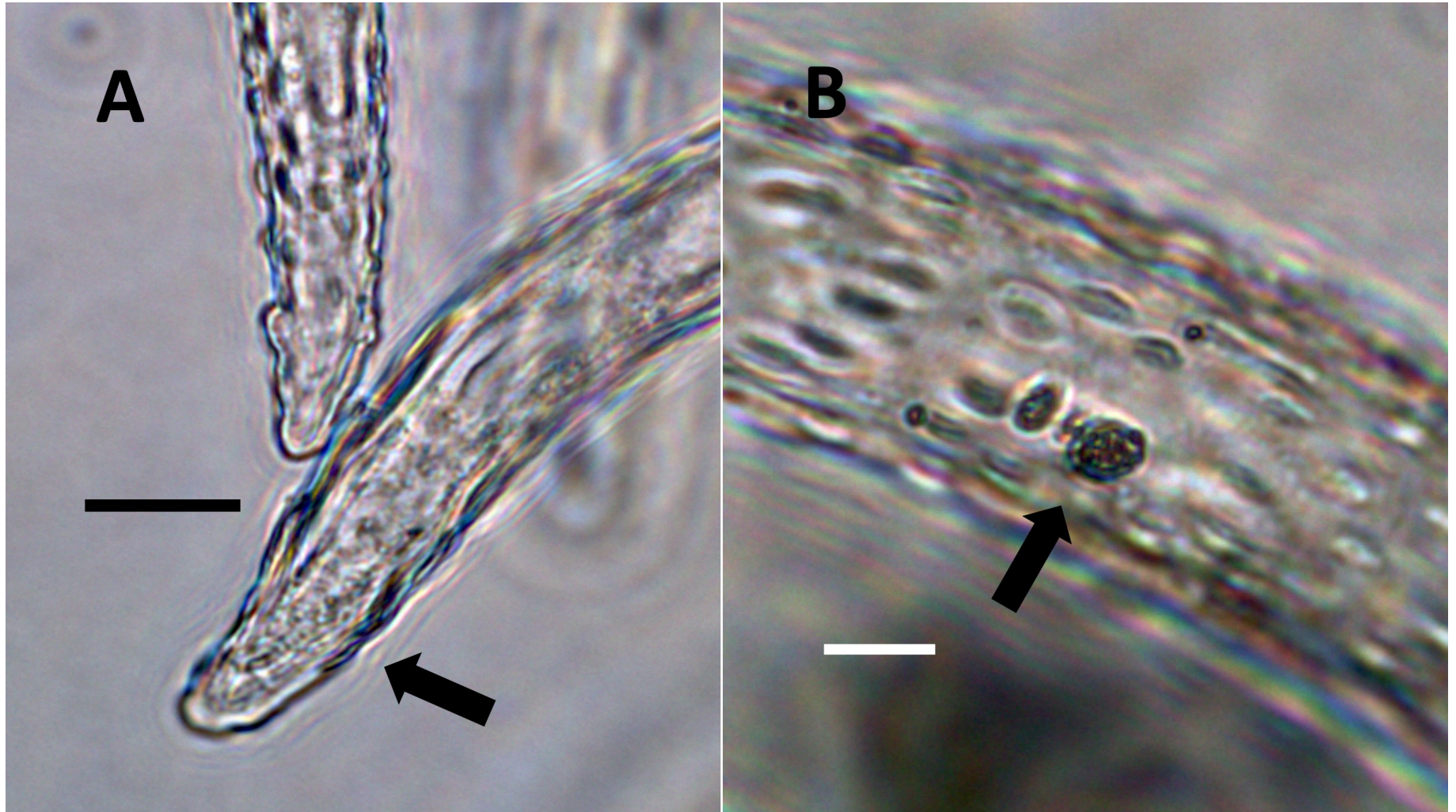
Tree-of-heaven (*Ailanthus altissima*)



A microscopic image showing numerous long, thin, needle-like trichomes (hairs) emerging from a dark, textured surface. The trichomes are translucent with a central canal and show some internal structure. They are arranged in a somewhat regular, upright pattern.

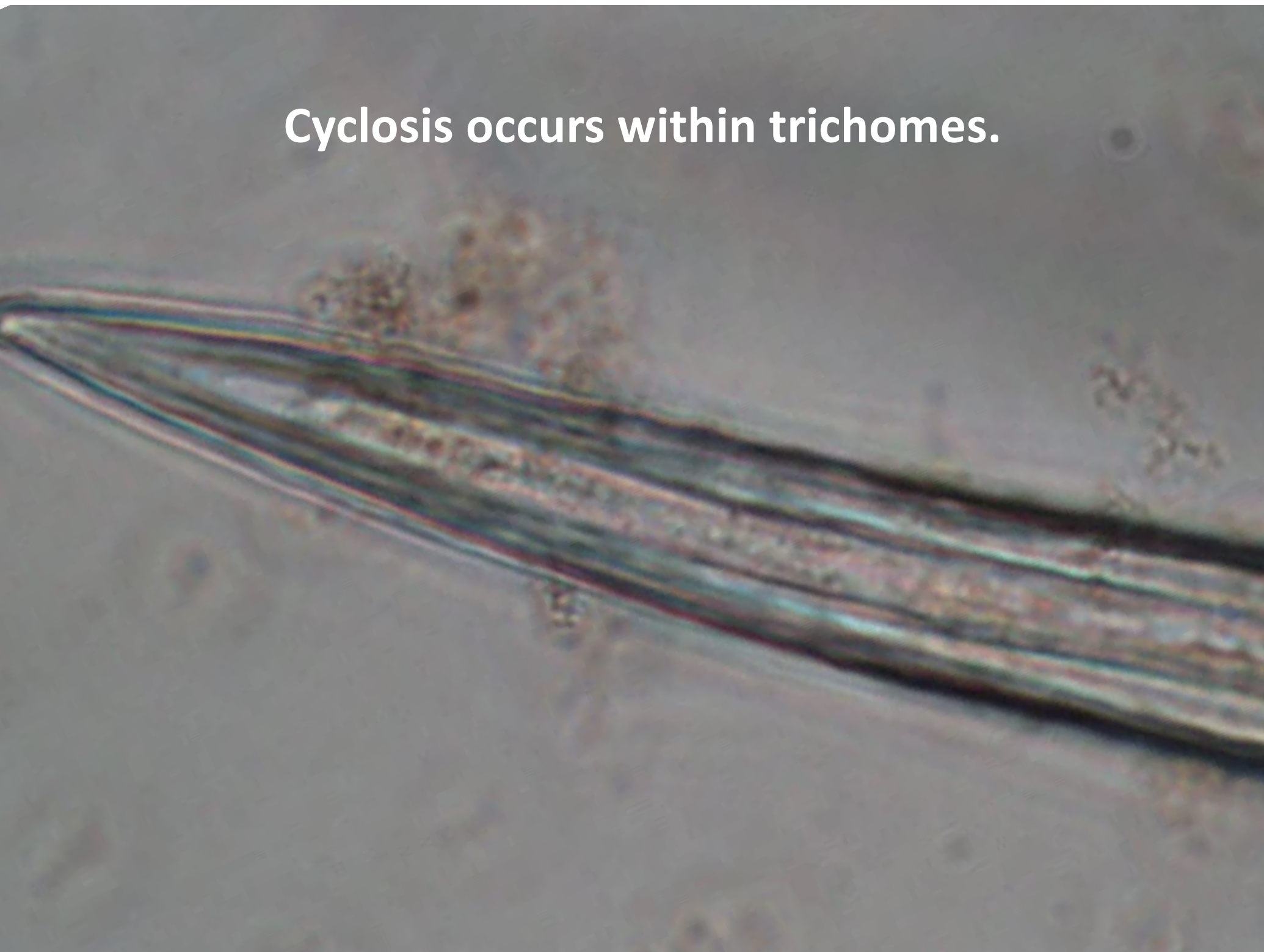
**Trichomes
of tree-of-heaven**

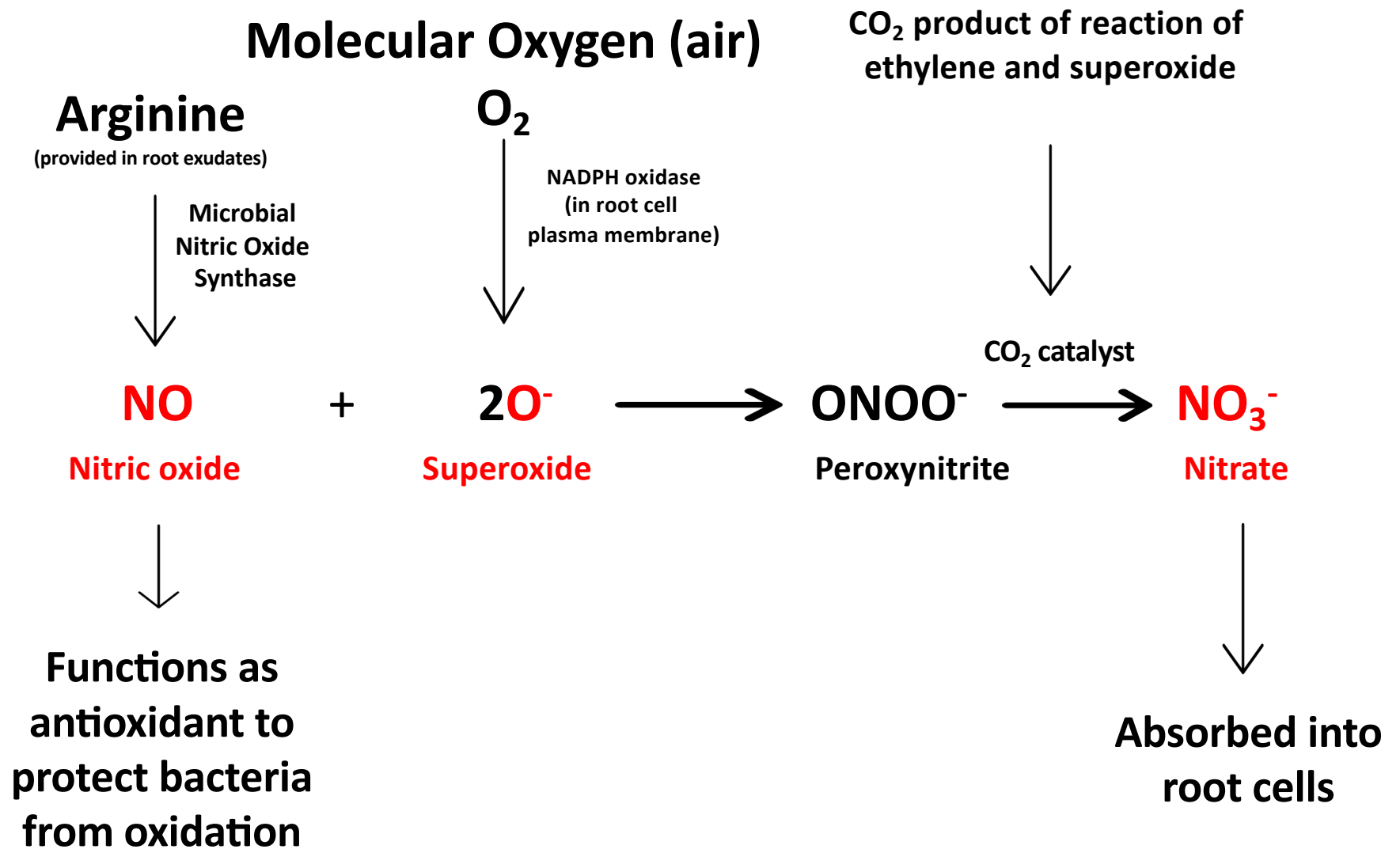
Symbiosis in pitted filamentous trichomes of tree-of-heaven



Pitted trichome of tree-of-heaven (*Ailanthus altissima*) showing bacteria. A. Developing trichome stained with acidified diphenylamine showing nitrate (blue color) around bacteria (arrow) in the tip of the trichome (Bar = 10 μm). B. Trichome stained with sulfur monochloride to show bacteria (arrow) emerging from lateral pits in wall (Bar = 10 μm).

Cyclosis occurs within trichomes.





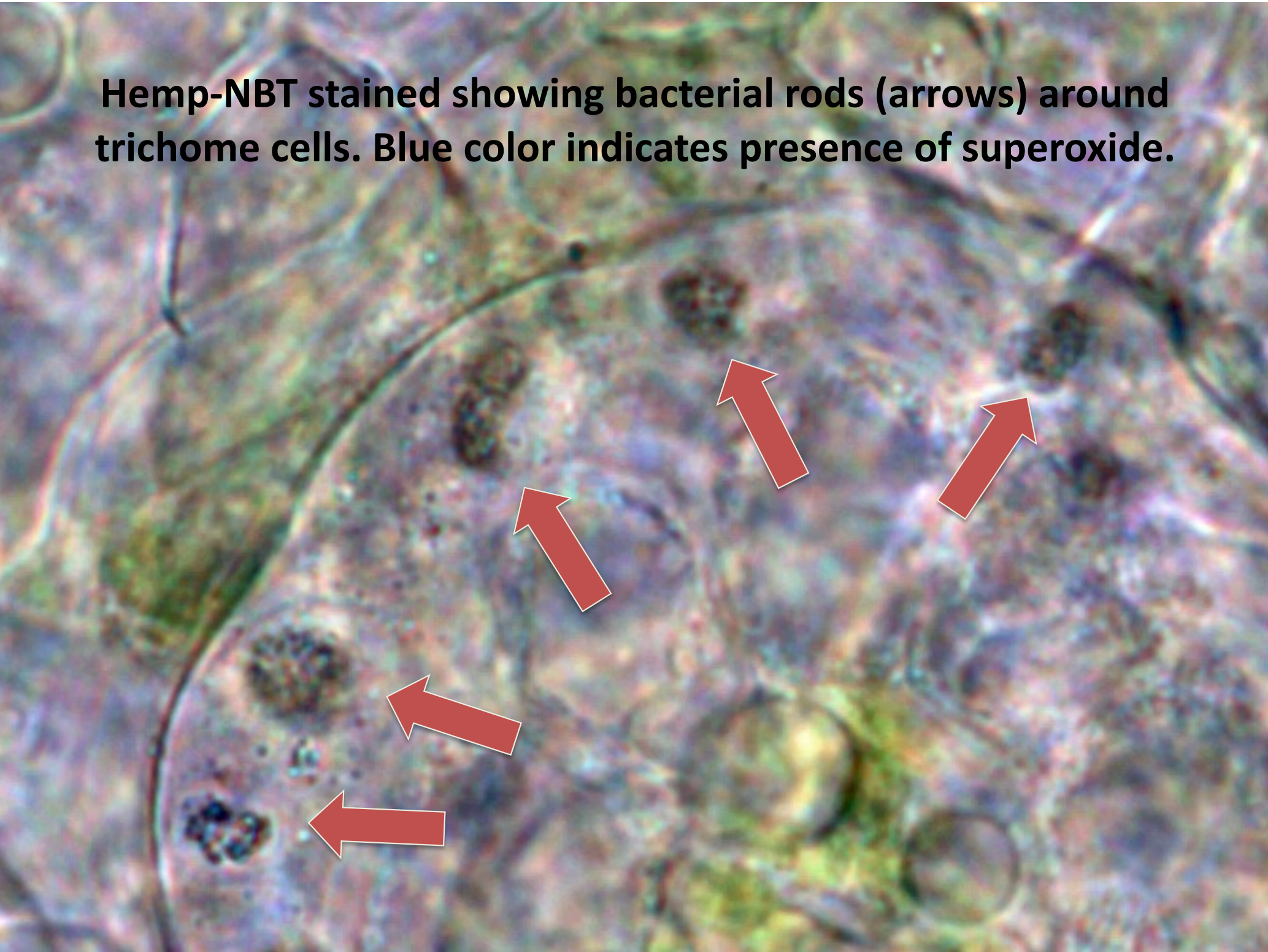
Hemp leaves bear trichomes (leaf hairs) that contain endophytic bacteria.



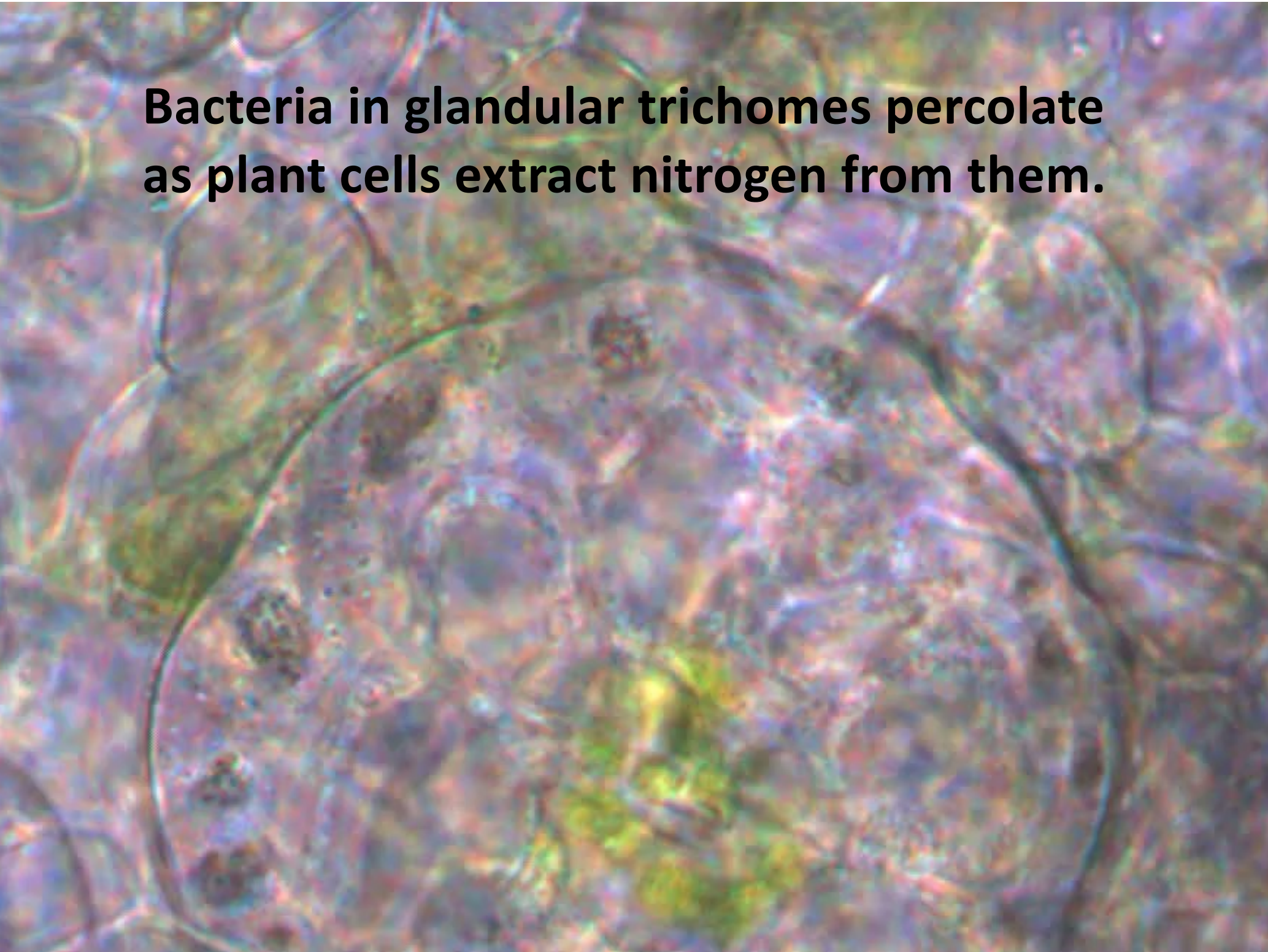
Hemp glandular trichomes unstained



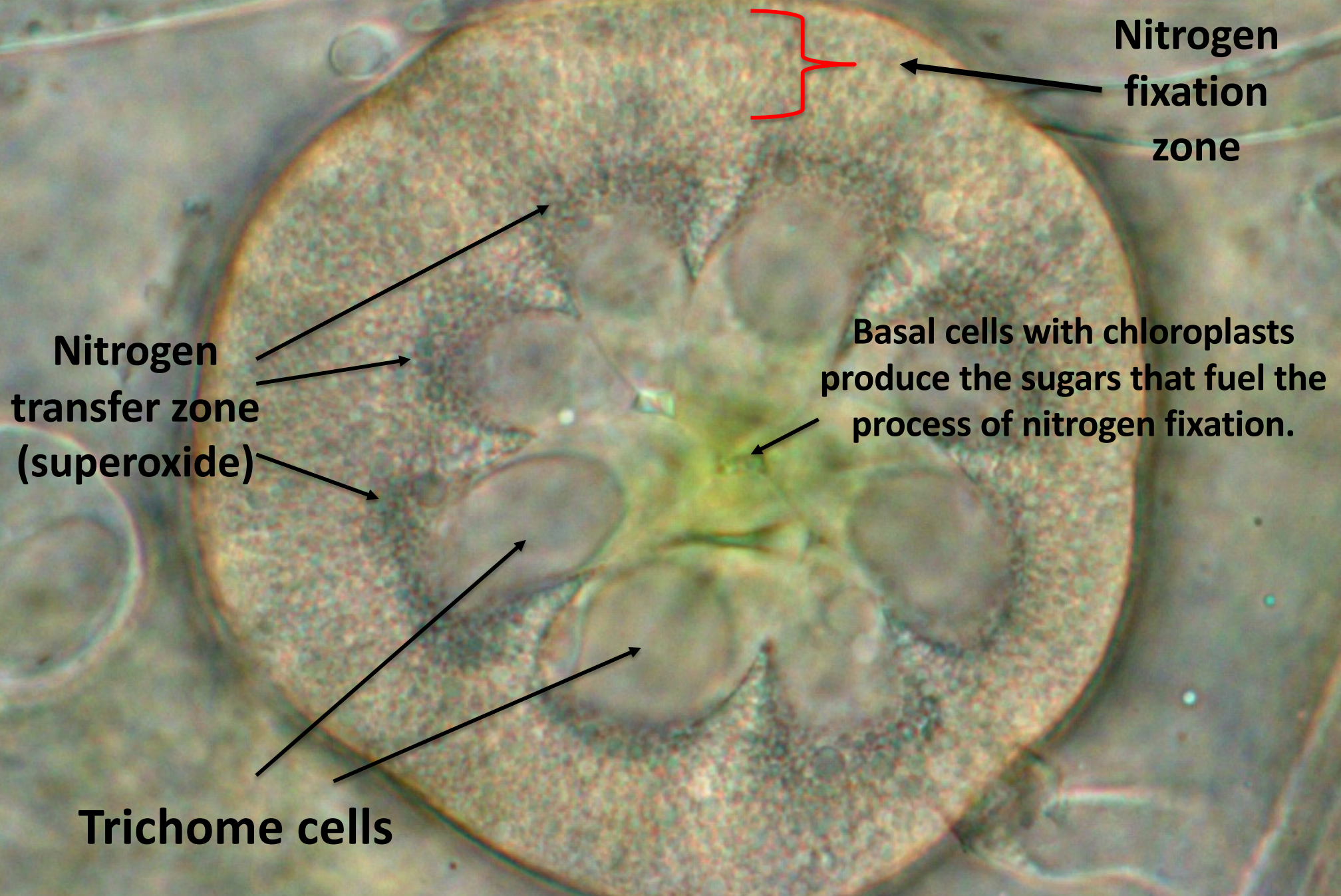
Hemp-NBT stained showing bacterial rods (arrows) around trichome cells. Blue color indicates presence of superoxide.



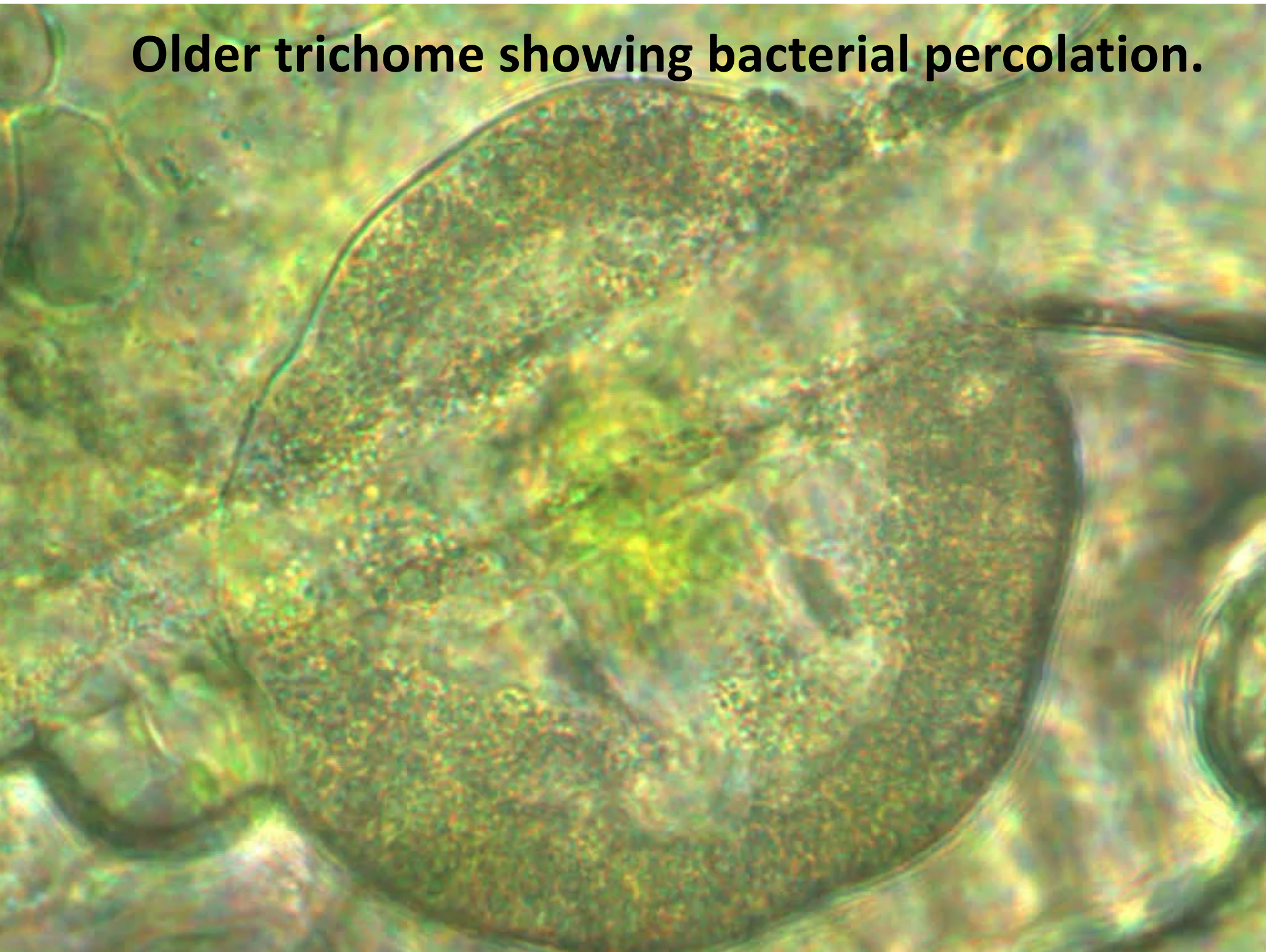
**Bacteria in glandular trichomes percolate
as plant cells extract nitrogen from them.**



Hemp Glandular Trichome



Older trichome showing bacterial percolation.



Developing N-fixing crops by microbiome transfer from land races and wild species.



Dr. Walter Goldstein (Plant Breeder)
Mandaamin Institute, WI

Nitrogen-fixing Peruvian landrace 'piricinco'.



Raquele Strickland
Rutgers University



Fig. Cob leaves dissected from a cob of a nitrogen-fixing Piricinco (from Walter Goldstein; Mandaamin Inst., Wisconsin).

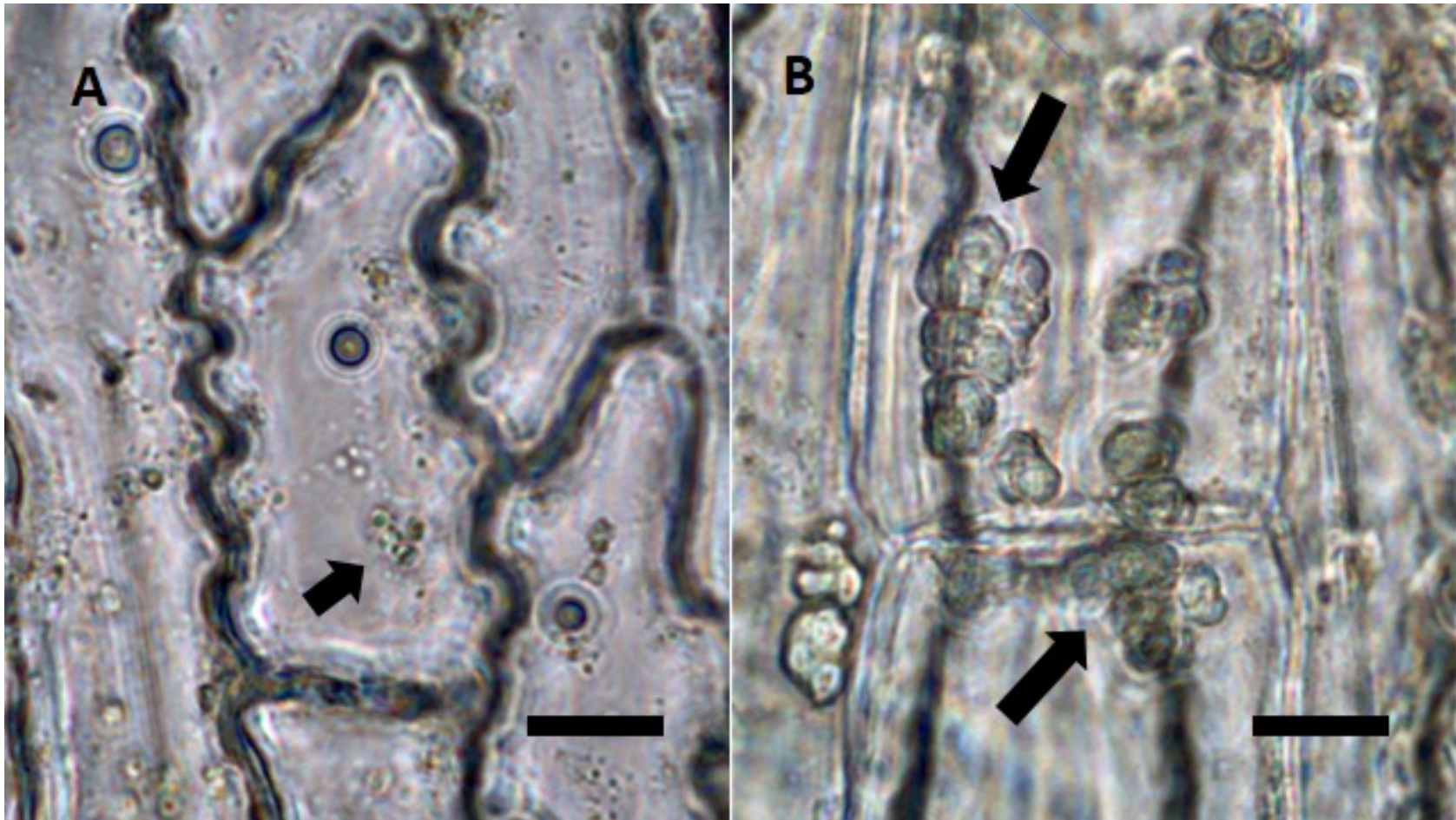


Fig. The microbial community in the husk leaves of a conventional sweet corn variety (A) and in the husk leaves of a nutrient dense, nitrogen efficient Mandaamin Institute cultivar derived from Peruvian 'Piricincó' (B). The arrows show the clusters of bacteria in the epidermal cells (Bar = 10 micrometers).

A

A. Piricinco cob leaf epidermis showing abundant trichomes (arrow). B. Bacteria (arrow) in cob leaf epidermal cells with convoluted walls (stained for nitrate, purple color; bar = 10 micrometers).

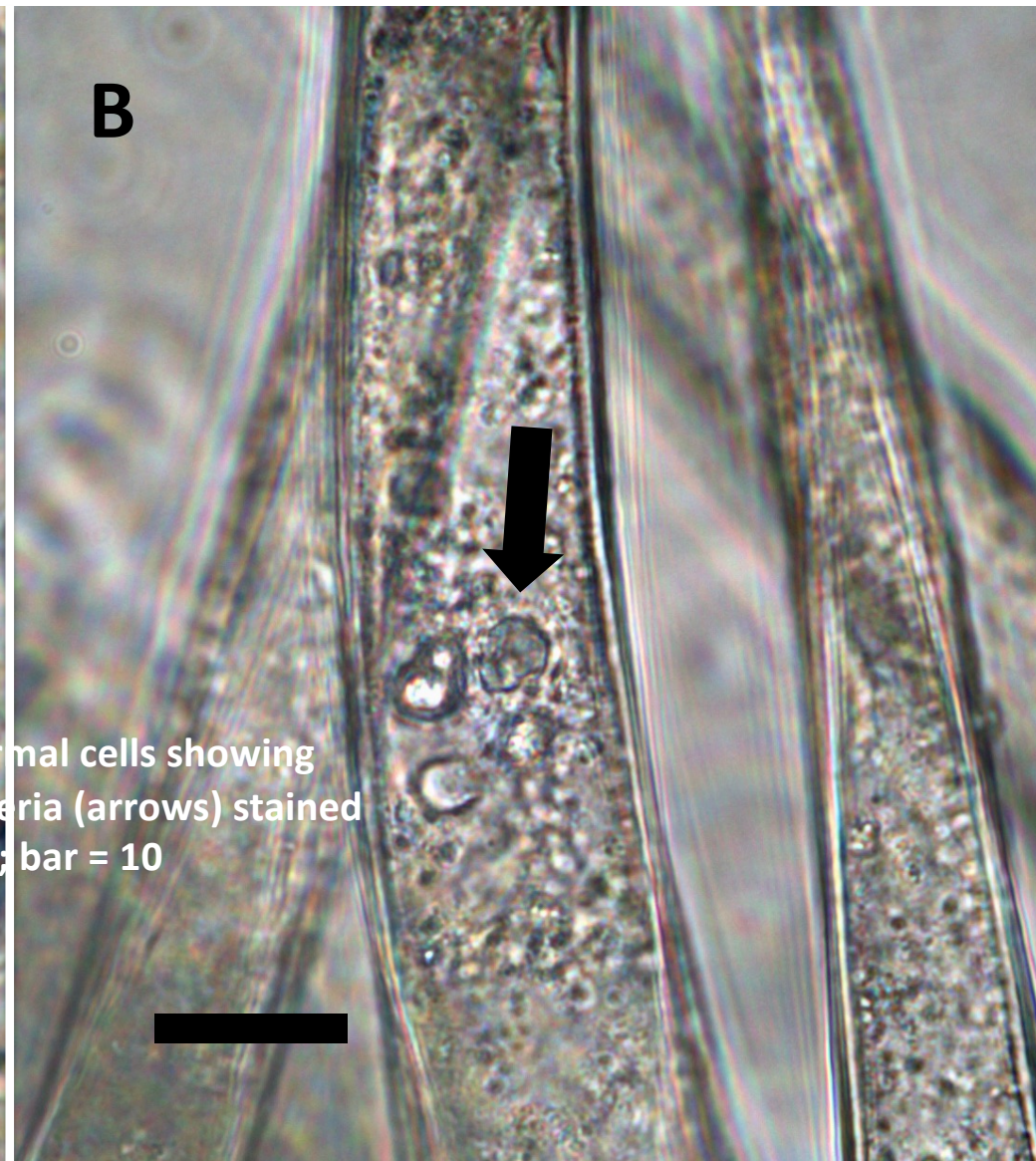
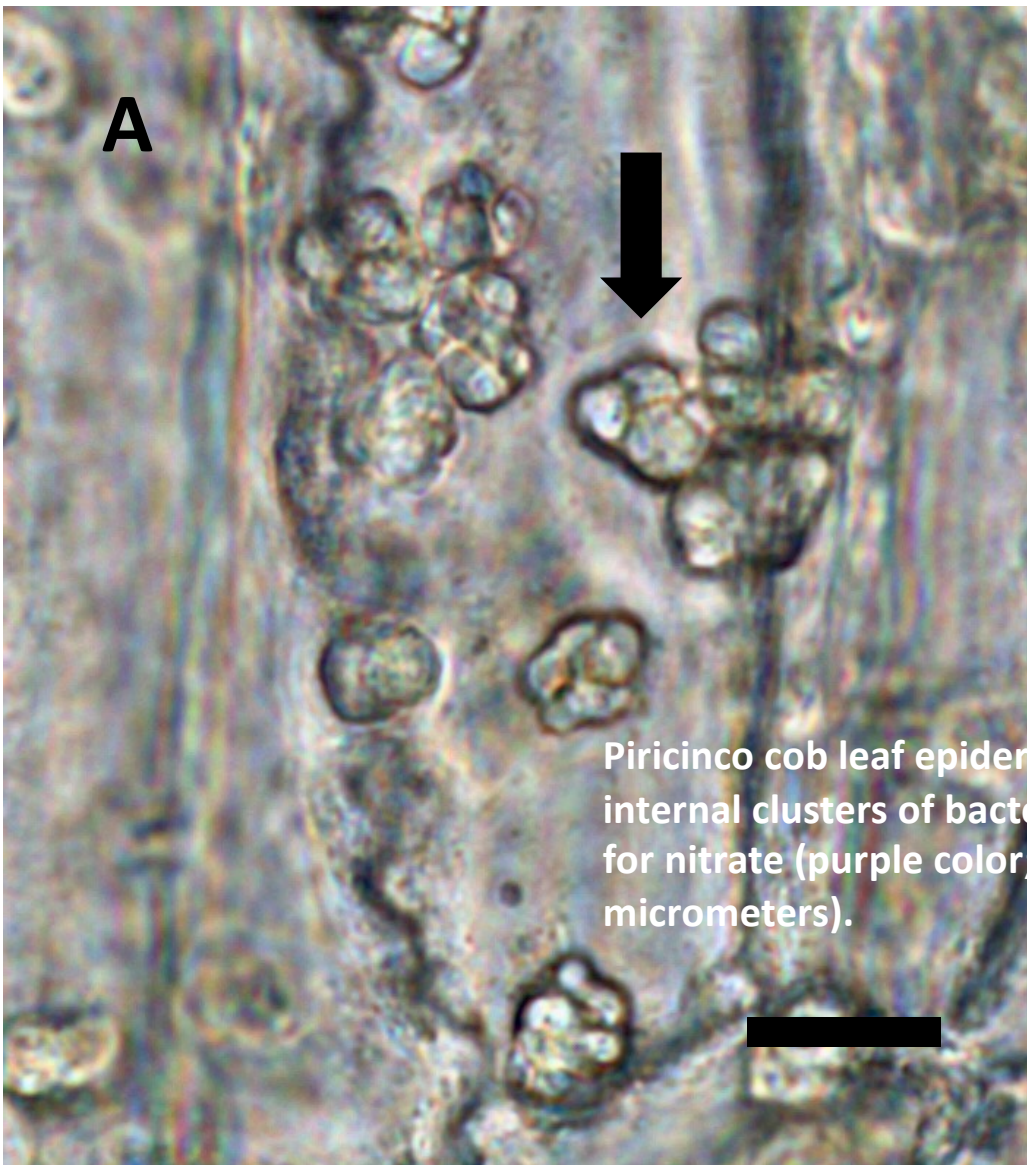
**B**

A



Piricinco cob leaf epidermal cells showing
internal clusters of bacteria (arrows) stained
for nitrate (purple color; bar = 10
micrometers).

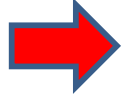
B



Protocol for microbiome transfer from corn to *Poa reptans*

We transfer endophytes between plants using variations of the technique used by the Iroquois Indians in their '**Iroquois corn medicine**'.

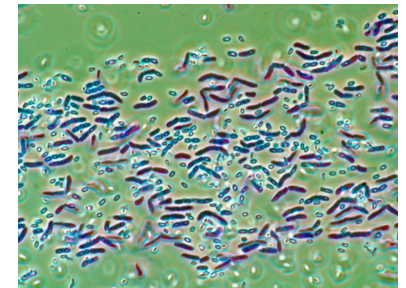
1) Microbiome source
(A = cob Leaves; B = roots)



2) Separate out cob leaves



3) Triturate young cob leaves
In H₂O to release bacteria

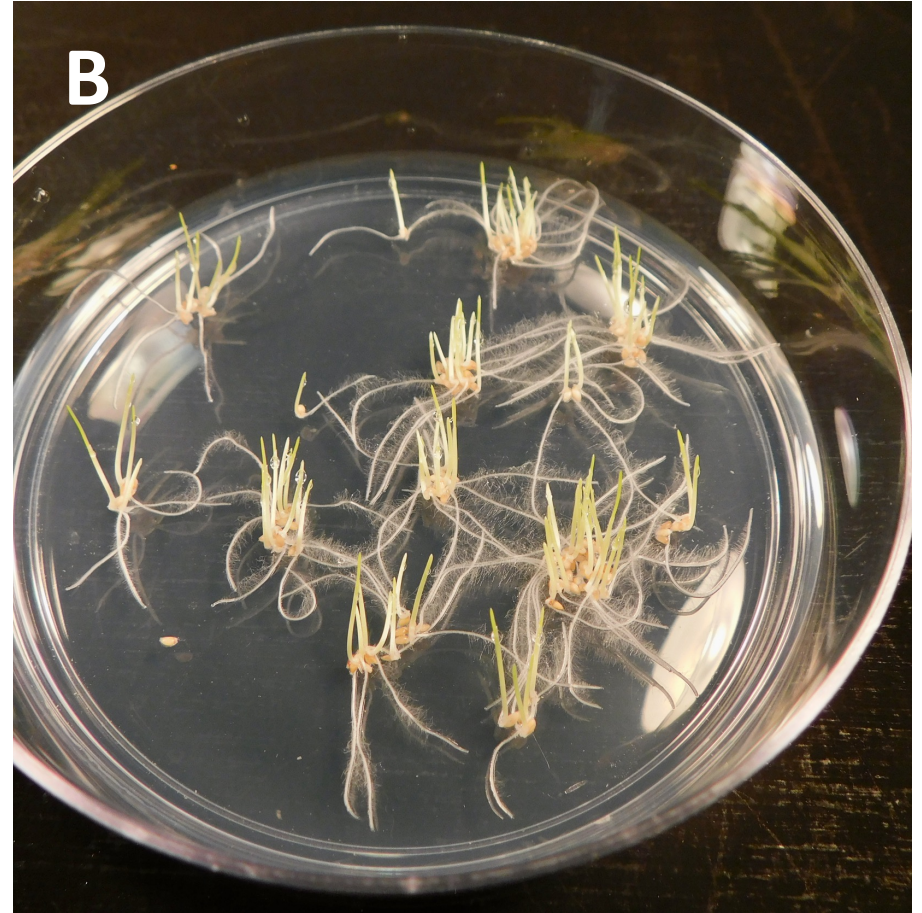
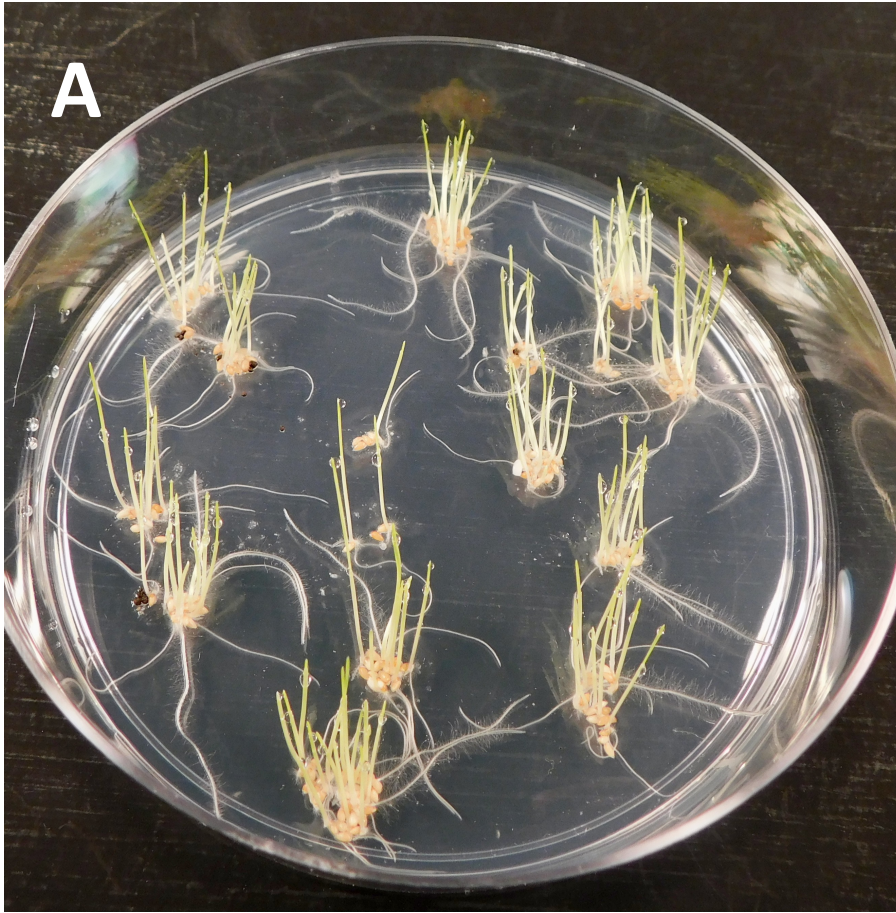


4) Strain out large tissue pieces of
plant tissues



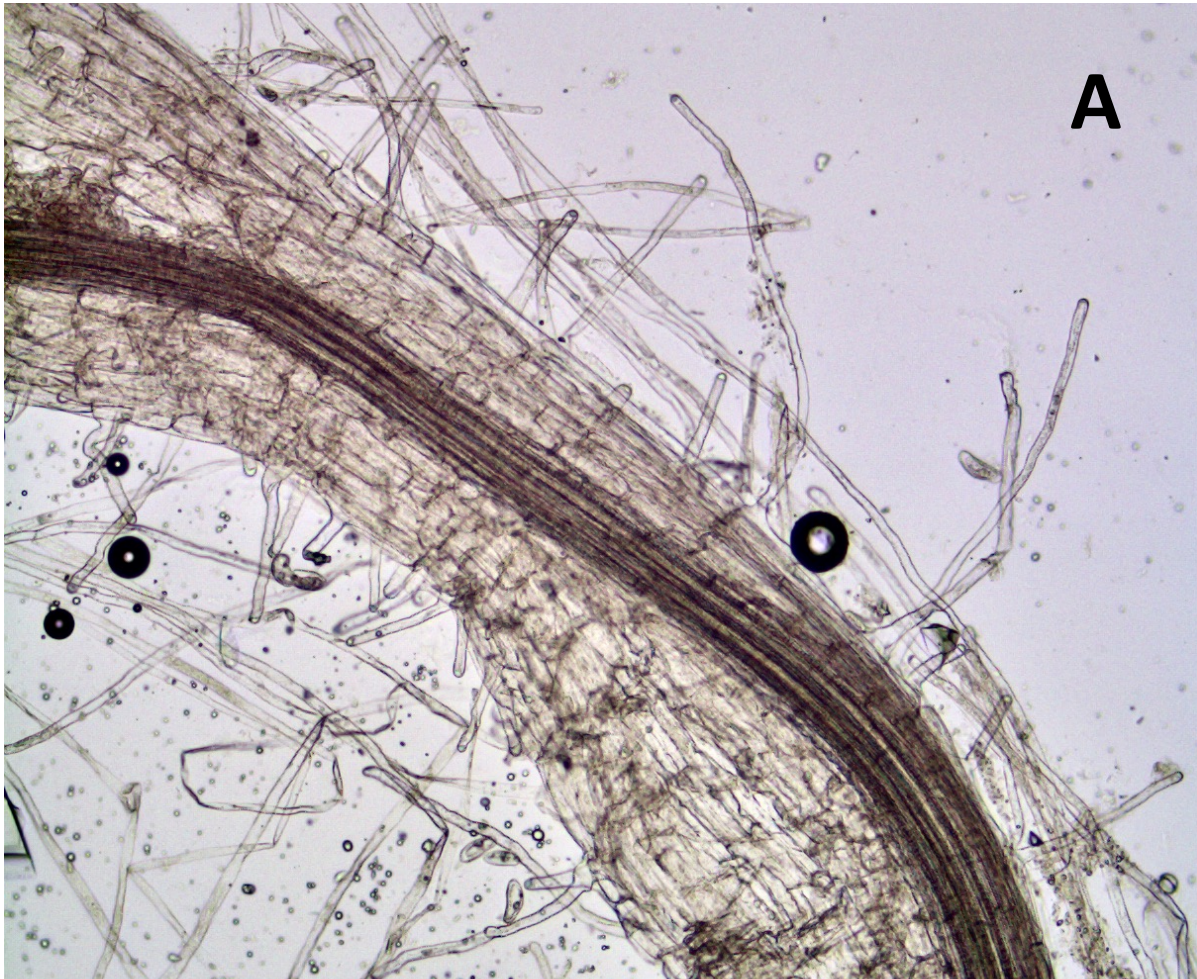
5) Inoculate cleaned seeds
on agarose using filtrate





5-day-old *Poa reptans* seedlings: A. Seedlings from seeds inoculated with microbiome extracted from 'piricinco' selection. B. Seedlings from seeds that were treated with water.

Plants with more nitrogen-fixing endophytes in them have more and longer root hairs and trichomes.



Figs. A. Roots of *Poa* seedlings from piricinco-inoculated seeds; B. Root from non-inoculated control.

Using a nitrate stain (acidified diphenylamine: purple color) more nitrate can be seen around bacteria (black arrows) in plant cells.

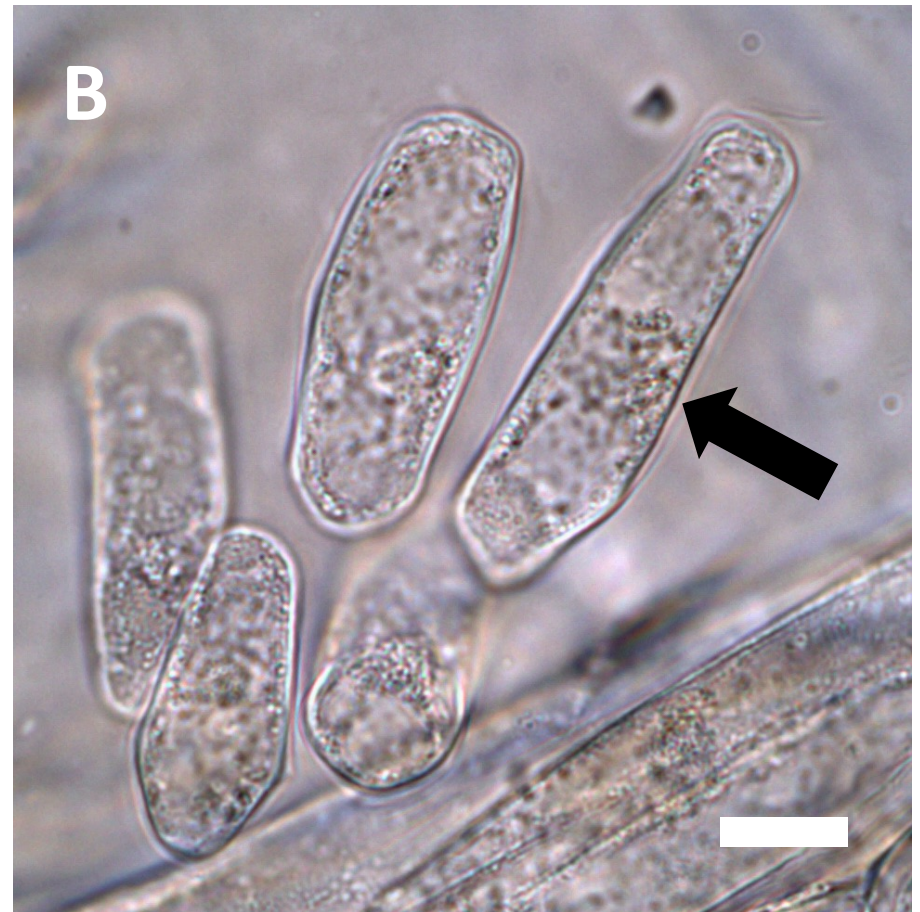


Fig. Root cap cells from 'Piricinco' treated and untreated *Poa reptans*. A. Cells showing dense nitrate staining (purple) of bacteria in cells; B. Cells from untreated seedlings showing absence of dark-staining masses of bacteria (bar = 10 microns).

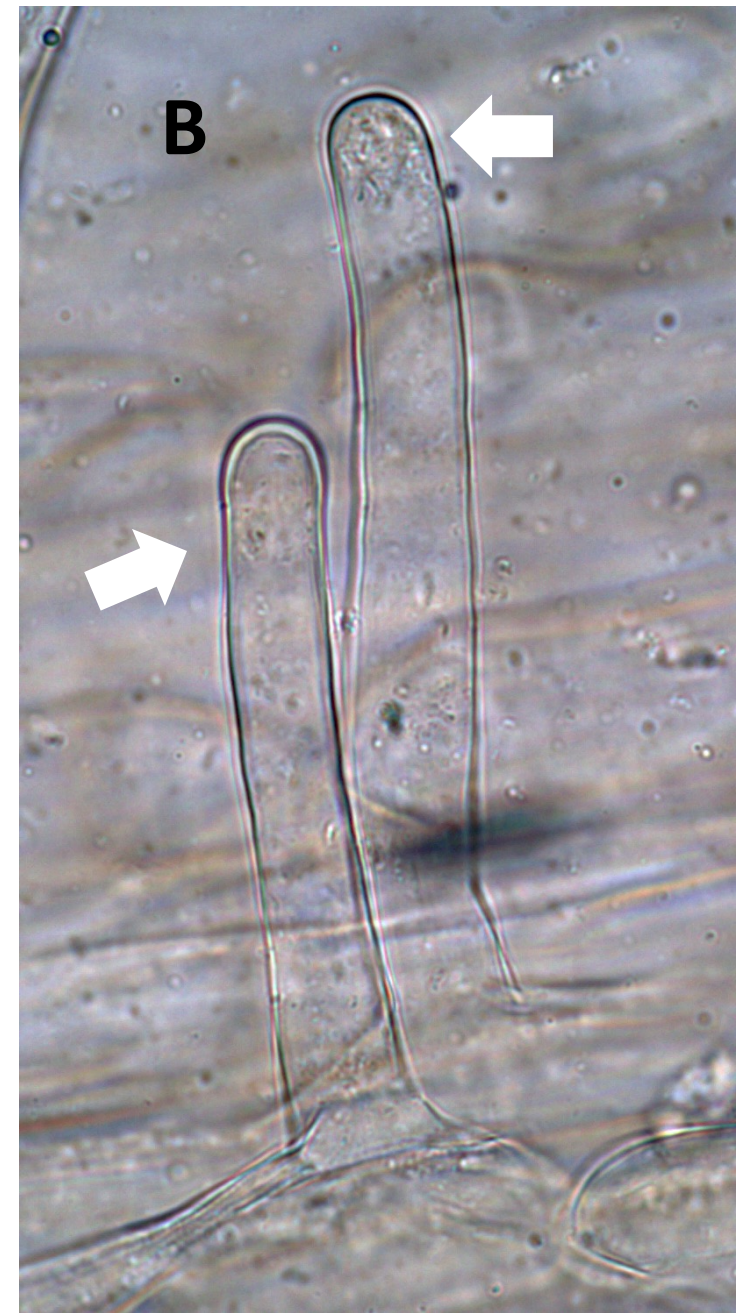
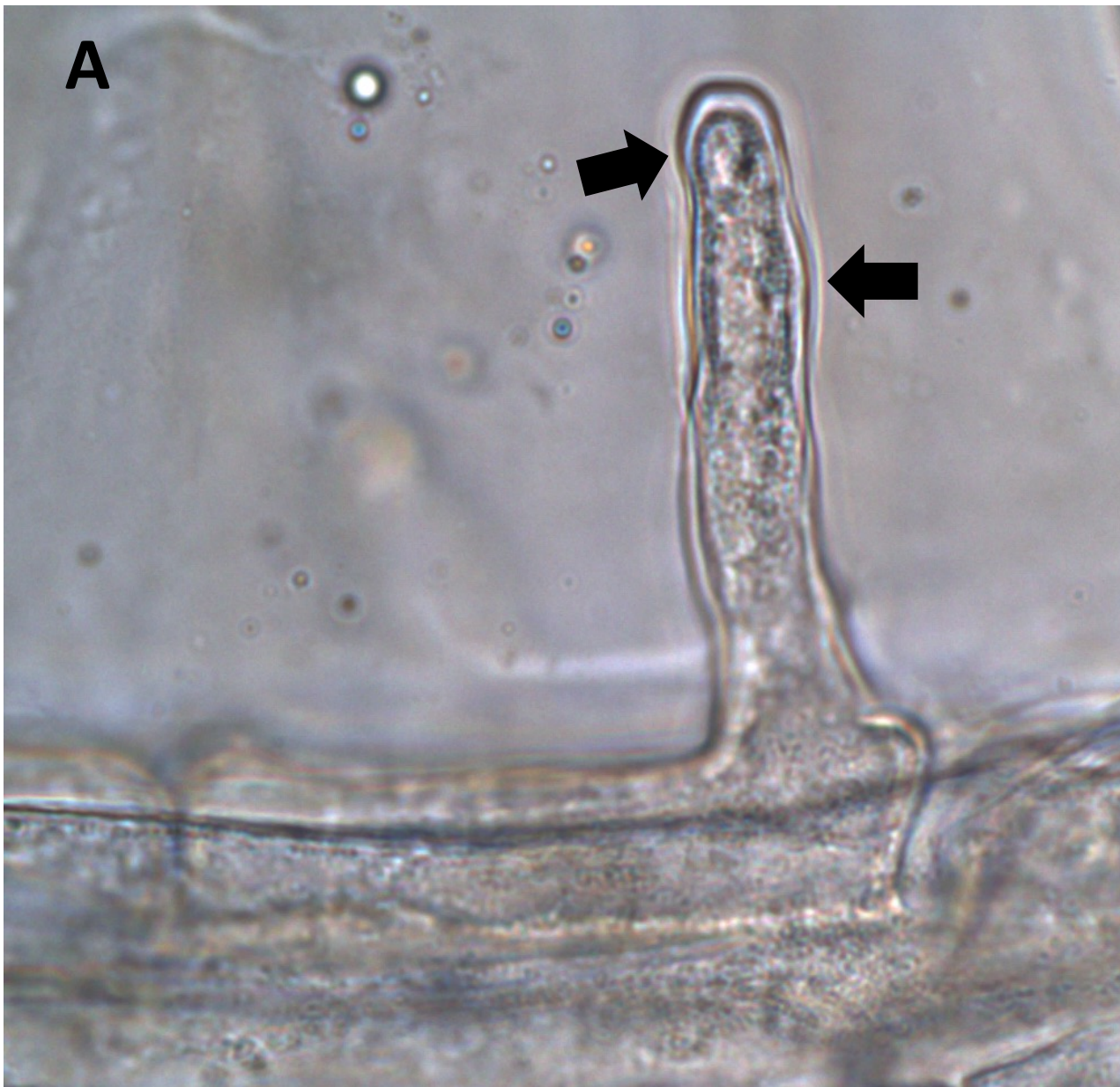


Fig. Root hairs (arrows) from piricinco-treated (A) and untreated control (B). Intense nitrate staining (purple color) can be seen in the piricinco treated seedlings (bar = 10 microns).

A

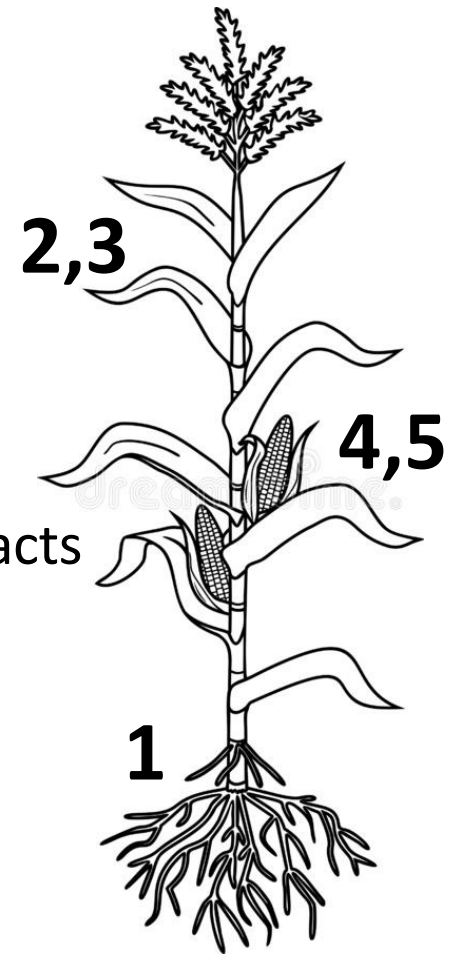
B

- A. 35-day-old *Poa reptans* seedlings inoculated with cob leaf microbiome from piricinco.
B. 35-day-old *Poa reptans* seedlings treated with water only (no microbe control).

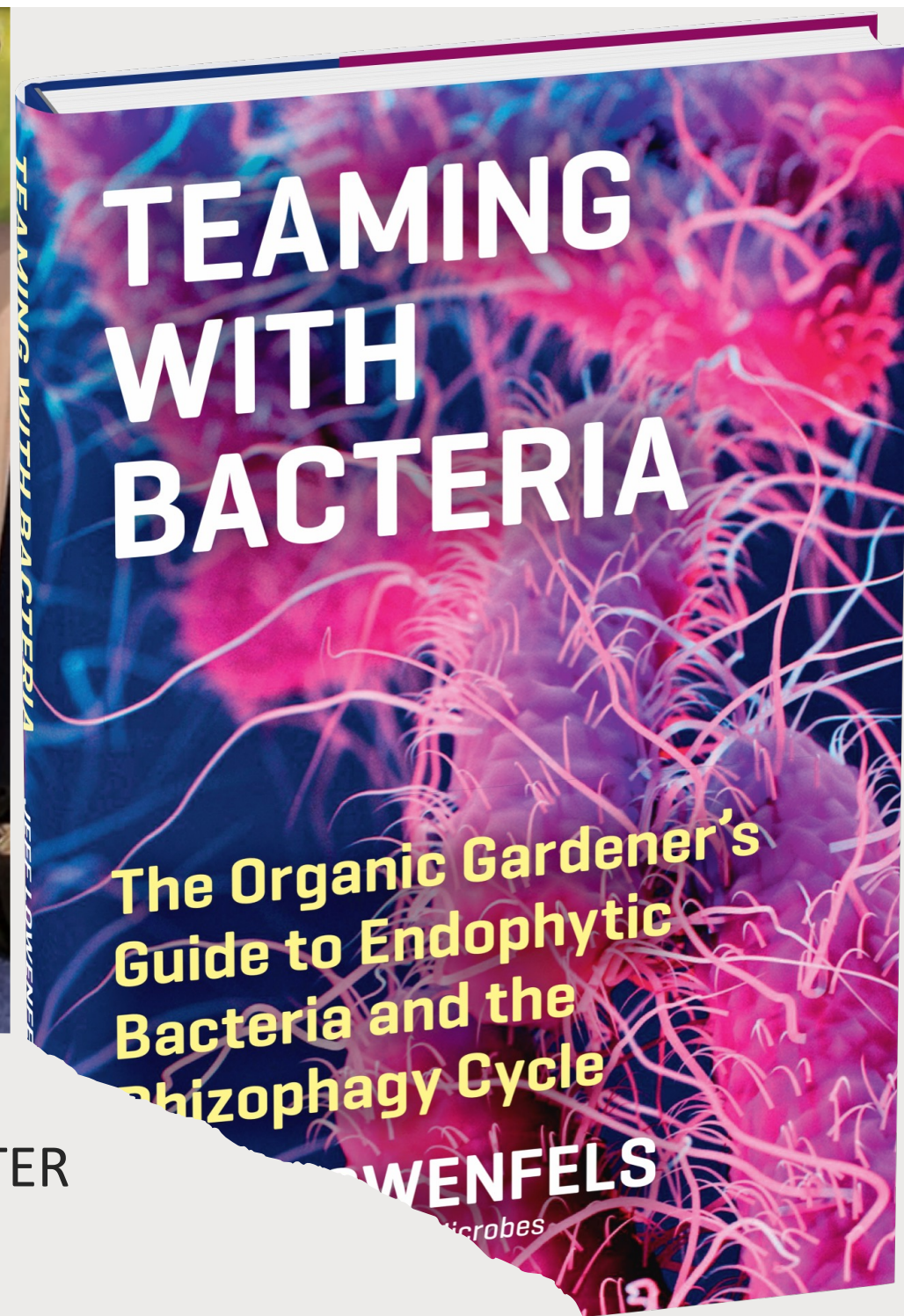
Endosymbiosis Stacking in Corn

- 1) Rhizophagy cycle in roots
- 2) Convoluted wall epidermal cell symbiosis in stem leaves
- 3) Trichome endosymbiosis on stem leaves
- 4) Trichome endosymbiosis in cob leaves/bracts
- 5) Convoluted epidermal wall symbiosis in the cob leaves/bracts

In corn plants without damage to endosymbiotic systems, microbes in several tissues provide nutrients to support growth. Rhizophagy cycle in roots delivers nutrients that are extracted oxidatively from microbes in root cells. As leaves grow, bacteria within the convoluted-walled epidermal cells and in trichomes deliver nitrogen to plants. In the cob leaves/bracts convoluted-walled epidermal cells and trichomes deliver nitrogen to plant cells that provides nitrogen for the developing ovaries/kernels on the cob. Use of chemical fertilizers may negate or damage these endosymbiosis in corn plants.



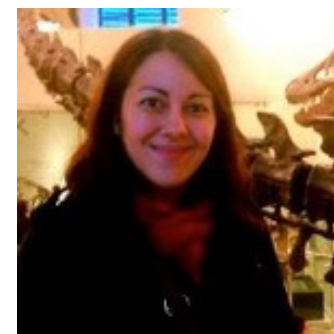
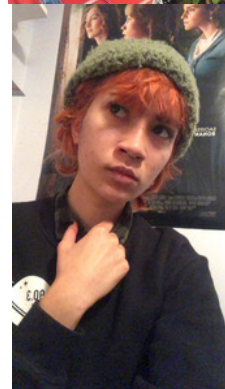
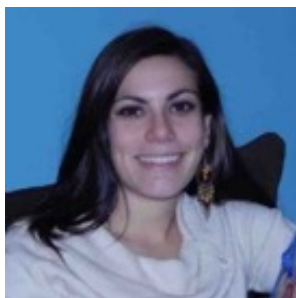
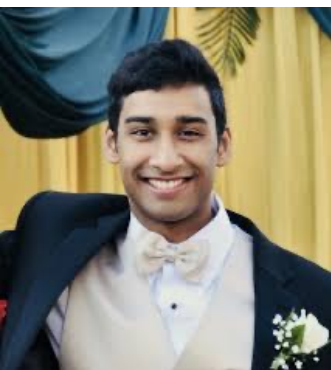
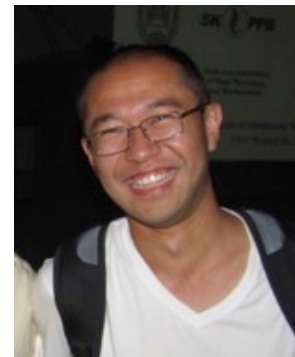
Reference: Micci, A.; Zhang, Q.; Chang, X.; Kingsley, K.; Park, L.; Chiaranunt, P.; Strickland, R.; Velazquez, F.; Lindert, S.; Elmore, M.; Vines, P.L.; Crane, S.; Irizarry, I.; Kowalski, K.P.; Johnston-Monje, D.; White, J.F. Histochemical Evidence for Nitrogen-Transfer Endosymbiosis in Non-Photosynthetic Cells of Leaves and Inflorescence Bracts of Angiosperms. *Biology* **2022**, *11*, 876. <https://doi.org/10.3390/biology11060876>



Jeff Lowenfels with MicroBIOMETER
founder Judy Fitzpatrick



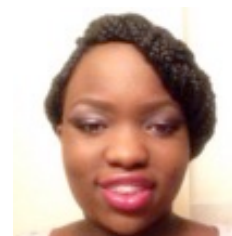
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Monica Torres
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Peerapol Chiaranunt
Celeste Zhang
Fernando Velazquez
Gianna Pecorella
Marshall Bergen
Chris Zambell
Mariusz Tadych
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New Jersey Agric. Exp. Sta.;
USDA NIFA Multistate 3147;
Rutgers Center for Turfgrass Science;
USGS-Rutgers U (CESU Study Agreement)



Questions?