"Methods for detecting and working with endophytes"

(A short-course given at Universidad del Valle in Cali, Colombia)

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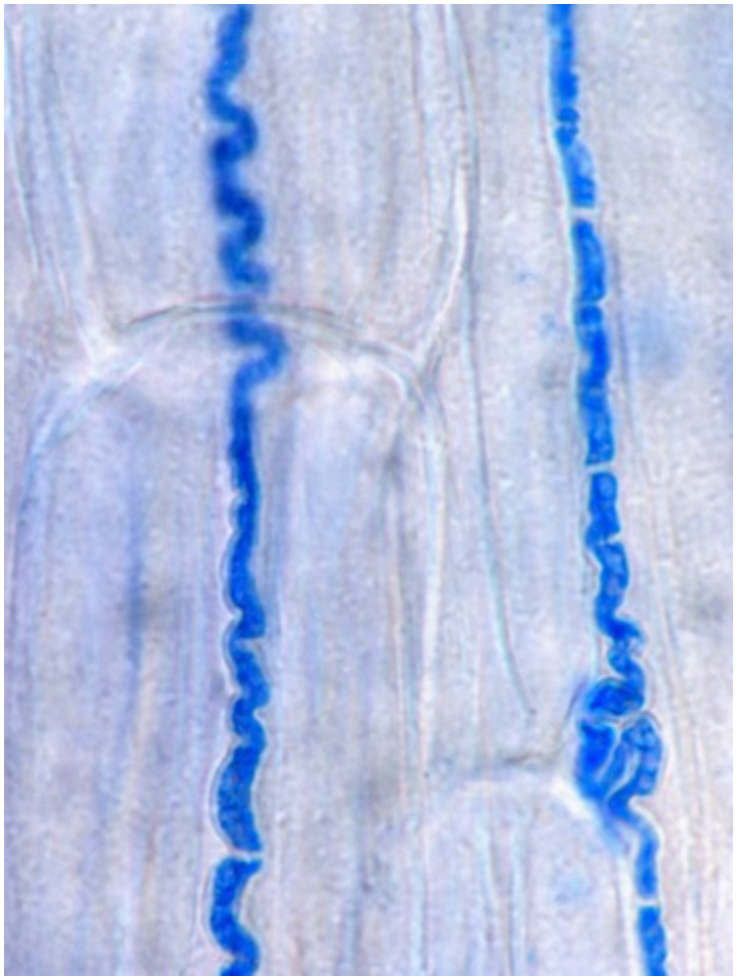
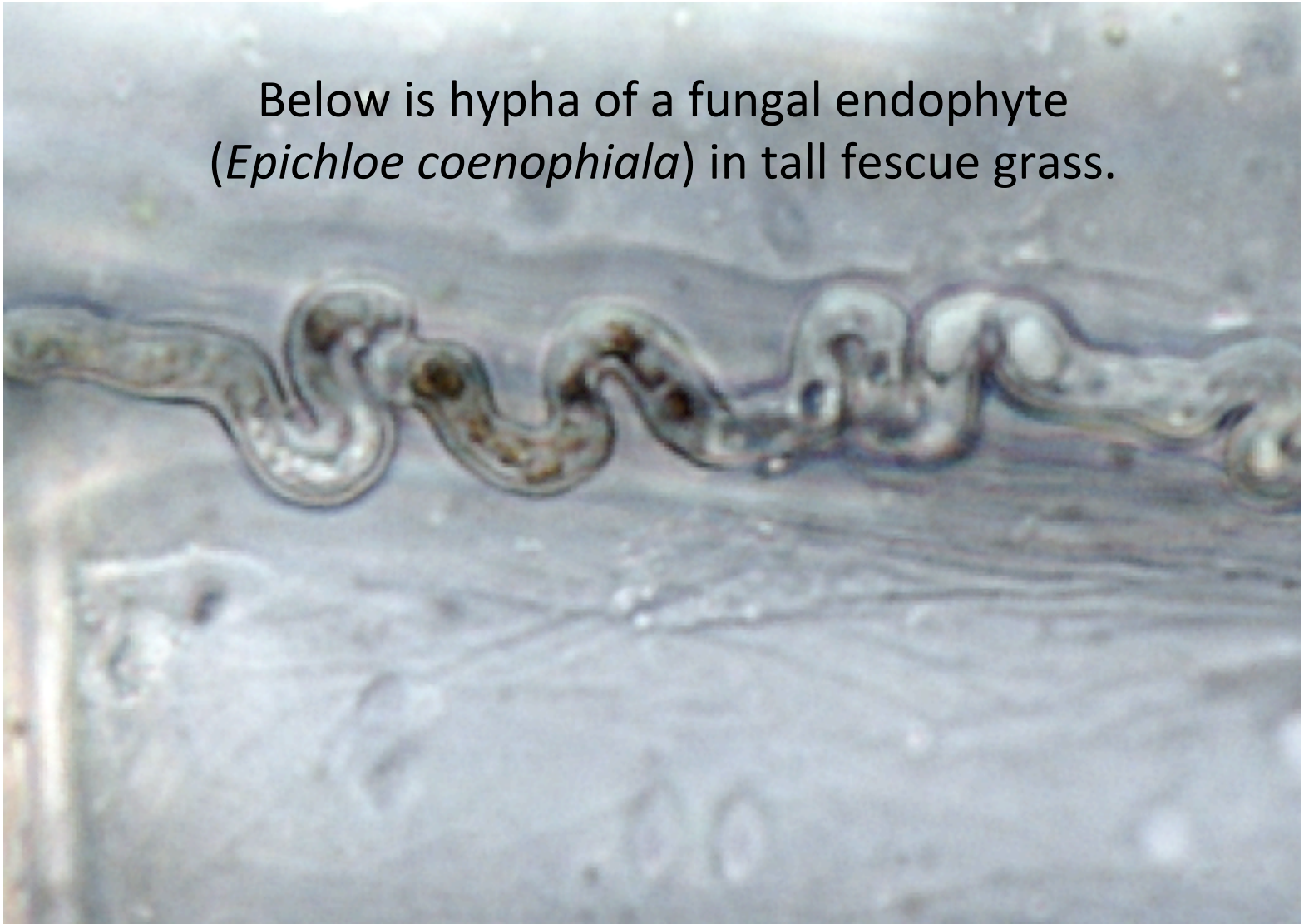
What are endophytes?

Endophytic/endosymbiotic non-pathogenic microbes (fungi or bacteria) are present asymptomatically in tissues of all plants



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

Microbial endophytes are typically fungi and bacteria that grow and reproduce within tissues of plants.



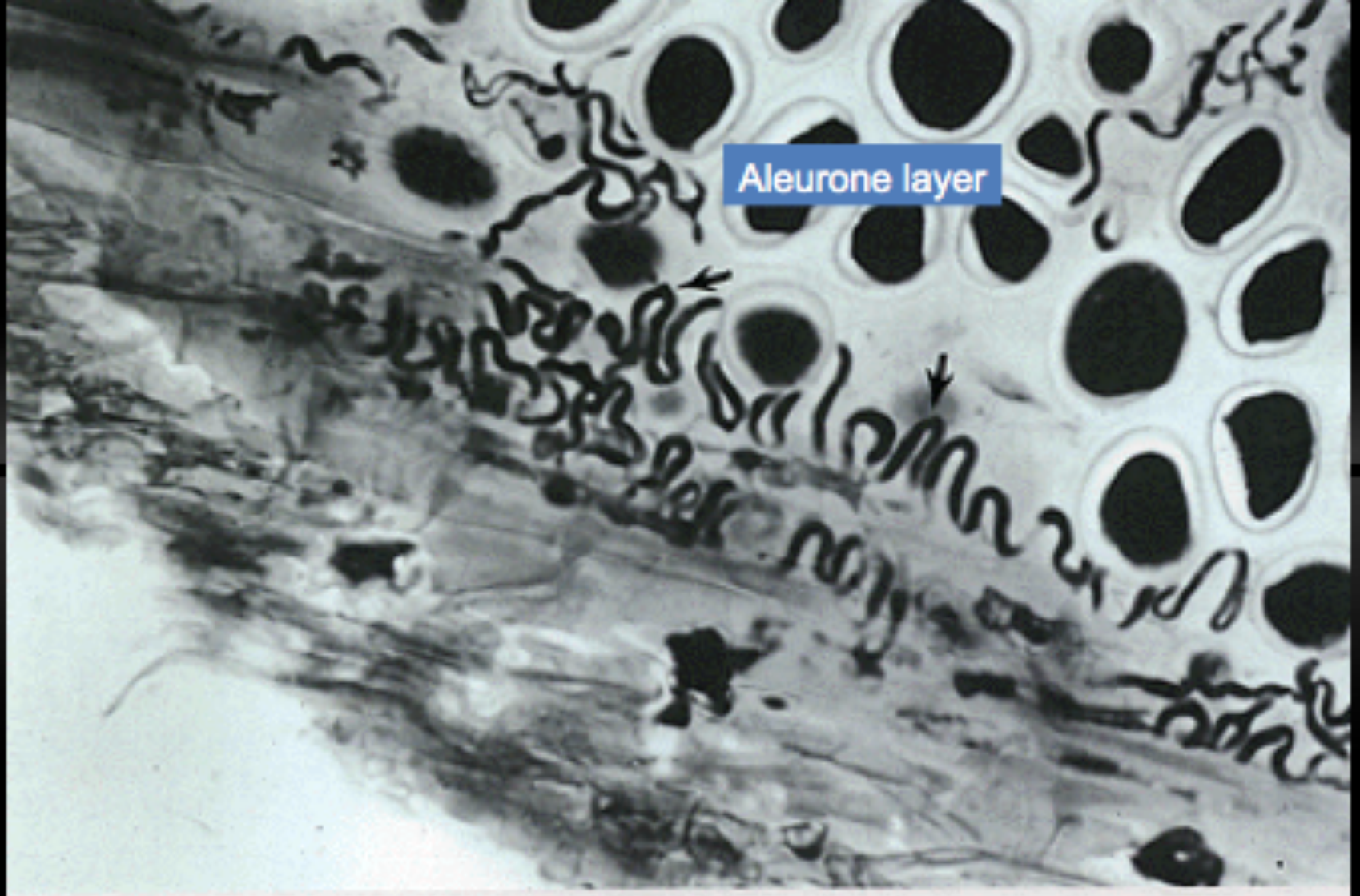


Fig. 2. Tangential section through seed of *Festuca arundinaceae* showing mycelium (arrows) within seed (X

Endophyte in seed of tall fescue

Methods Literature

Bacon, C. W., and J. F. White, Jr. (editors). 1994. Biotechnology of *Acremonium* endophytes of grasses. CRC Press, Boca Raton, FL. 226 pages; ISBN-10: 0849362768; ISBN-13: 978-0849362767. Book out of print (chapter available on ResearchGate).

Chapter 4

Stains, Media, and Procedures for Analyzing Endophytes

Charles W. Bacon and James F. White, Jr.

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In Book: 2011. Prospects and Applications for Plant Associated Microbes: A Laboratory Manual, Part B: Fungi. Chapter: Isolation and identification of fungal endophytes. Editors: Anna Maria Pirttila, Seppo Sorvari. Publisher: Paimo, BBI (Biobien Innovations), Turku, Finland. Out of print.
Presently available on research gate.

Prospects and Applications for Plant-Associated Microbes. A laboratory Manual, Part B: Fungi"

2. Theory

2.1. Isolation and identification of fungal endophytes

2.2. Experimental procedure: "Dilution-to-extinction cultivation of endophytic fungi" and "isolation and detection of grass endophytic fungi in different plant parts"

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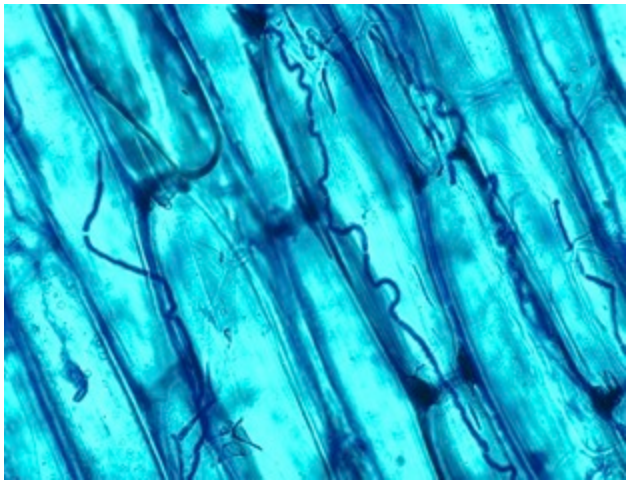
Part 1. Detection of endophytes

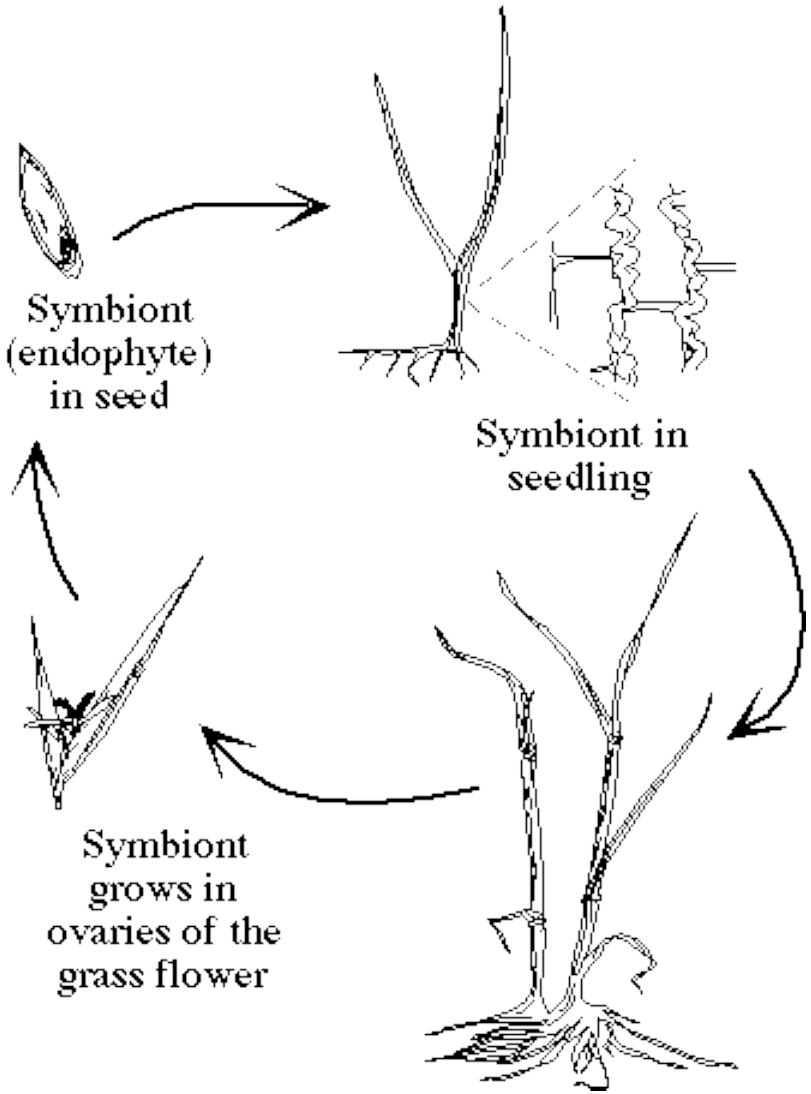
- Culture methods
- Visualization methods
- Immuno-detection methods

Epichloë (on various species of grasses)

Endophytic with perithecial stroma enveloping and aborting the inflorescence (choke disease)

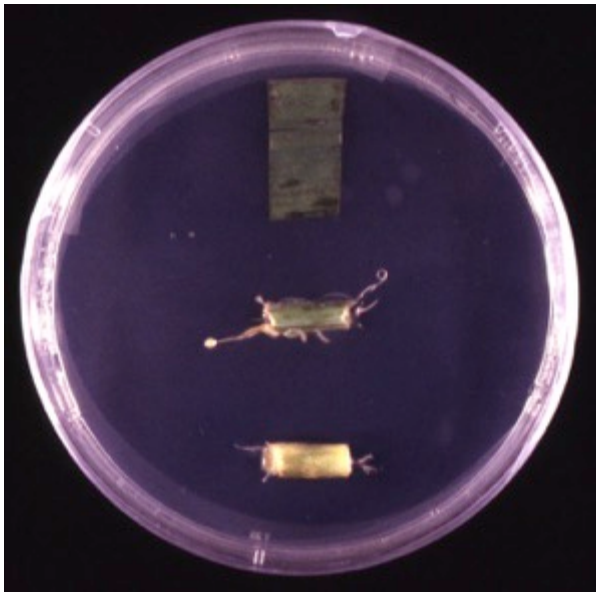


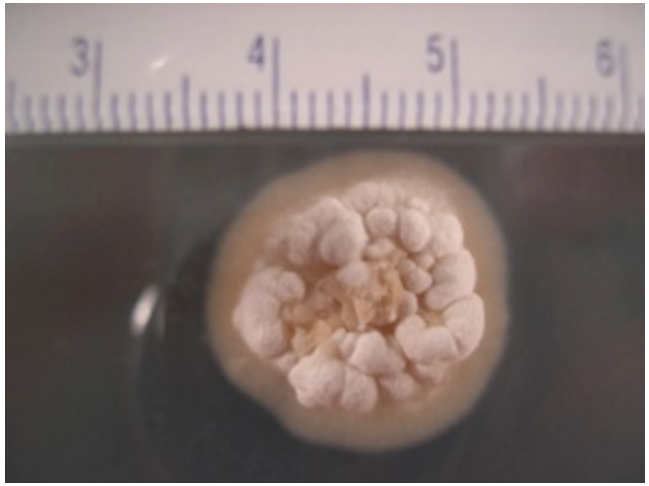
Vertical (seed) transmission of *E. coenophiala*

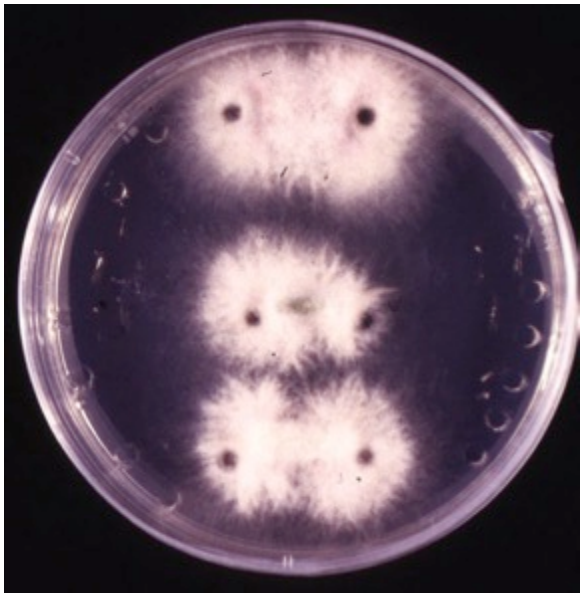


A Broad Definition of “Endophyte”

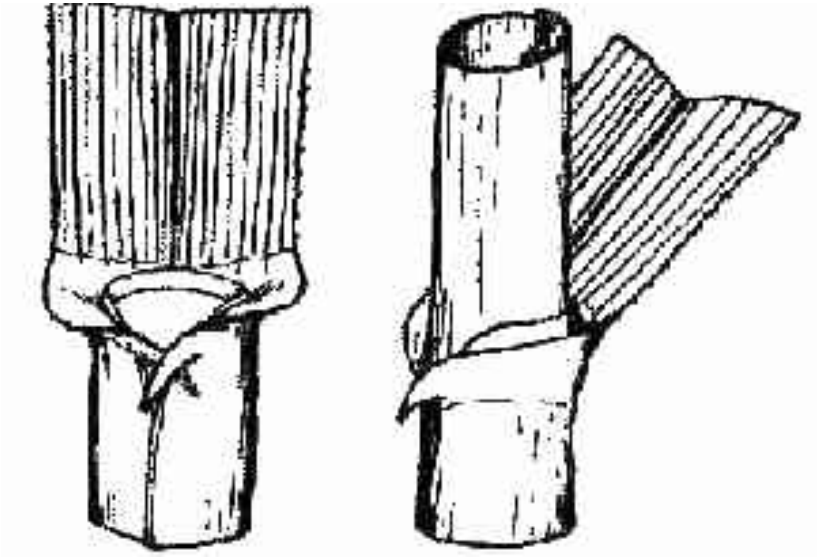
- Functional definition of “endophyte”
  - Recovery from surface-sterilized plant material (onto Potato Dextrose Agar)
  - Microscopically demonstrated to reside within plant tissue
  - Term used for fungi and bacteria





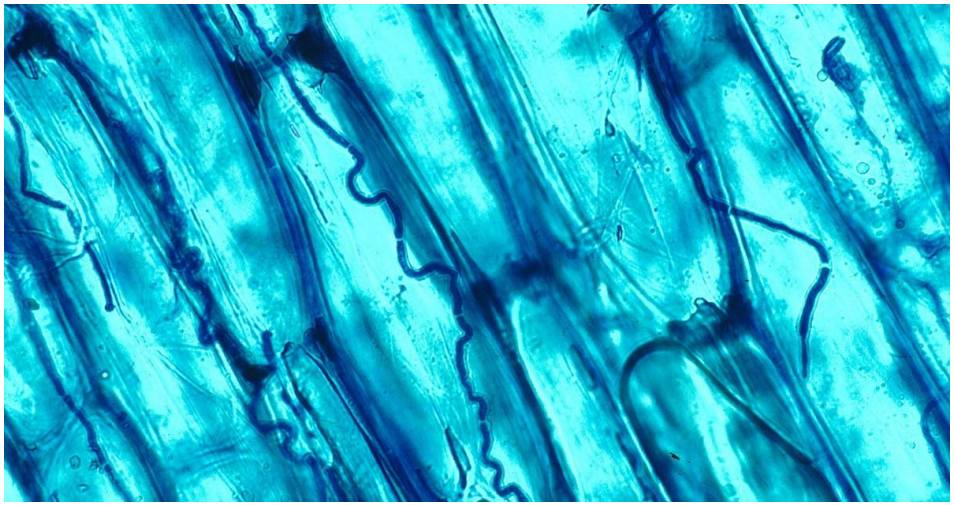


Observation of fungal endophytes in tall fescue tillers



Culm examination:

1. Culm split.
2. Exposed inner tissue moistened with aniline blue stain (0.1% aniline blue + lactophenol)
3. Tissue scraped onto glass slide and macerated
4. Tissue examined under compound microscope for typical convoluted intracellular mycelium

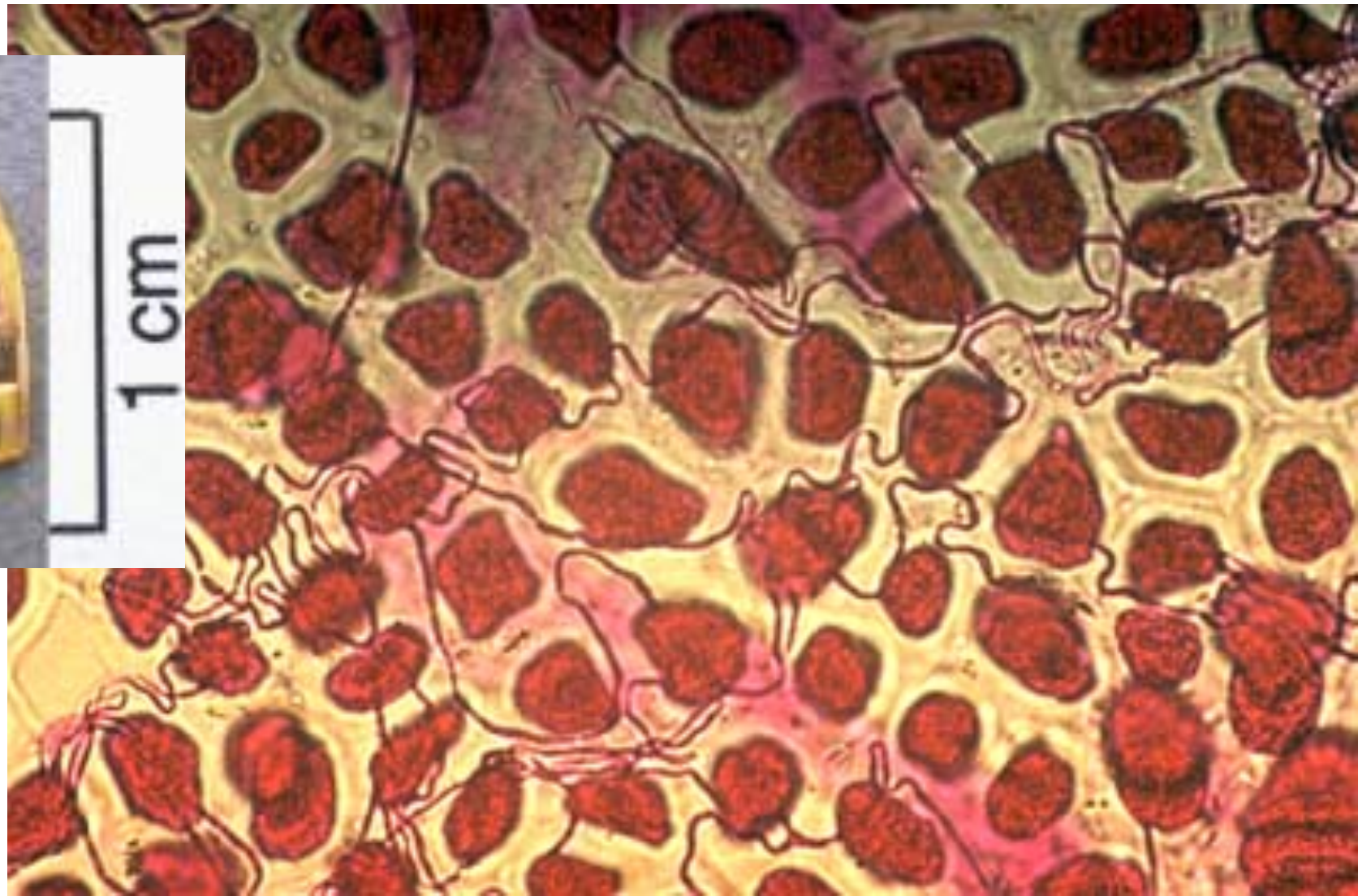


Leaf sheath examination:

1. Leaf sheath split
2. Aniline blue applied
3. Inner parenchyma scraped out and examined

1. **Observation of fungal endophytes in grass seeds:**

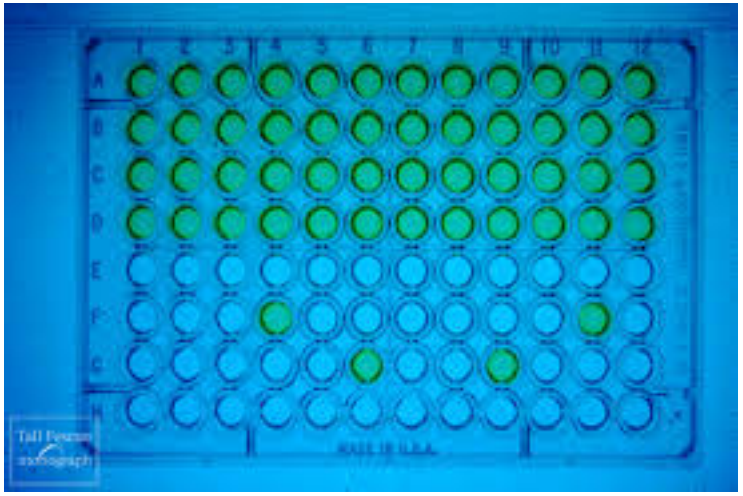
- pretreatment with NaOH (overnight)
- Squash—stain with aniline blue or rose bengal stain
- Examine microscopically
-

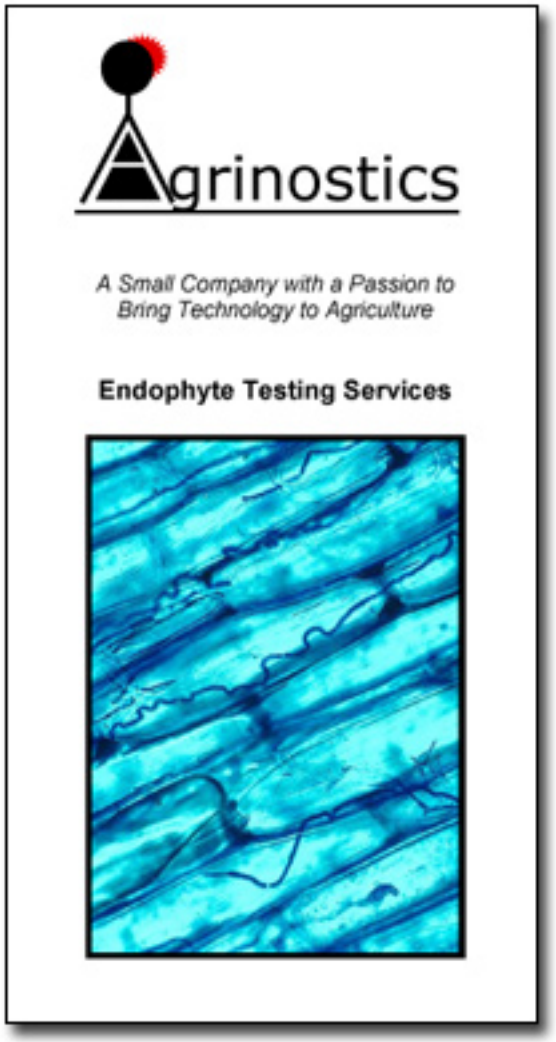


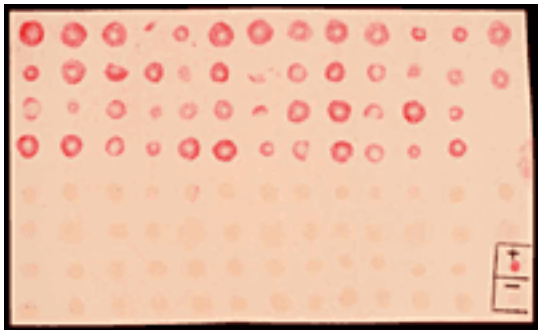
Agrinostics Ltd. Co. (Dr. Nick Hill, Univ. Georgia)

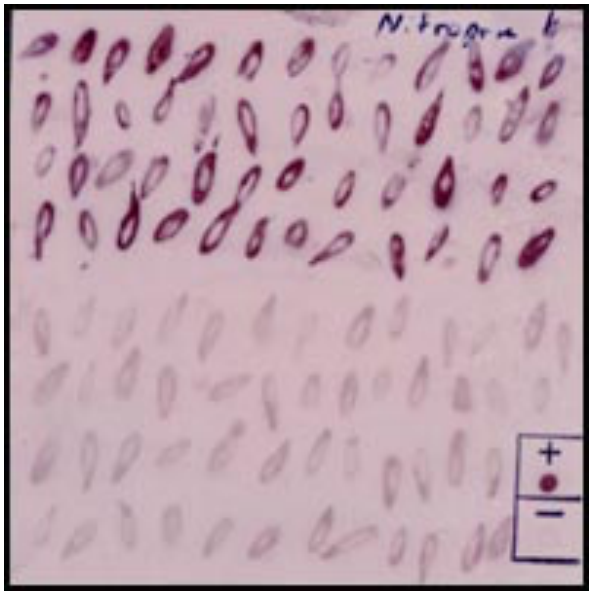
Immuno-detection kits
(based on antibodies against mycelium
or alkaloids)

Elisa









Immuno-blot

Bacterial endophyte detection:

1. Isolation methods

2. Visualization methods

Natural Populations of *Vanilla phaeantha* Grow in Cypress Swamps of South Florida

Vanilla phaeantha is an epiphytic orchid vine.

Bacillus amyloliquefaciens is the endophyte widespread in this species of vanilla orchid.

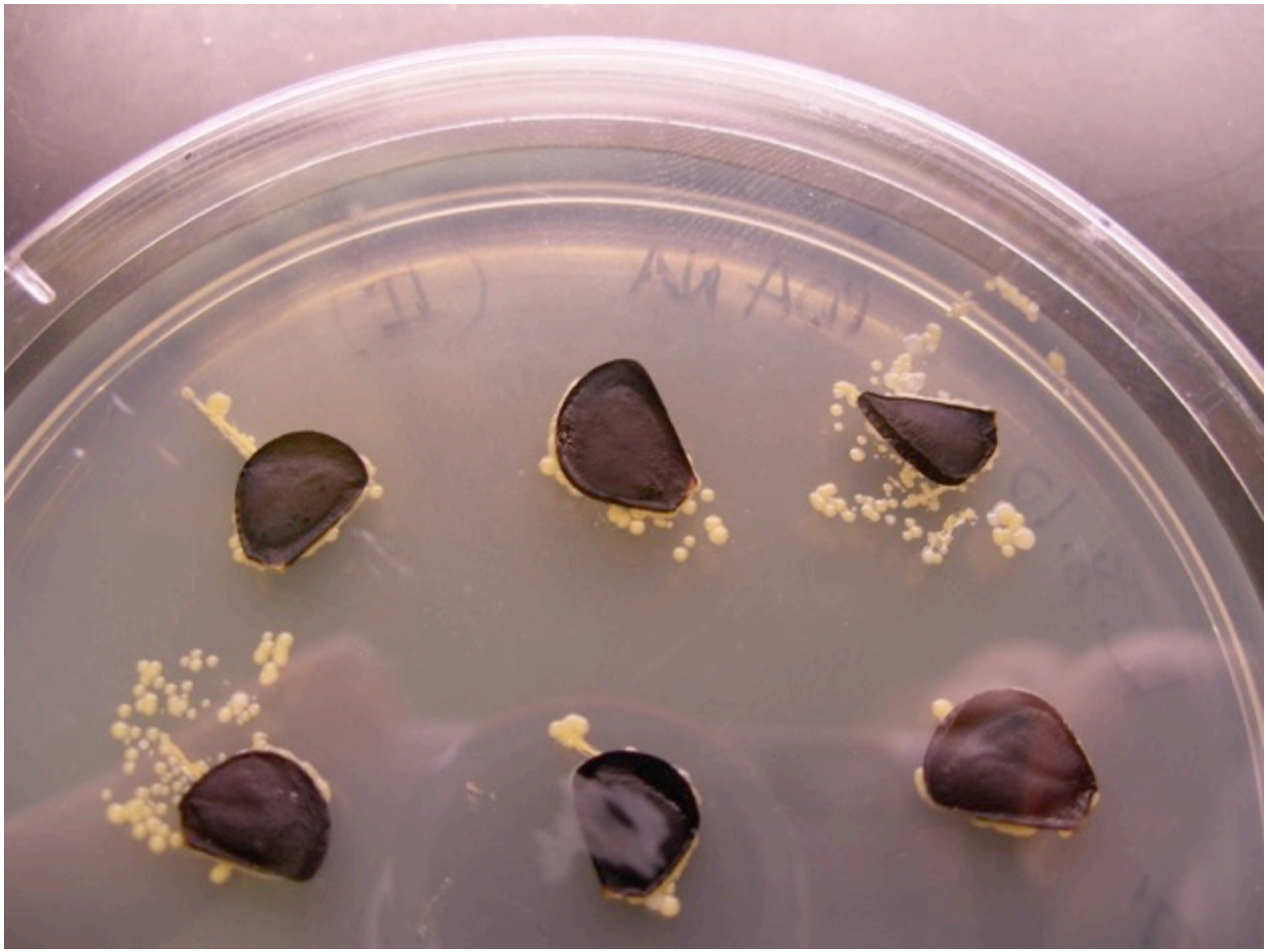
Media useful for endophyte isolation:

- 10% TSA (Tripticase Soy Agar; good for Bacillus)
- YES agar (1% yeast extract + 1% sucrose + agar)

Isolation only after rigorous surface disinfection (e.g. 20 + minutes with 4% NaOCl for most seeds; shorter disinfection for soft plant tissues)

Agave palmeri on rocks. Plants flourish with minimal soil and water.

Systemic Endophytic/Epiphytic Bacteria



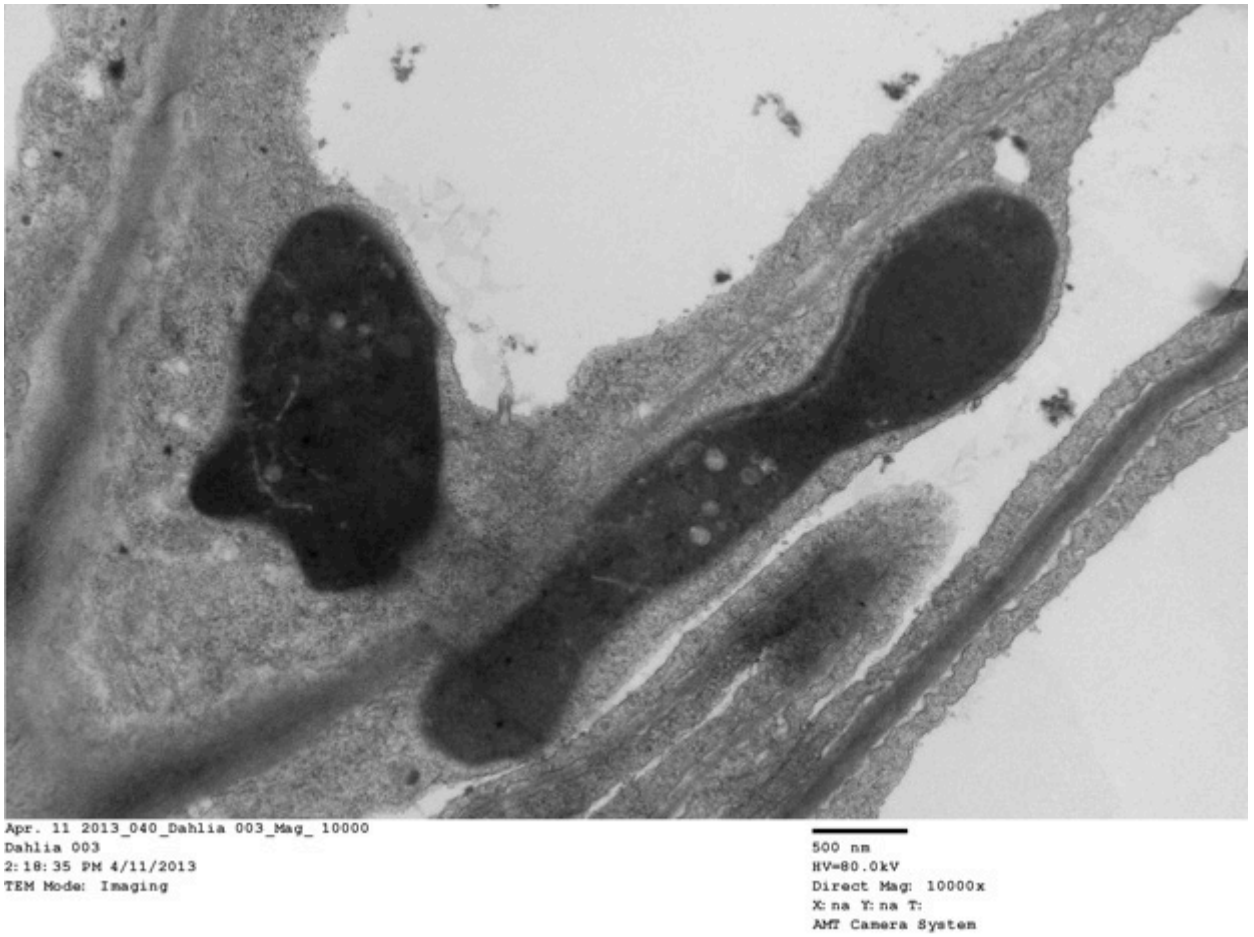
Systemic endophytic bacteria are common in uncultivated species of agaves and Agaveceae. This bacterium is a nitrogen-fixing Klebsiella oxytoca from Yucca sp. (N-fixation in vitro demonstrated by acetyene reduction test).

Visualization methods:

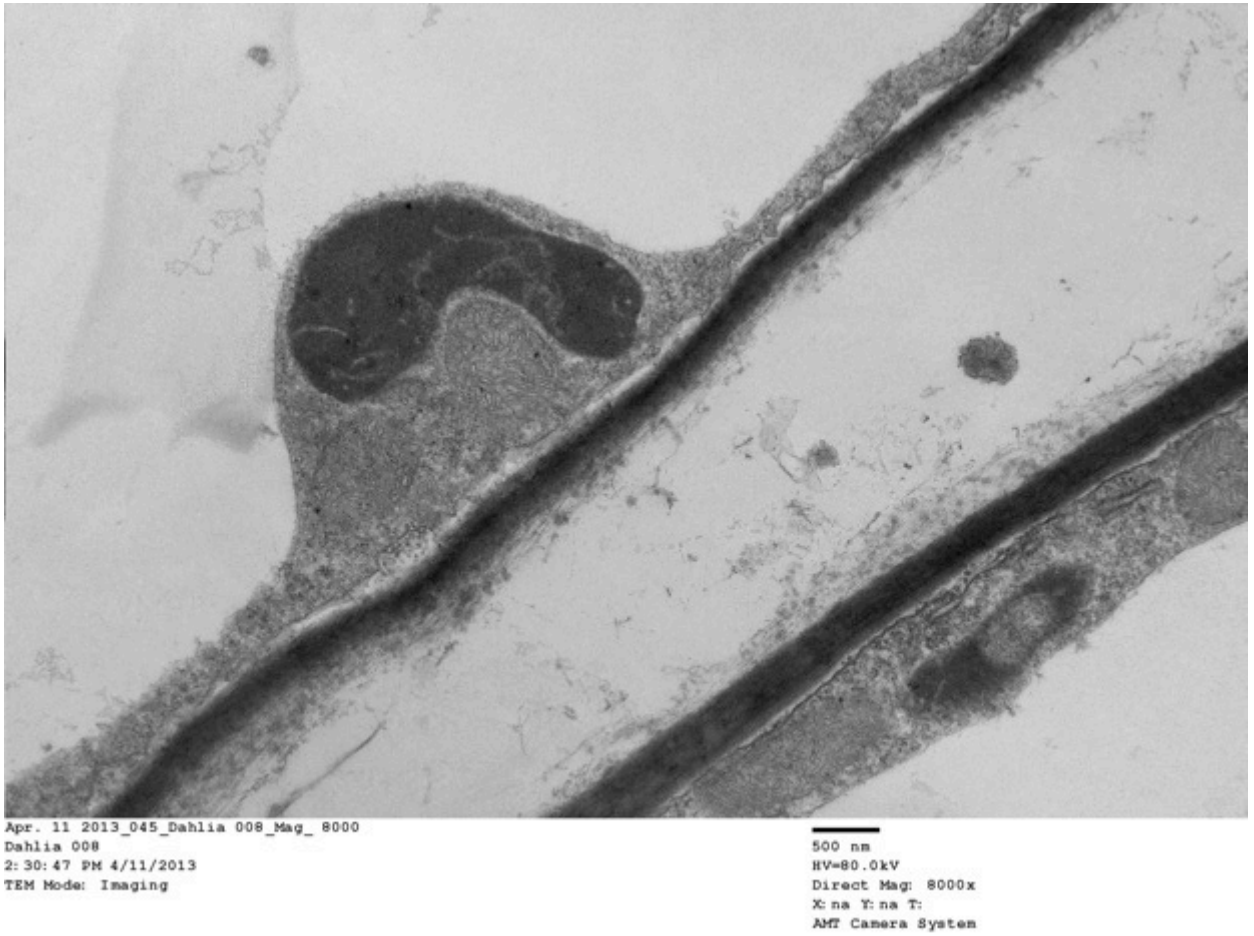
1. Transmission Electron Microscopy
2. Reactive oxygen staining
3. Fluorescence microscopy

Dahlia variabilis (Asteraceae)

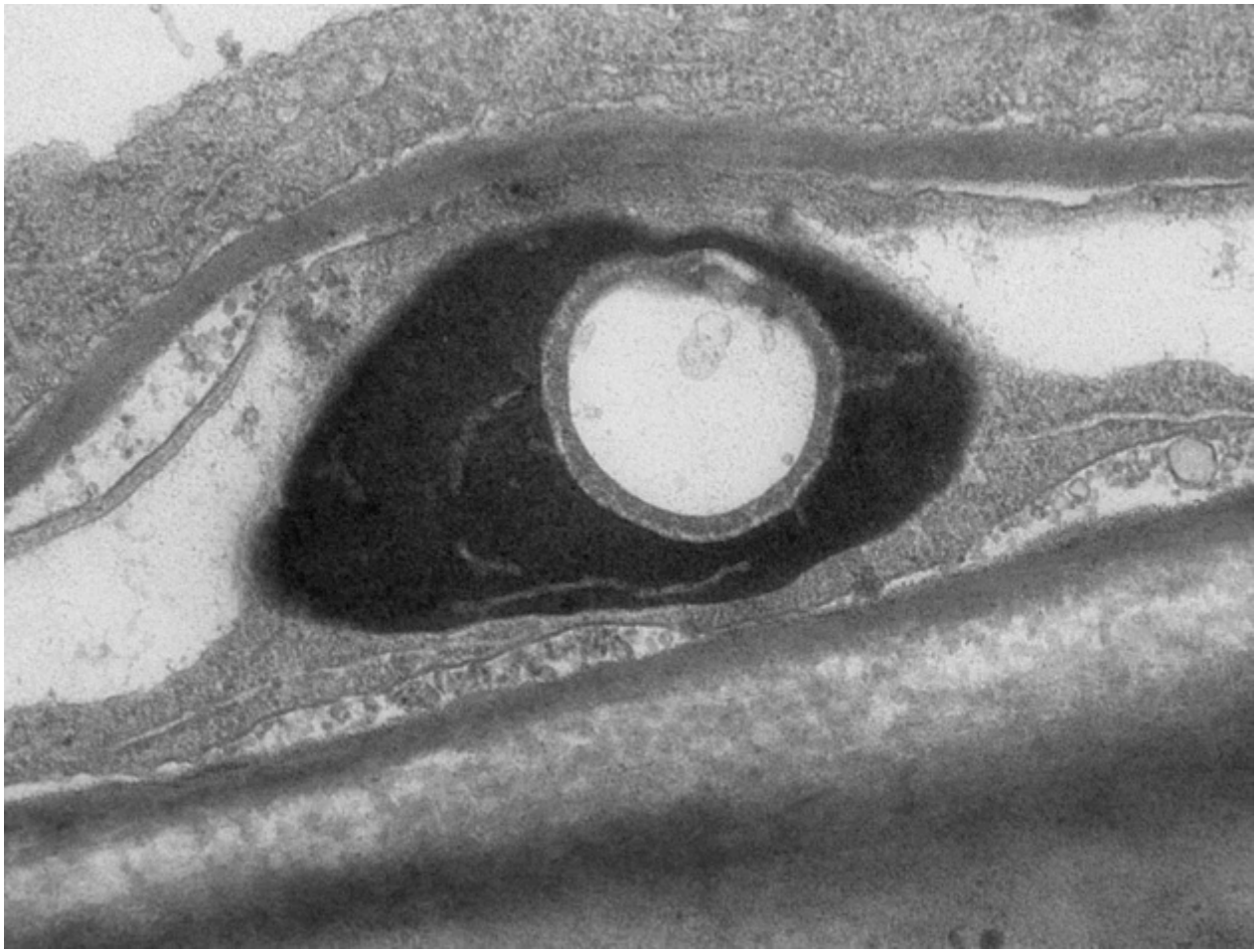
Dahlia seedling root ultrastructure

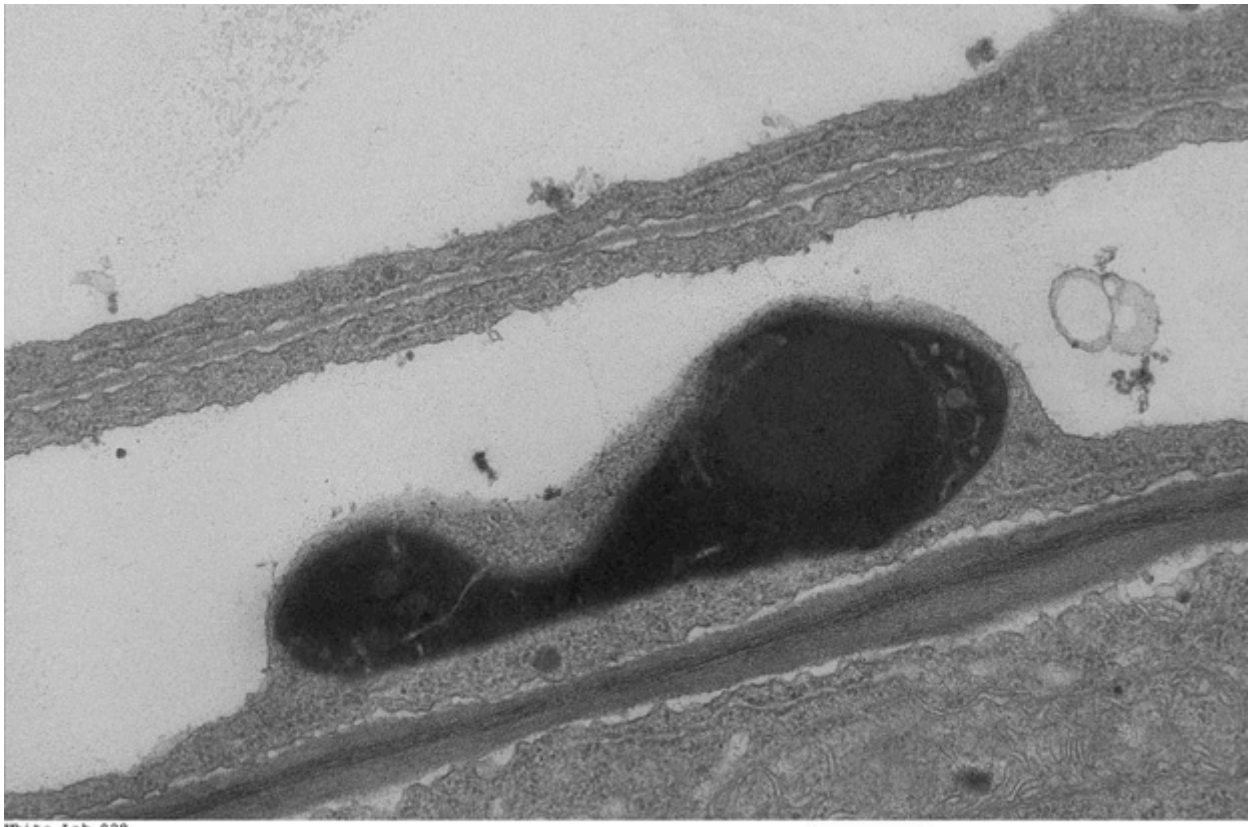


Bacterial endophytes in root cells



Endospores evident in many bacteria





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Dahlia-4
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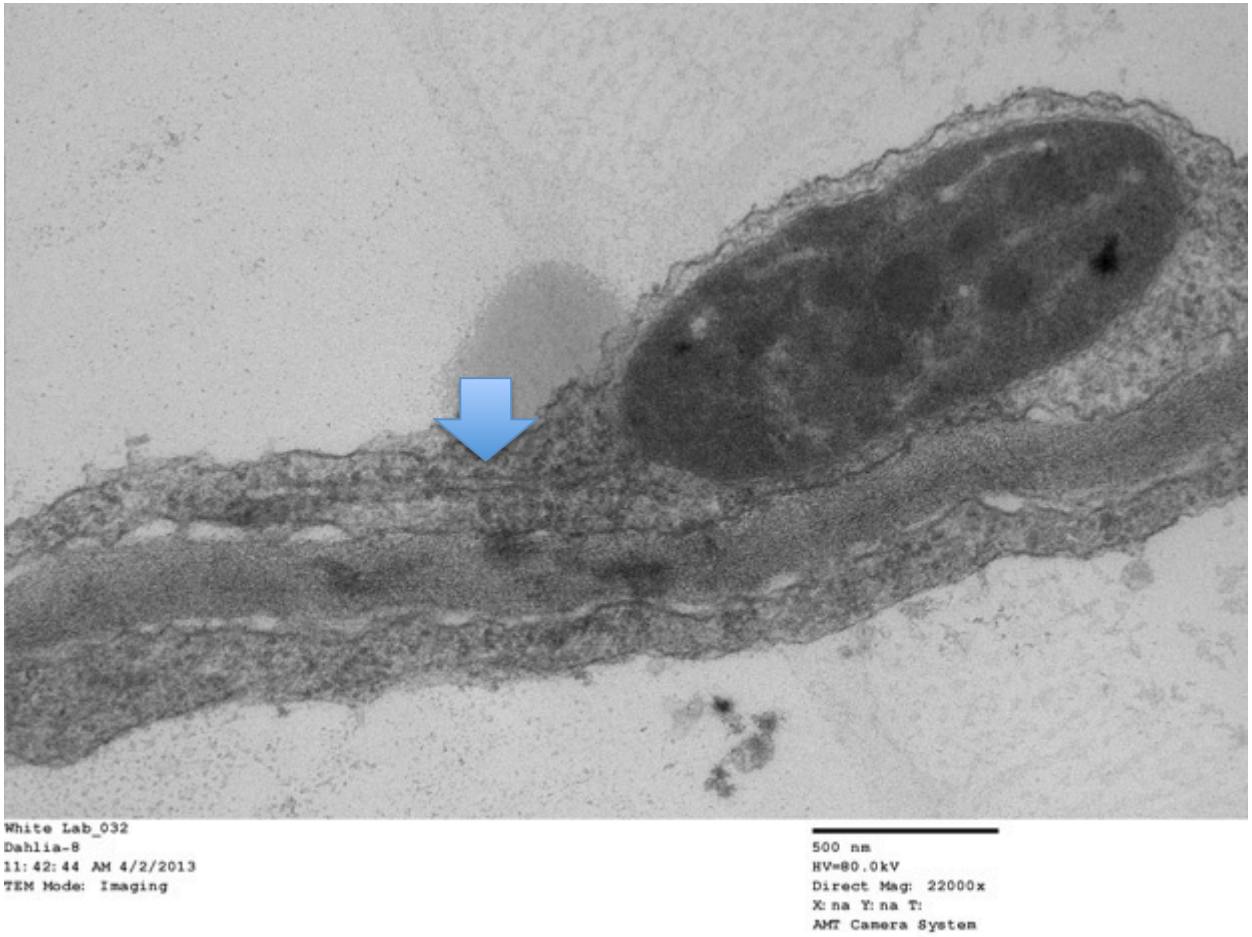
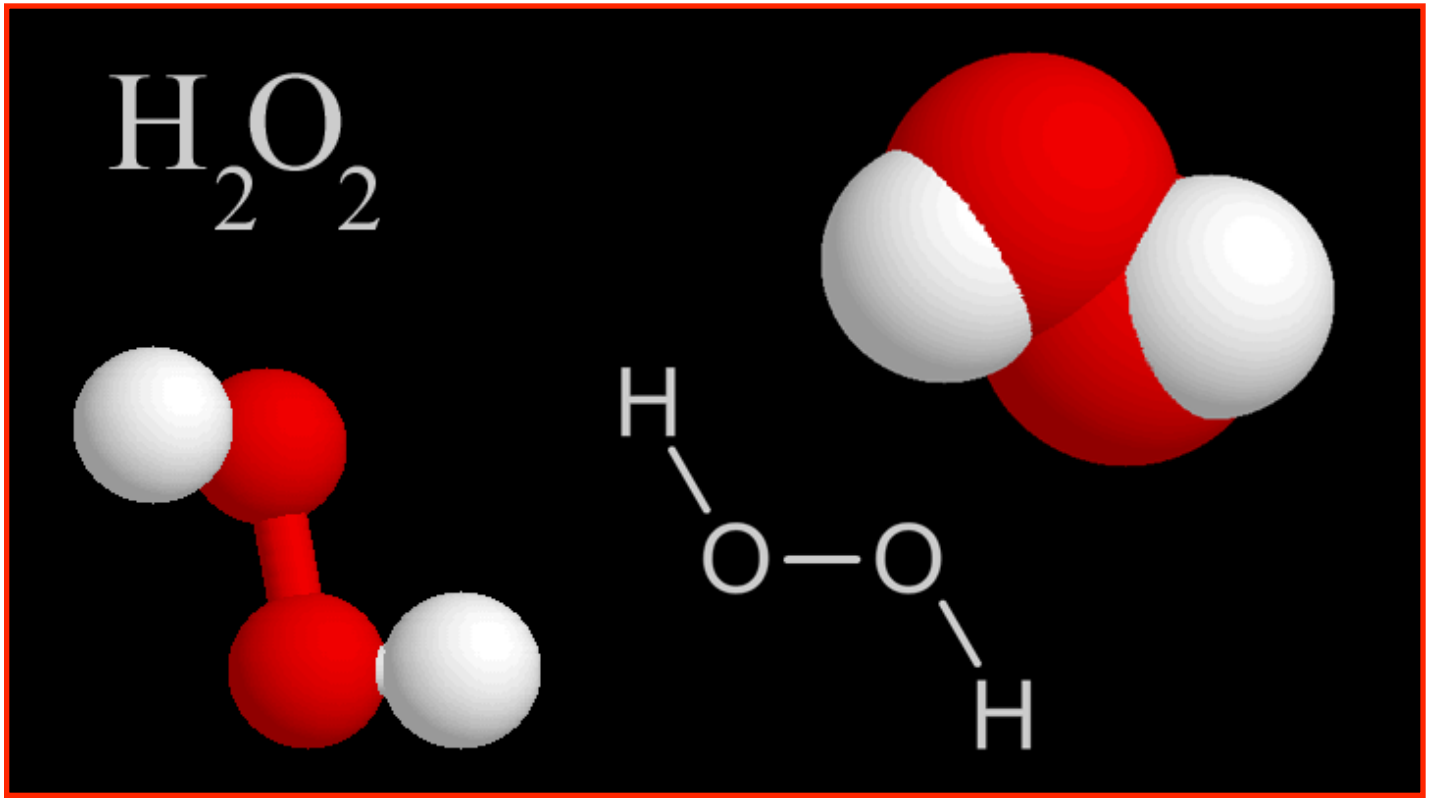
Intracellular bacterium with flagellum



Reactive Oxygen Species (ROS)



Oxidation of organic molecules (e.g., proteins) may be a key mechanism for Disarticulation prior to absorption.

Reactive oxygen staining procedure

MICROSCOPY RESEARCH AND TECHNIQUE 77:566–573 (2014)

Hydrogen Peroxide Staining to Visualize Intracellular Bacterial Infections of Seedling Root Cells

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KEY WORDS intracellular bacteria; symbiosis; hydrogen peroxide staining; light microscopy

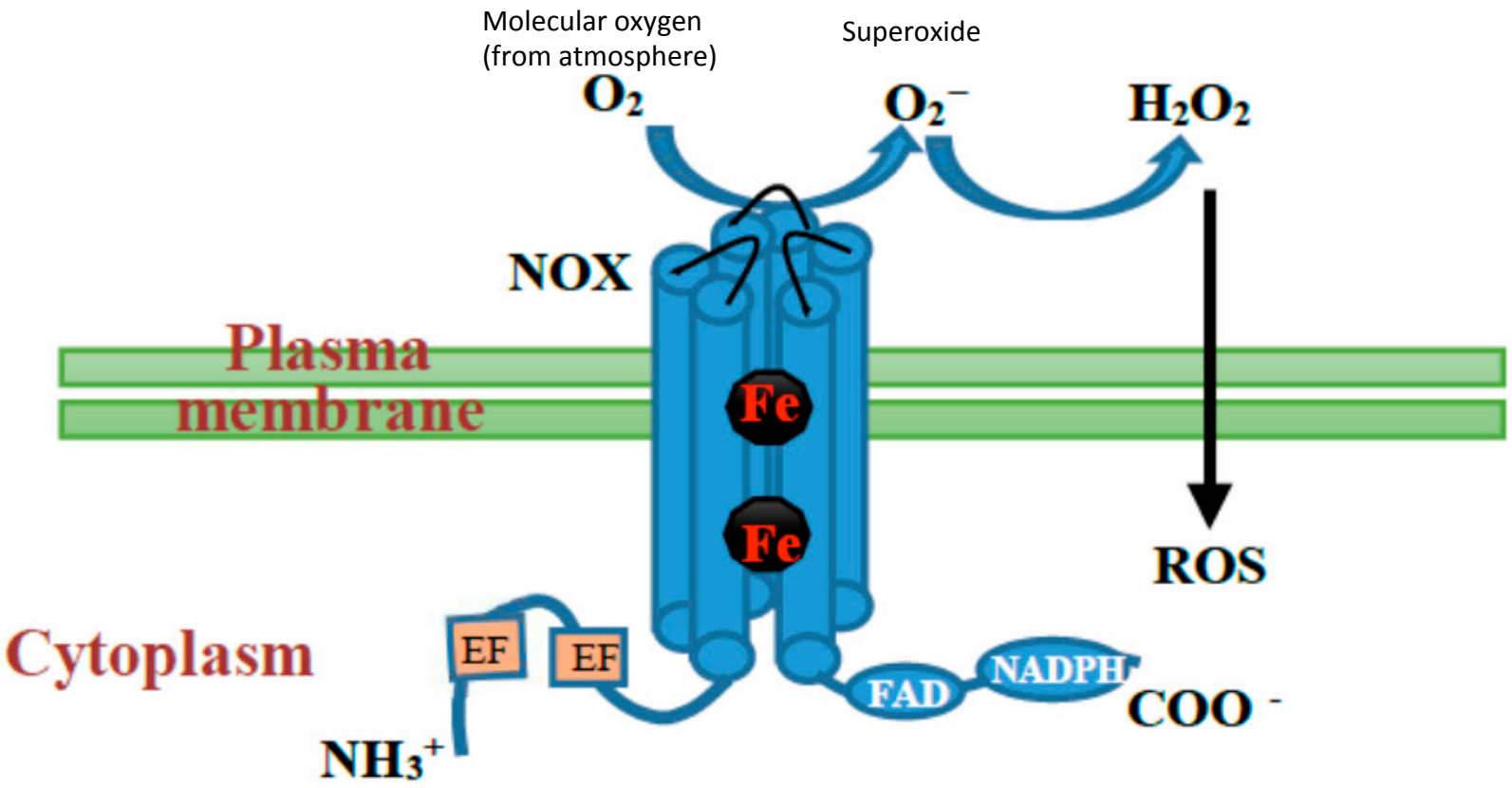
ABSTRACT Visualization of bacteria in living plant cells and tissues is often problematic due to lack of stains that pass through living plant cell membranes and selectively stain bacterial cells. In this article, we report the use of 3,3′-diaminobenzidine tetrachloride (DAB) to stain hydrogen peroxide associated with bacterial invasion of eukaryotic cells. Tissues were counterstained with aniline blue/lactophenol to stain protein in bacterial cells. Using this staining method to visualize intracellular bacterial (*Burkholderia gladioli*) colonization of seedling roots of switch grass (*Panicum virgatum*), we compared bacterial free seedling roots and those inoculated with the bacterium. To further assess application of the technique in multiple species of vascular plants, we examined vascular plants for seedling root colonization by naturally occurring seed-transmitted bacteria. Colonization by bacteria was only observed to occur within epidermal (including root hairs) and cortical cells of root tissues, suggesting that bacteria may not be penetrating deeply into root tissues. DAB/peroxidase with counter stain aniline blue/lactophenol was effective in penetration of root cells to selectively stain bacteria. Furthermore, this stain combination permitted the visualization of the bacterial lysis process. Before any evidence of H_2O_2 staining, intracellular bacteria were seen to stain blue for protein content with aniline blue/lactophenol. After H_2O_2 staining became evident, bacteria were often swollen, without internal staining by aniline blue/lactophenol; this suggests loss of protein content. This staining method was effective for seedling root tissues; however, it was not effective at staining bacteria in shoot tissues due to poor penetration. *Microsc. Res. Tech. 77:566–573, 2014.*

INTRODUCTION

There is growing evidence that all plants are inhabited by a plethora of nonpathogenic or weakly pathogenic microbes (Arnold and Lutzoni, 2007; Johnston-Monje and Raizada, 2011; Magnani et al., 2010; Rosenblueth and Martinez-Romero, 2006; Stone et al., 2000). How microbes interact with host plants is sur-

microbes. Prieto et al. (2011) demonstrated that the biological control bacteria *Pseudomonas fluorescens* and *Pseudomonas putida* enter roots via root hairs. Nitrogen-fixing rhizobial bacteria become endophytic in plant roots through infection of root hairs (Perrine-Walker et al., 2007). The fungal pathogen *Thielaviopsis basicola* uses root hairs to enter roots and initiate

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



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

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.

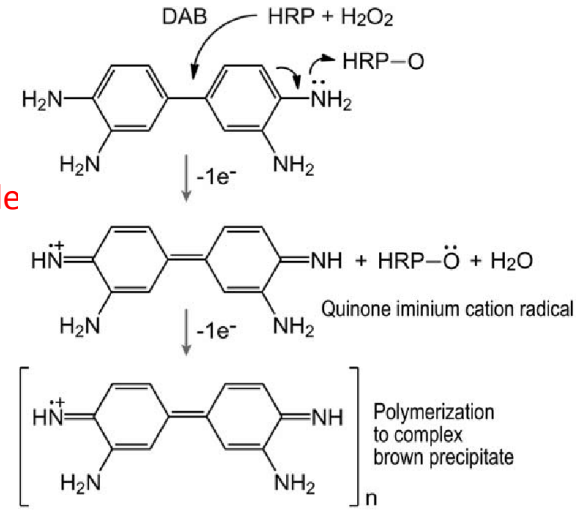
How DAB Works:

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

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

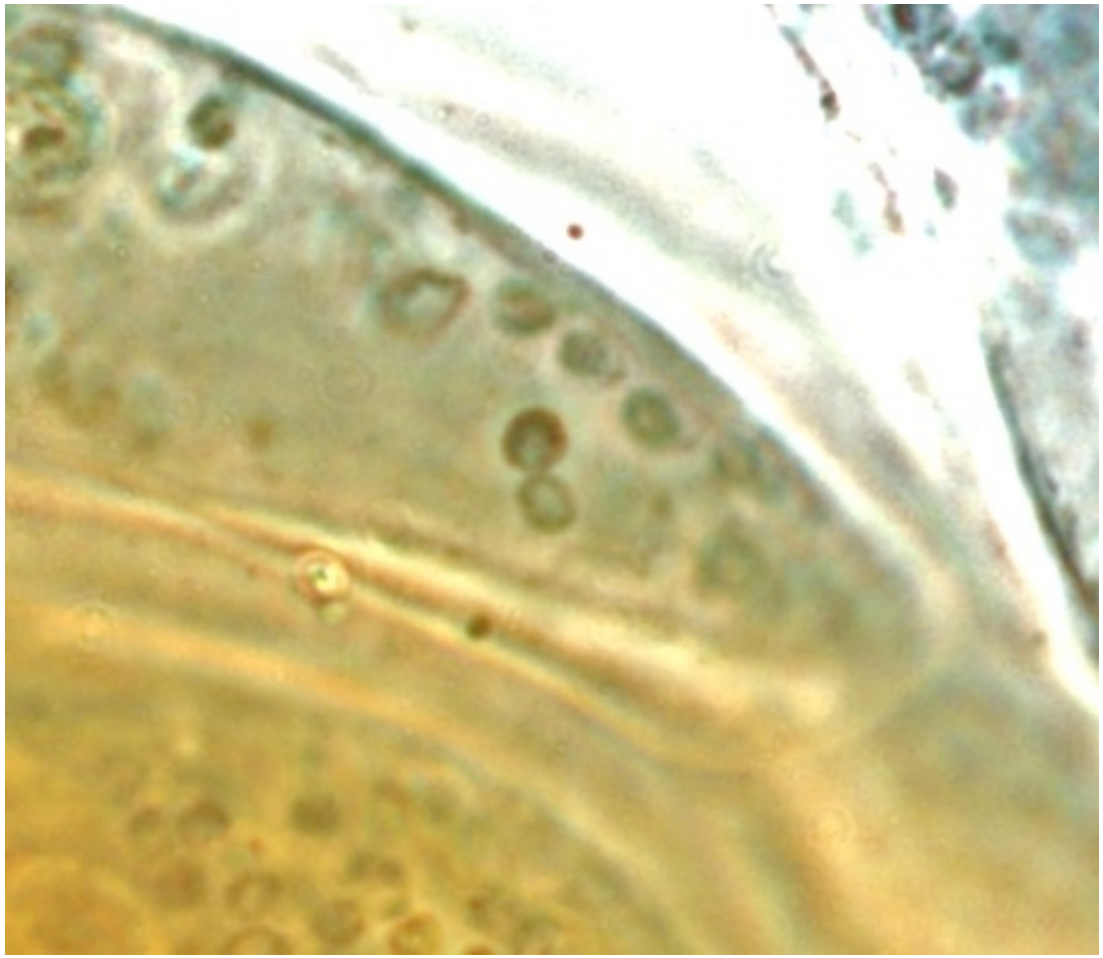
3) DAB reacts with Hydrogen peroxide to form Brown/red coloration

HRP is peroxidase

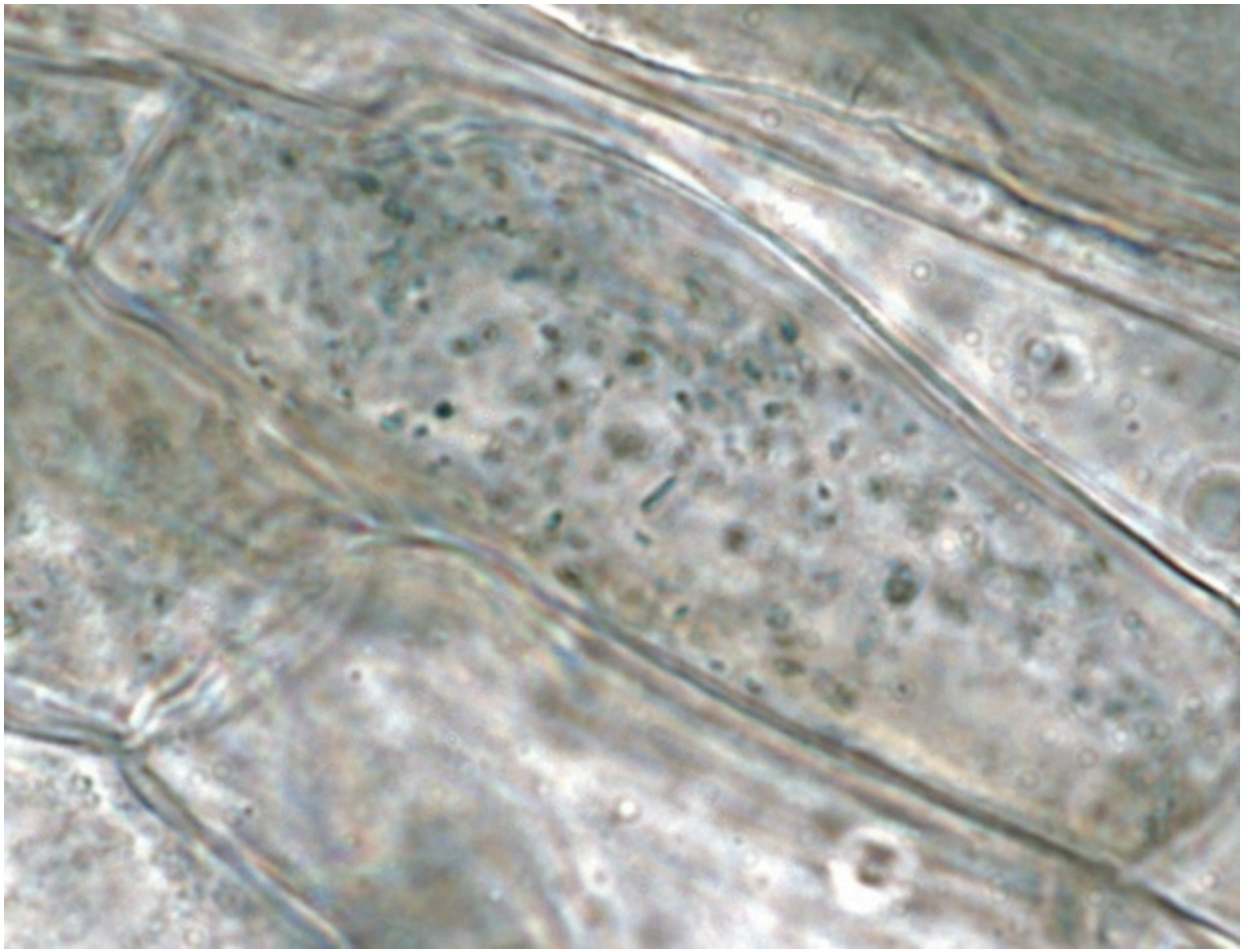


Agave desertii in rocks

Bacteria present in root cells of Agavaceae

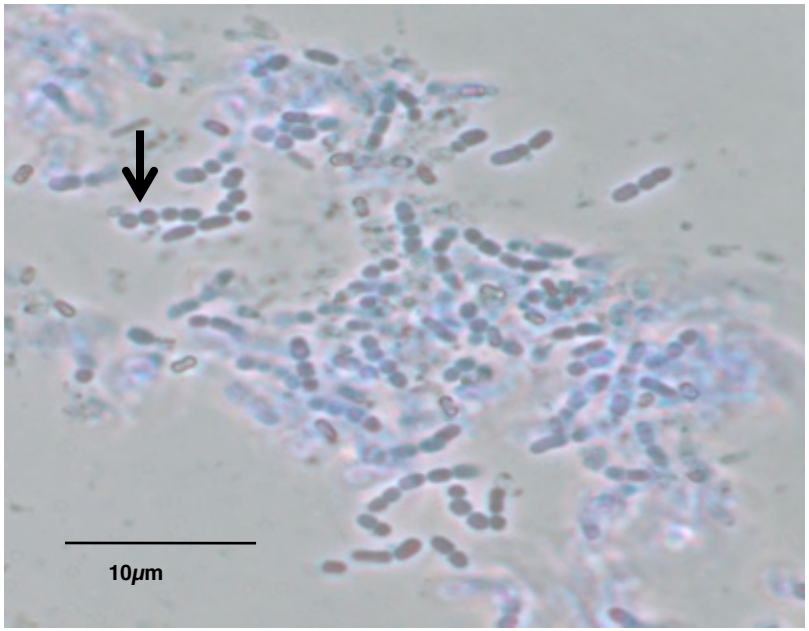


Yucca parenchyma cell with endobiotic bacteria

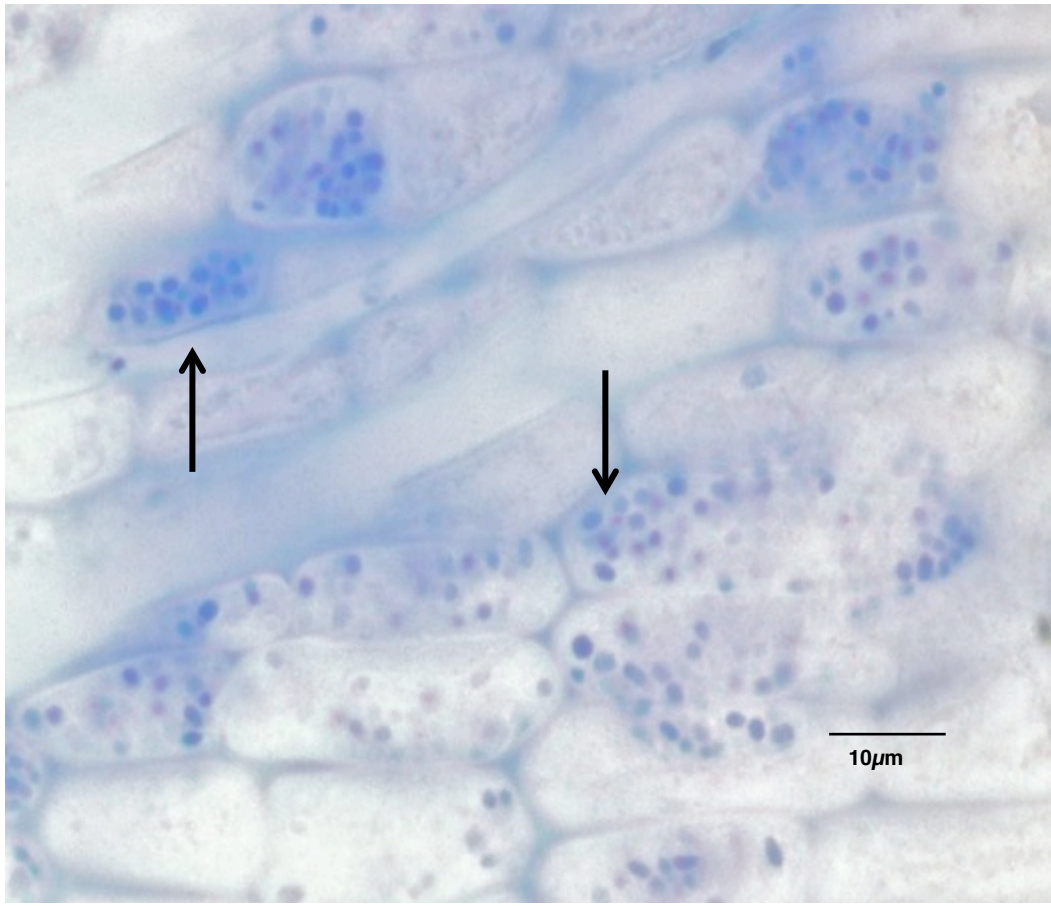


Agave tequilana or blue agave

Bacillus on agar



Bacillus in meristematic root cells

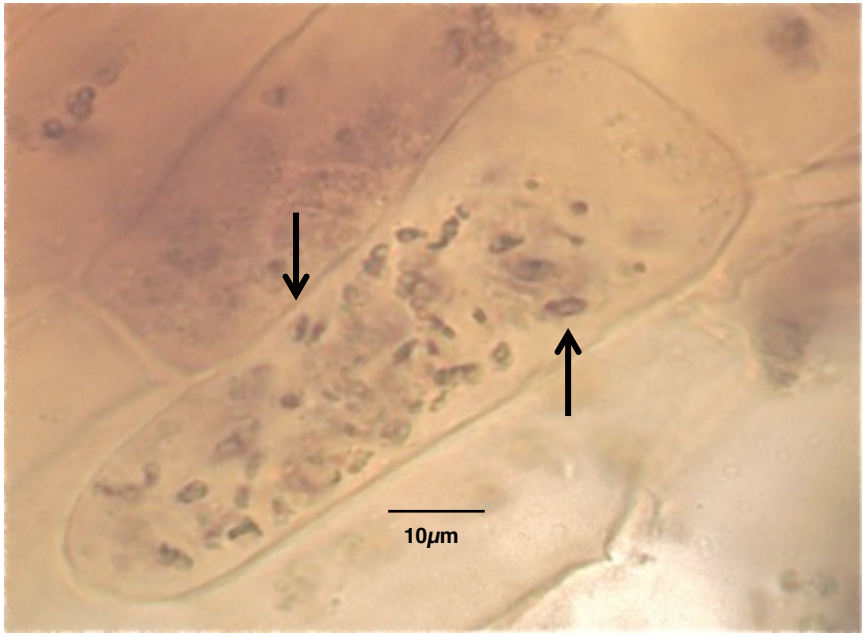


Aniline blue stain (0.01% aqueous)

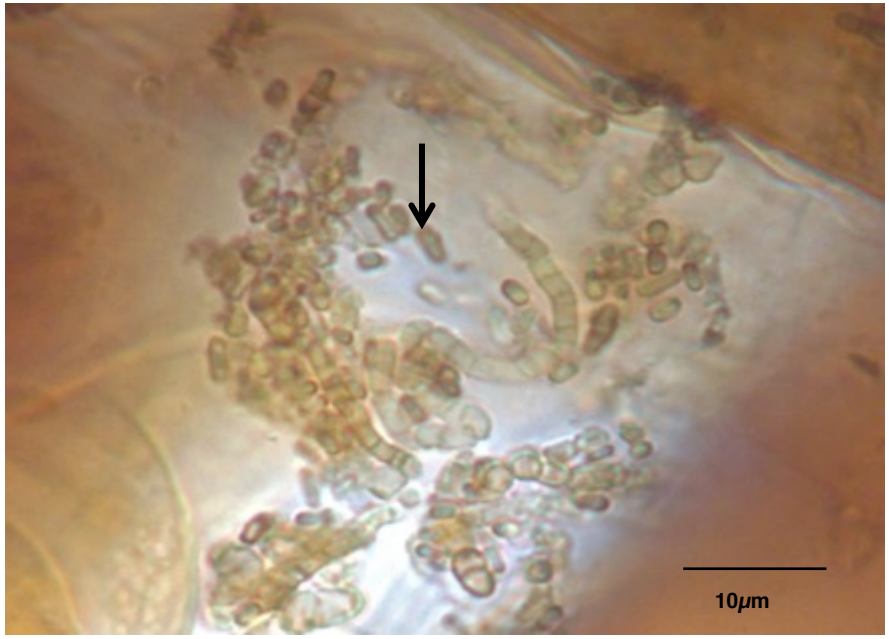
Bacillus oxidation in Agave tissues

Stained with DAB

Bacteria oxidizing in root cells

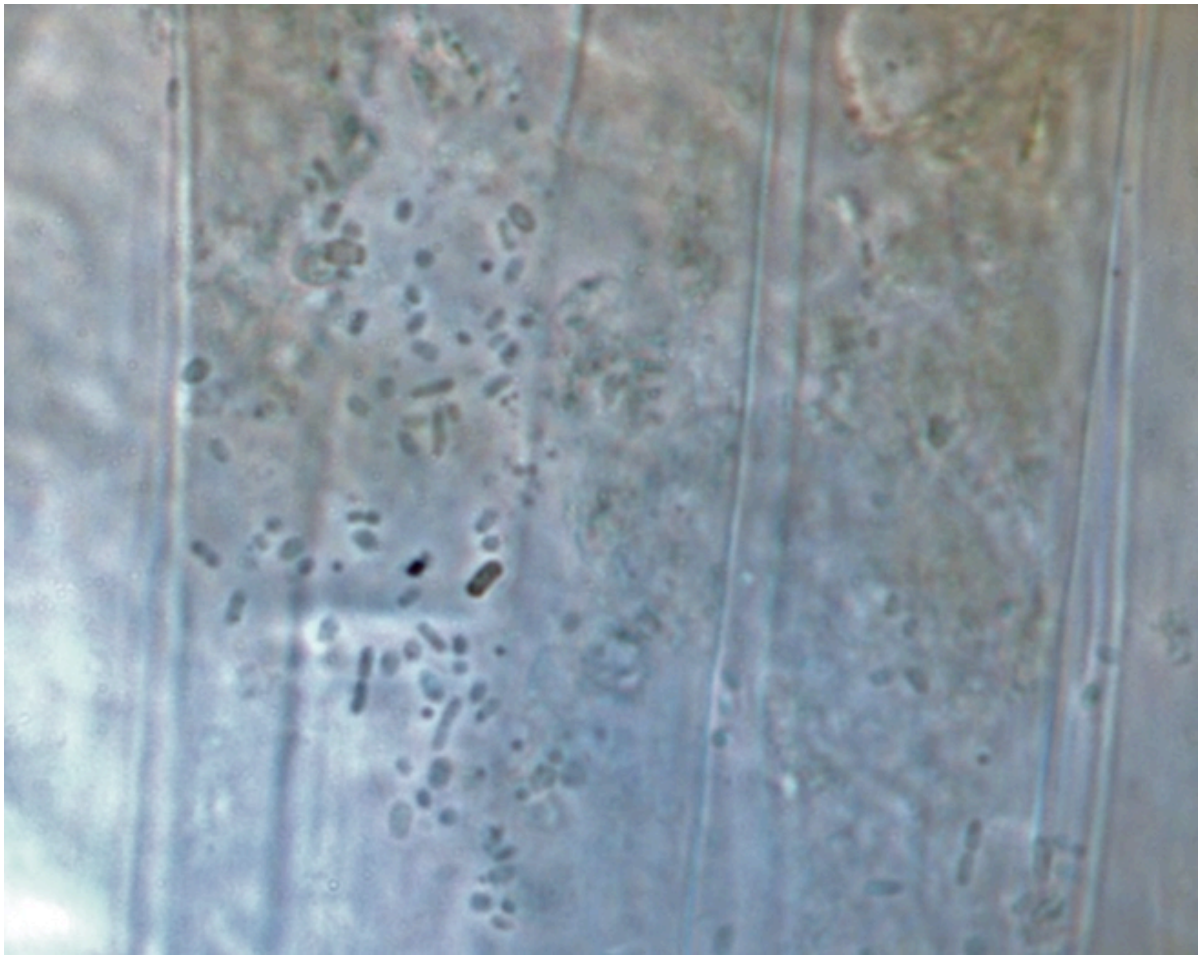


Bacteria oxidizing on the surface of root cells



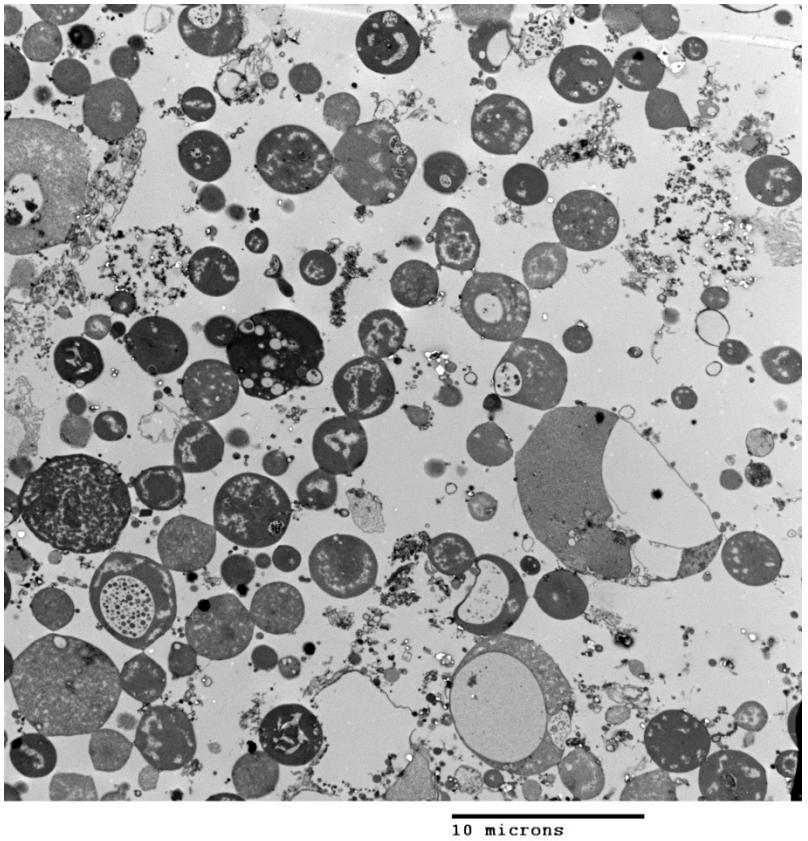
Agave schottii in the Sonoran desert

Bacteria on root cells (rods)
Bacteria have cell walls and characteristic shapes



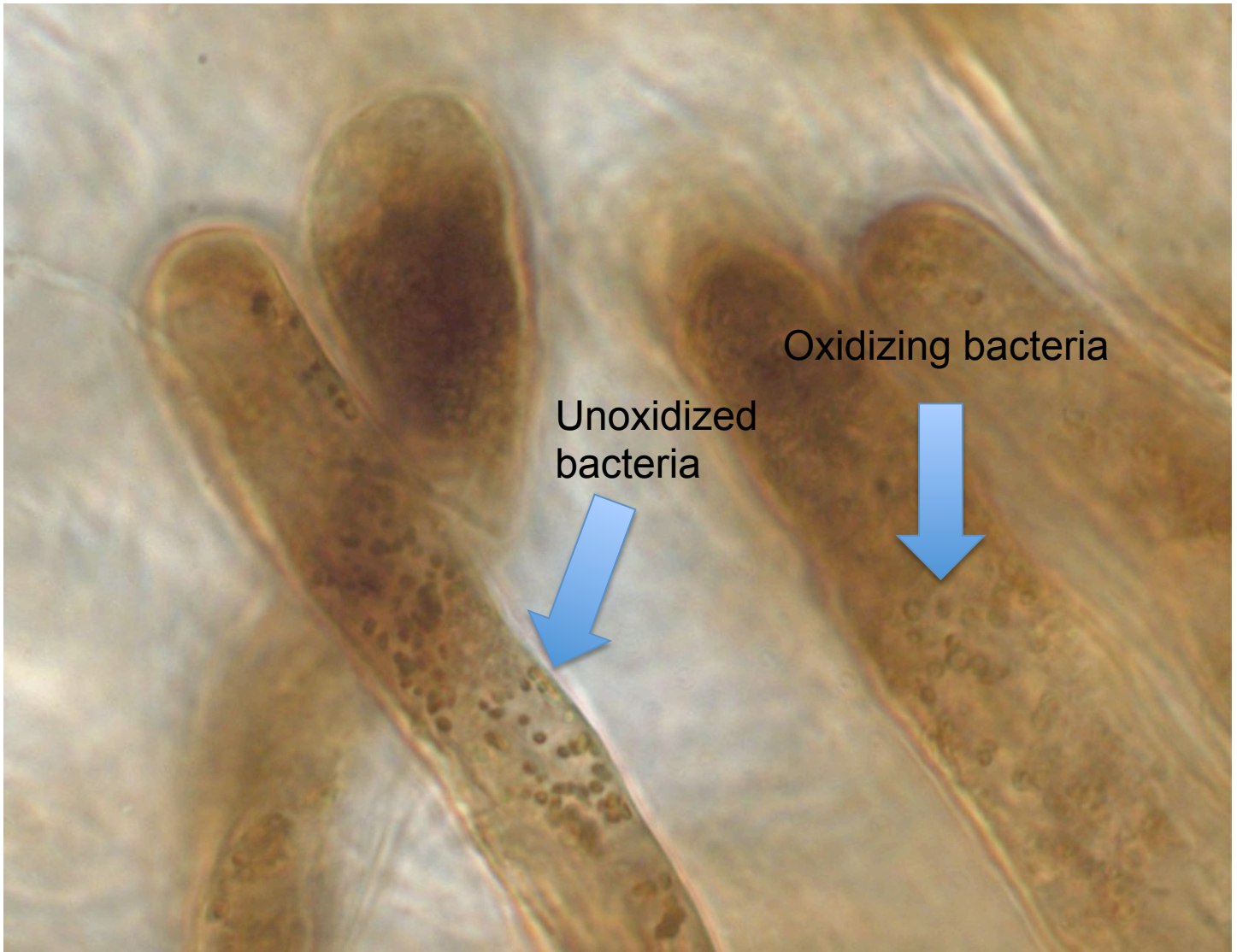
Inside root cells bacteria lose cell walls and assume a spherical shape.

L-forms = Protoplasts

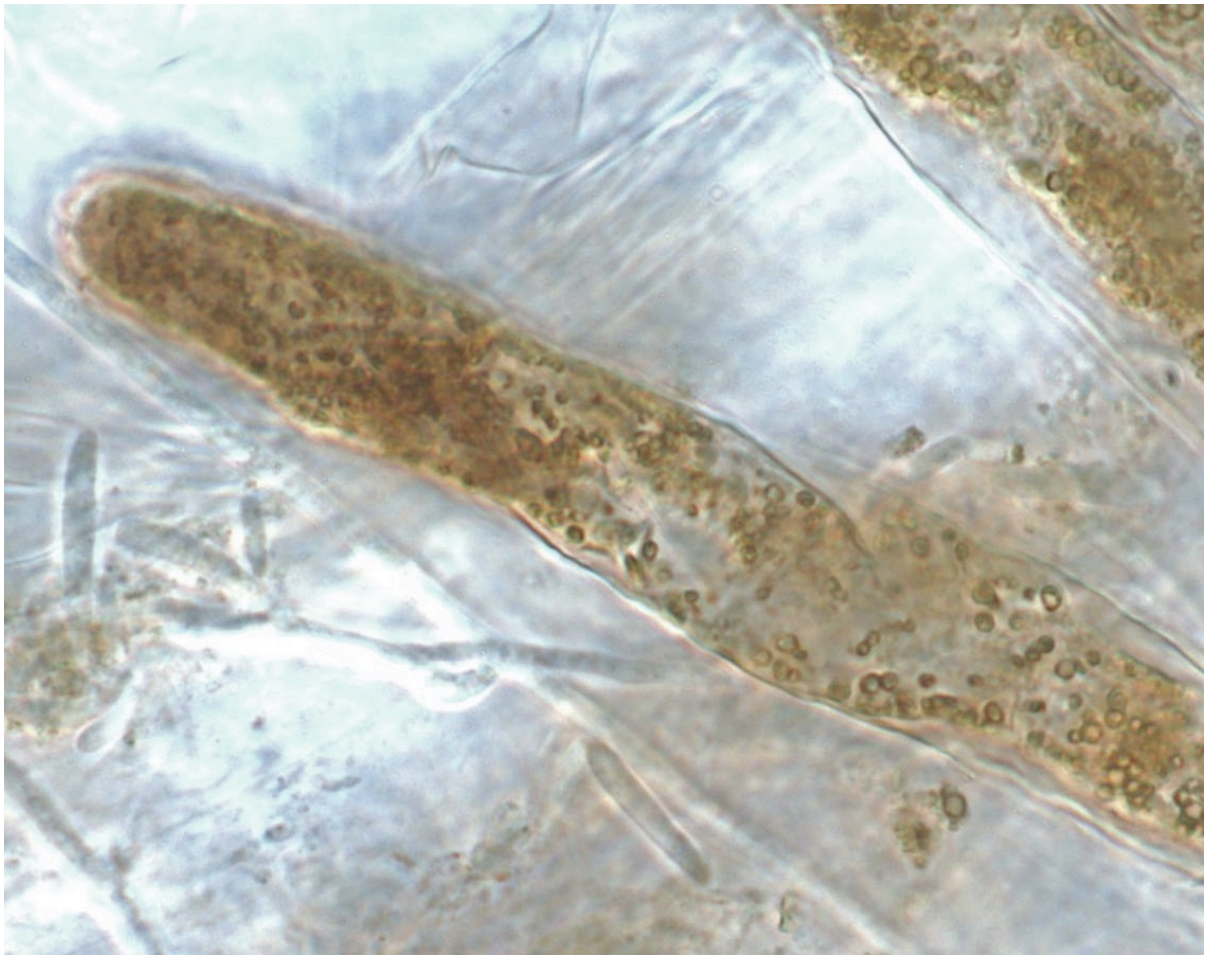


L-forms are bacterial cells that do not form cell walls (also called ‘cell wall deficient bacteria’). L-forms typically are seen inside eukaryotic cells. They are thought to be a mechanism to evade host defense response. L-form bacteria are typically variable in size.

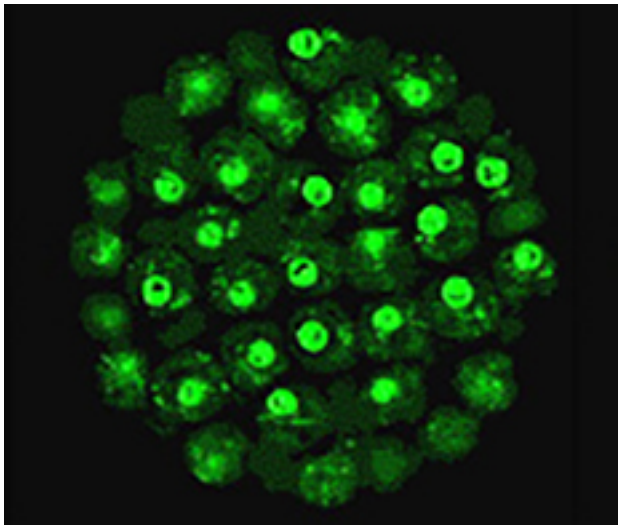
Bacterial protoplasts in root hairs of Agave schottii (stained with DAB/peroxidase; counterstained with aniline blue)



Agave schottii root hair containing bacteria



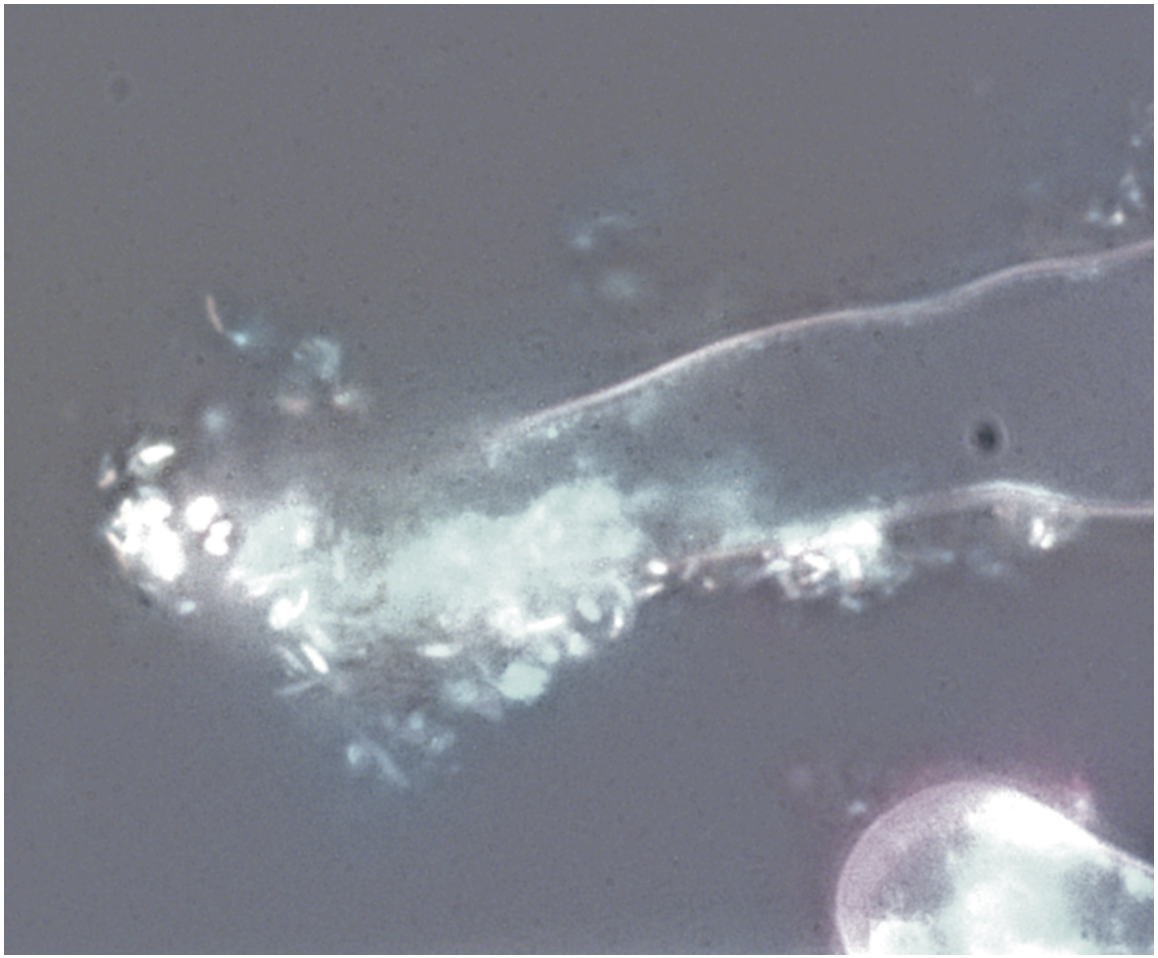
Fluorescent Stains



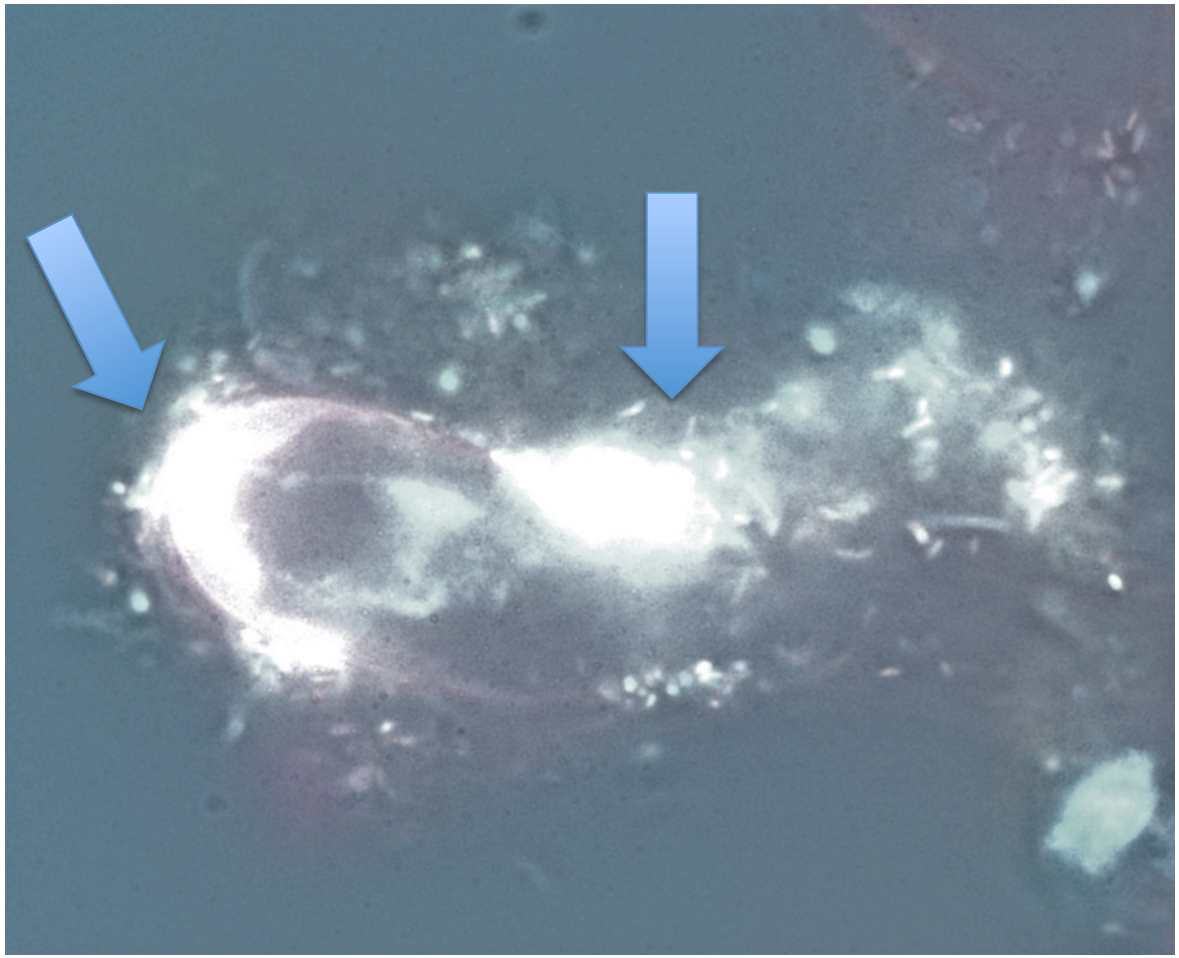
- Syto 9 nuclear stain for both live and dead Gram-positive and Gram-negative bacteria.

- GFP (Green Fluorescent Protein). GFP can be used to track the fate of individial strains of microbes in plant tissues. The down side is that microbes must be transformed with GFP, and microbes may not express the GFP within plant tissues and cells.

Bacteria on Yucca root hairs stained with florescent nuclear stain SYTO 9



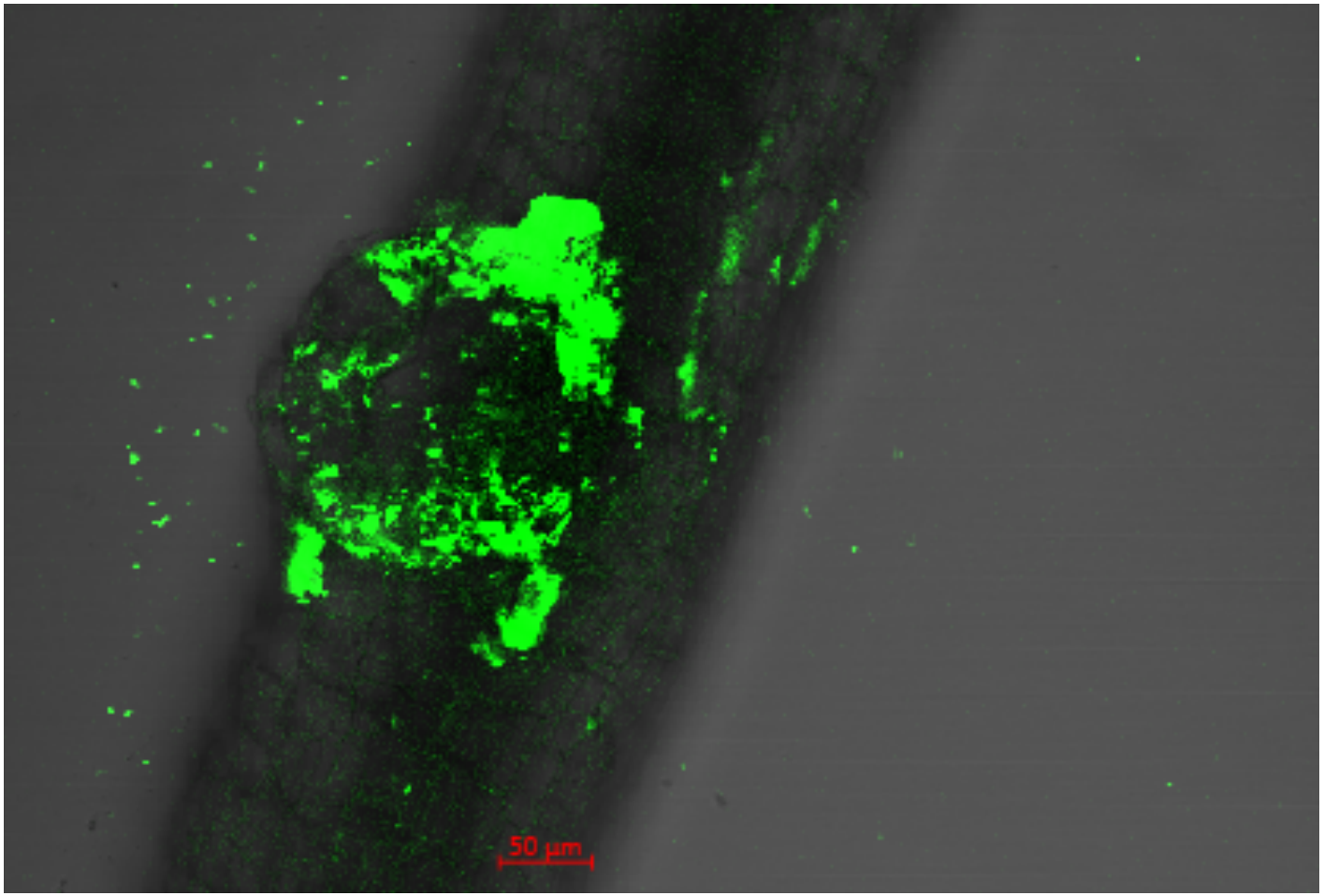
Bacteria appear to be within root hairs.



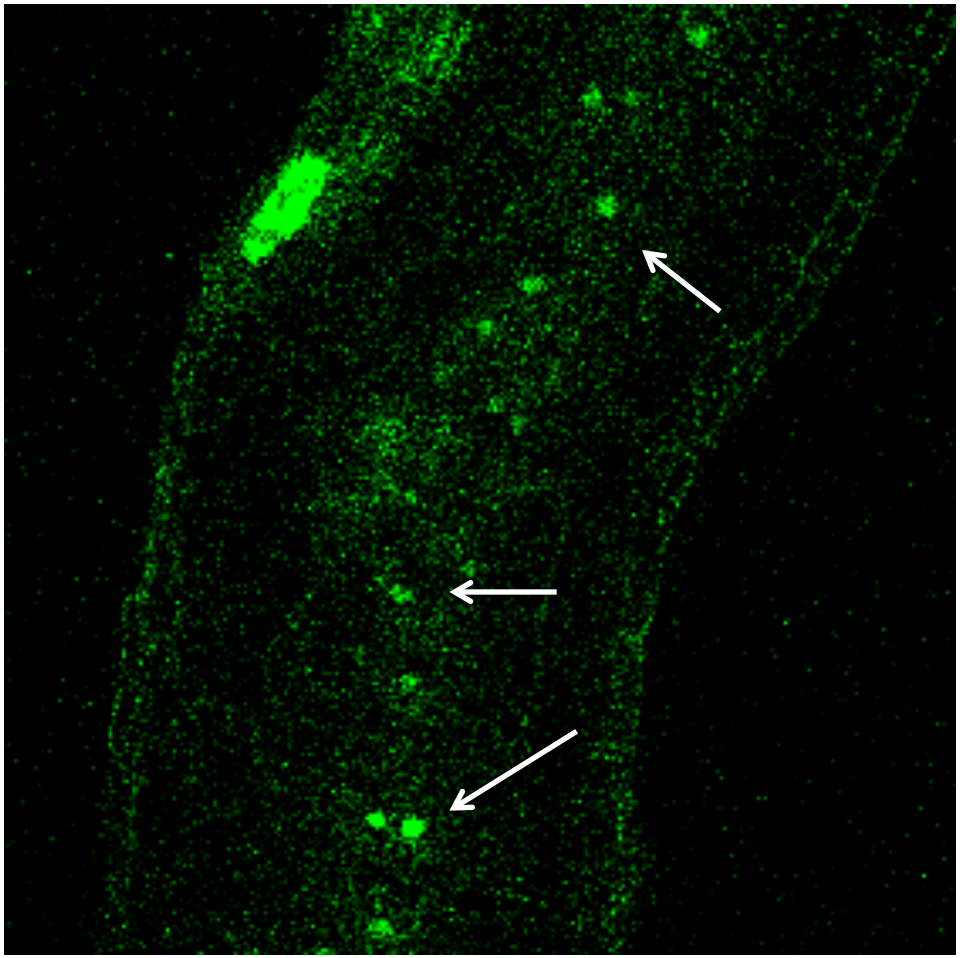
Localization of GFP-labeled *E. coli* in Bermuda grass seedling root tissues using confocal microscopy

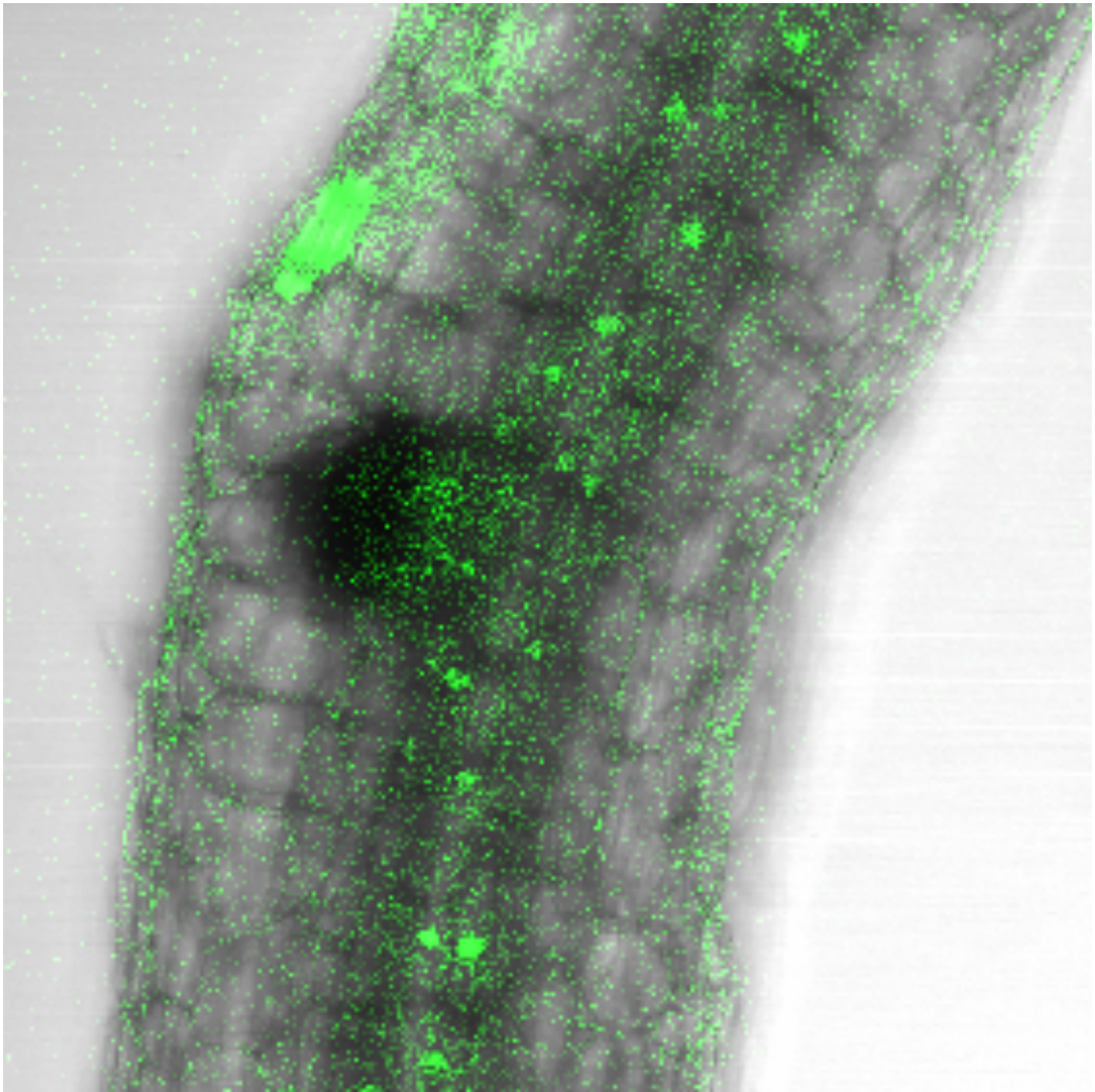
Satish Kumar Verma, Qiang Chen, James White

Lateral root showing GFP labeled-*E. coli* on tissues



E. coli colonizes internally all parts of the root. Arrows show the bacterium within the vascular tissue.





Bonaire: A Desert Island in the Caribbean

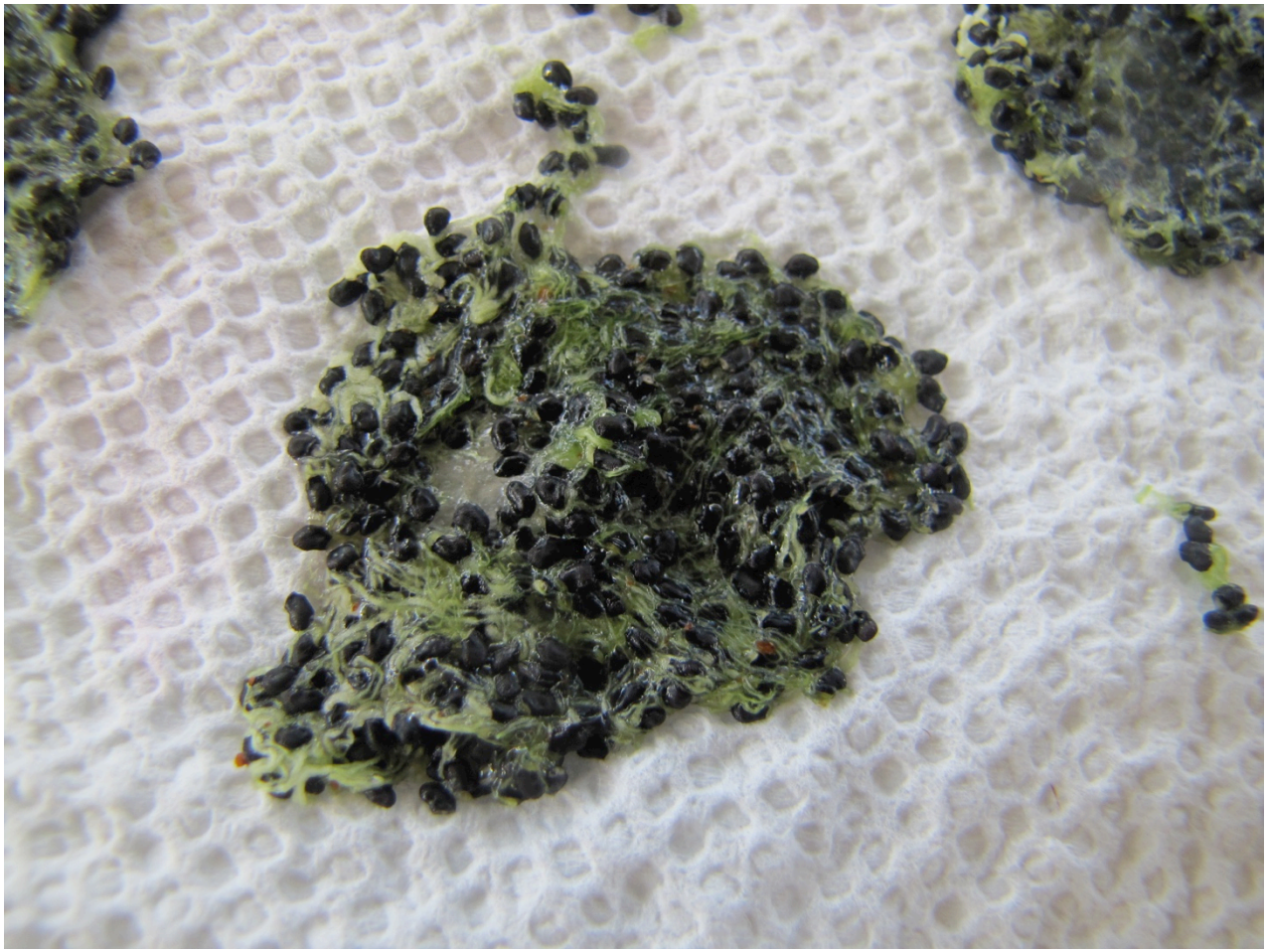
Cacti are ‘big boyz’ of desert

‘Cadushy’ cactus: Subpilocereus repandus

Cadushy fruits

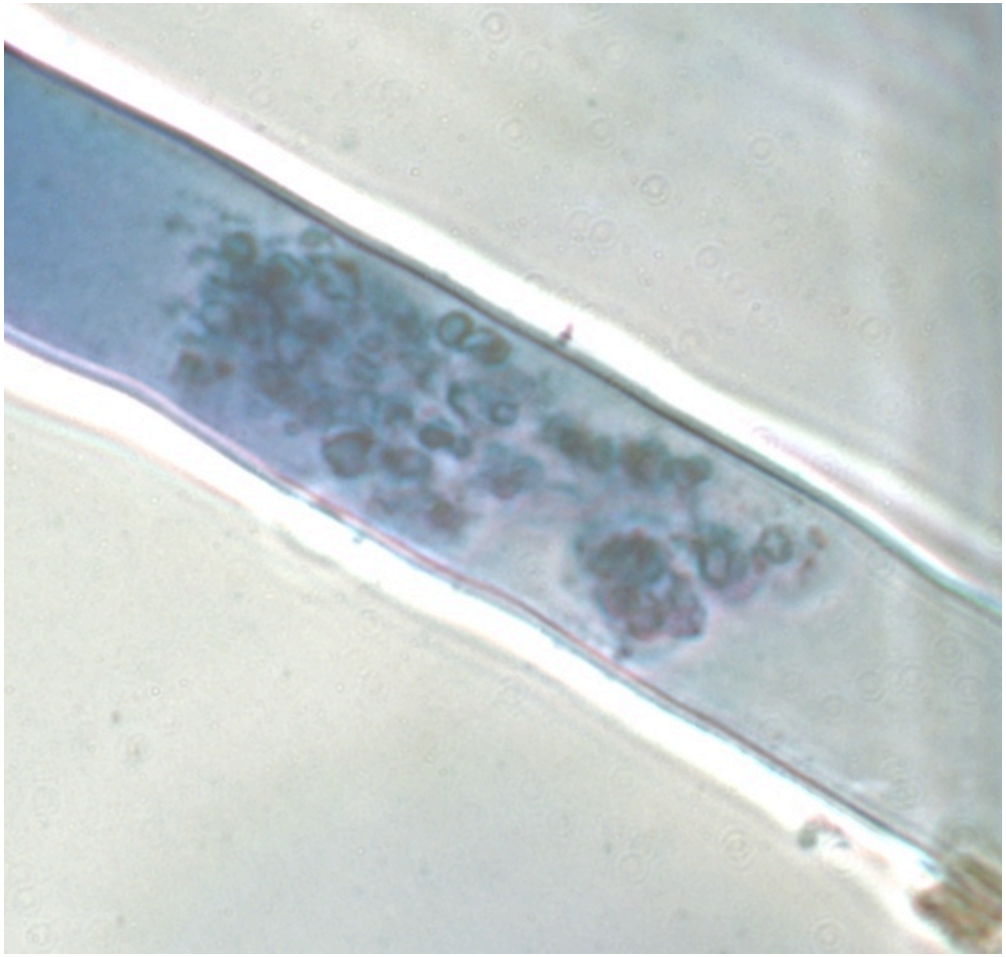
Fruit with seeds

Seeds spread on paper to dry

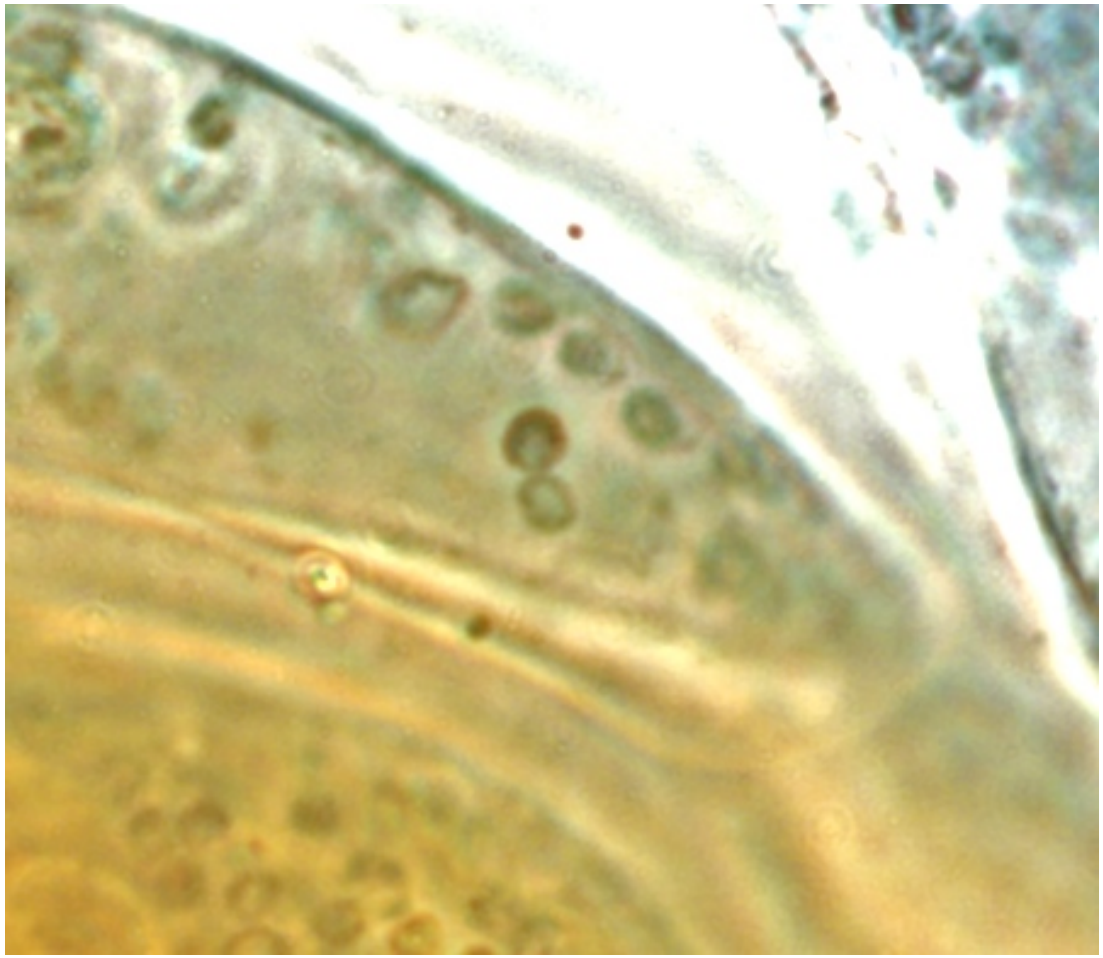


Cadushy seedling germinated on agarose, then stained with DAB

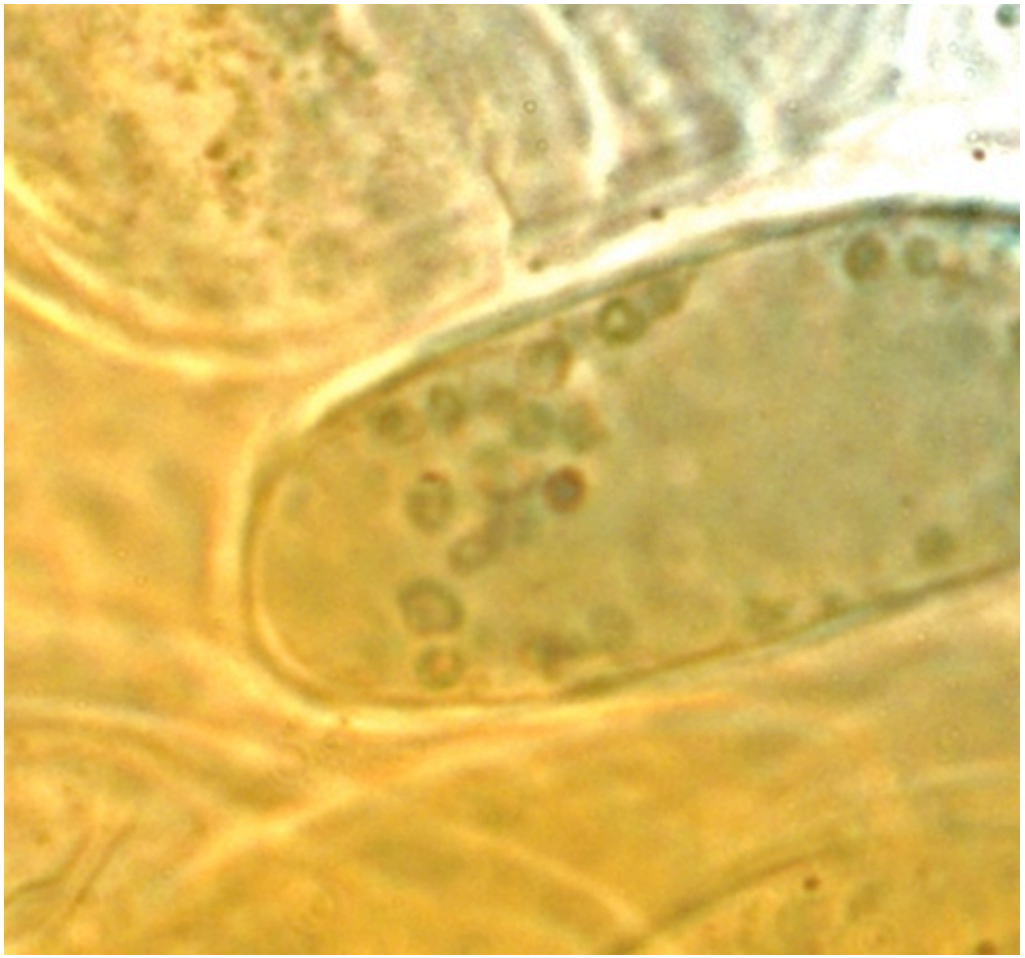
Oxidized bacteria in root hair



Oxidizing bacteria



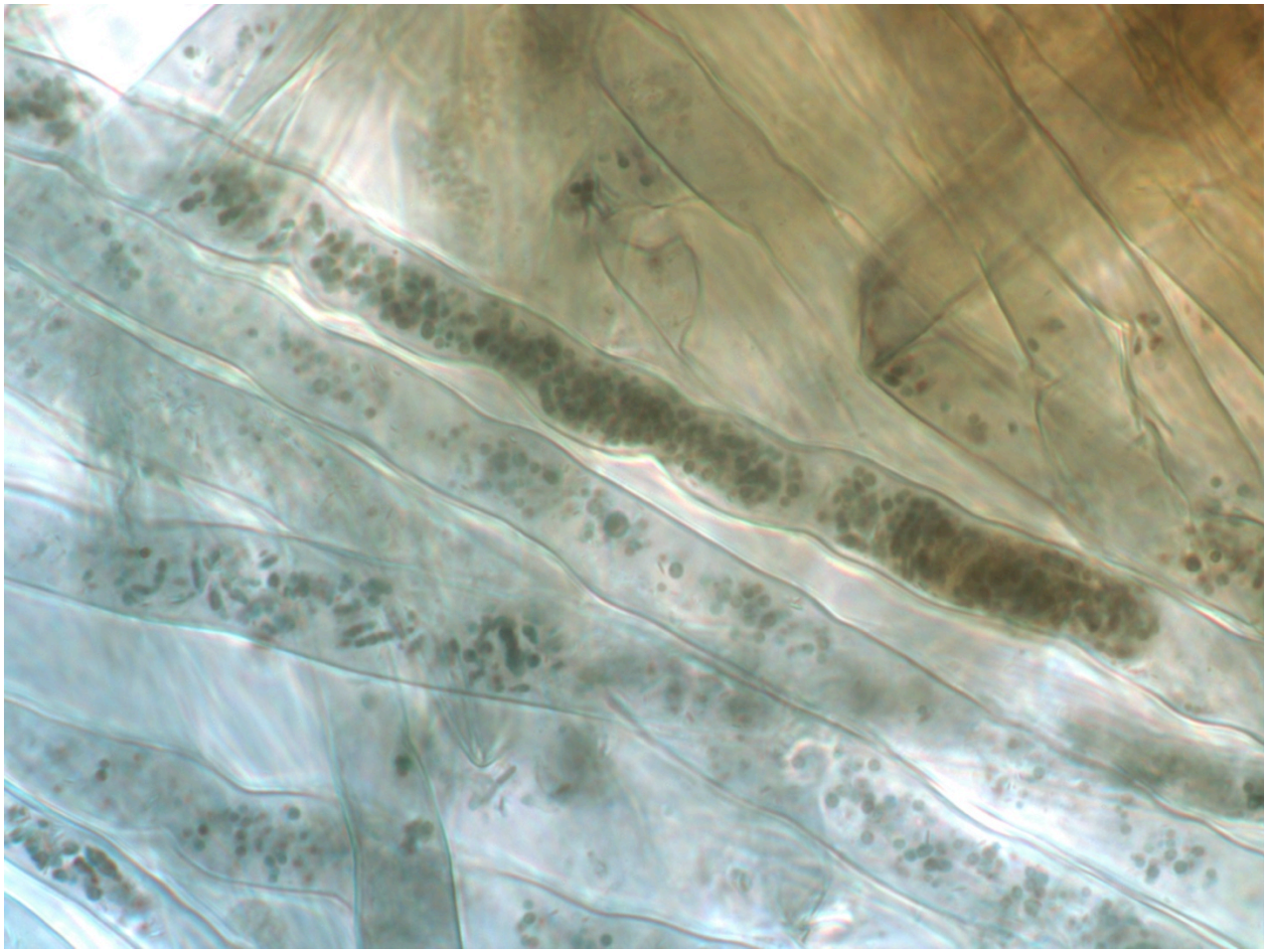
Oxidized and oxidizing bacteria in vesicles of root cap cell



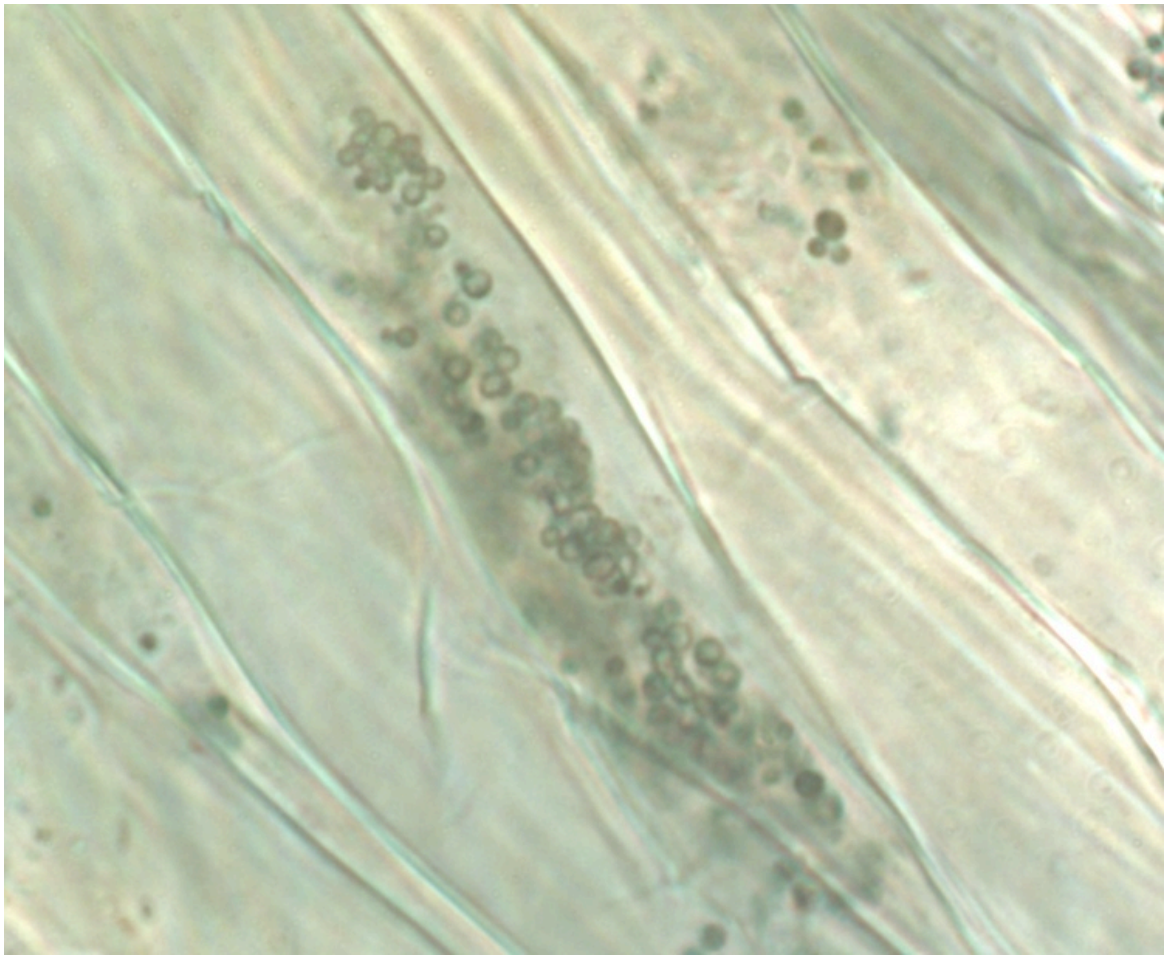
‘Yatu cactus’ : Ritterocereus griseus

Yatu fense

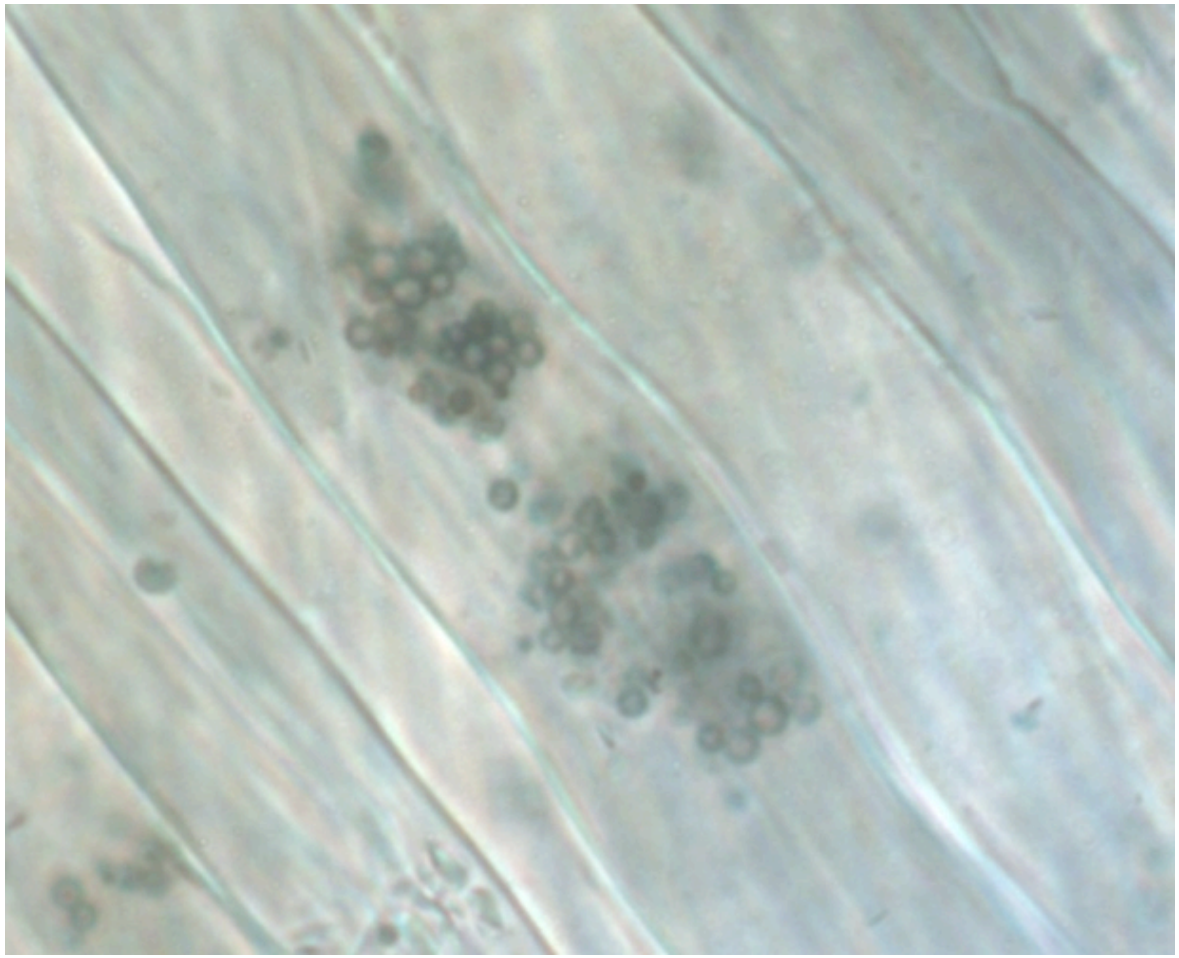
Bacteria in root hairs (Stain in DAB for 1 wk in refrigeration hours followed by aniline blue).



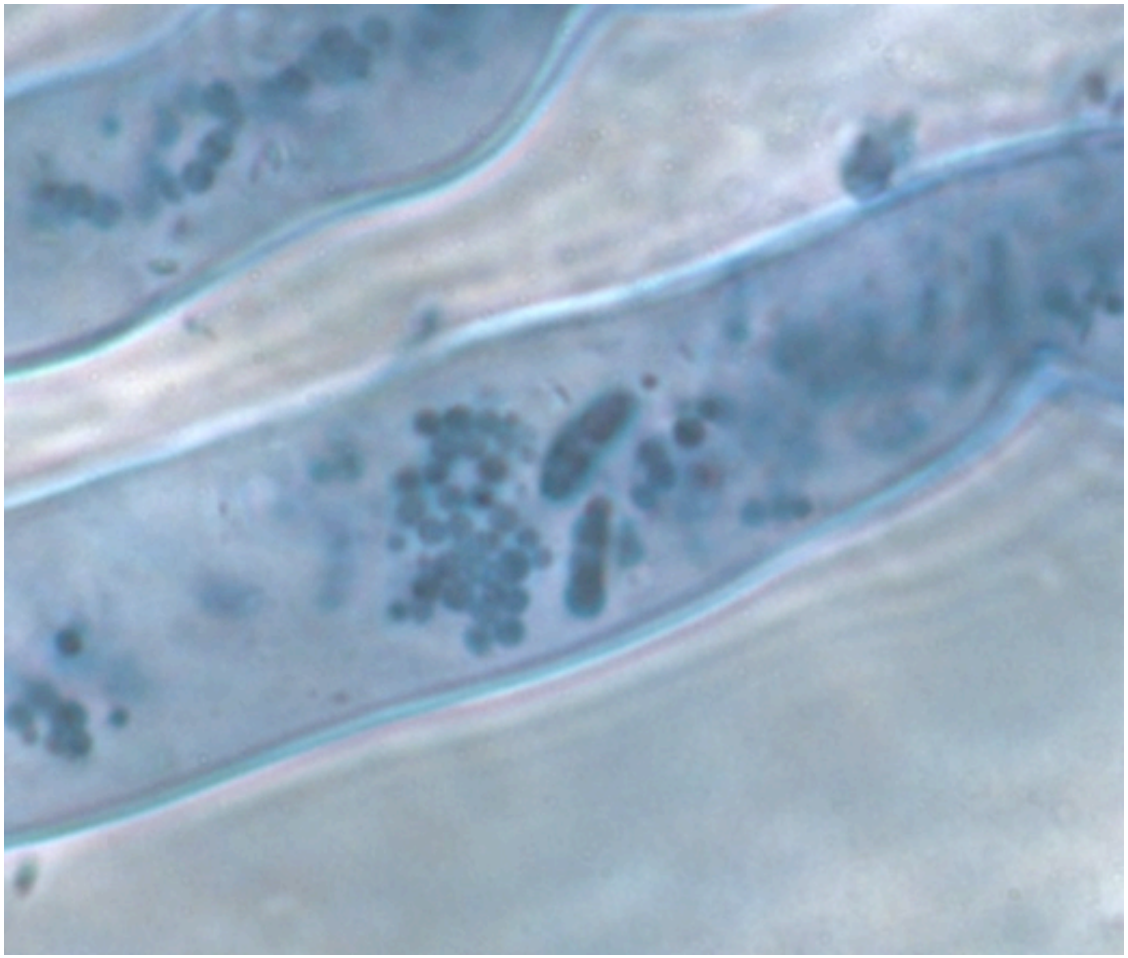
Oxidized bacteria in root hair



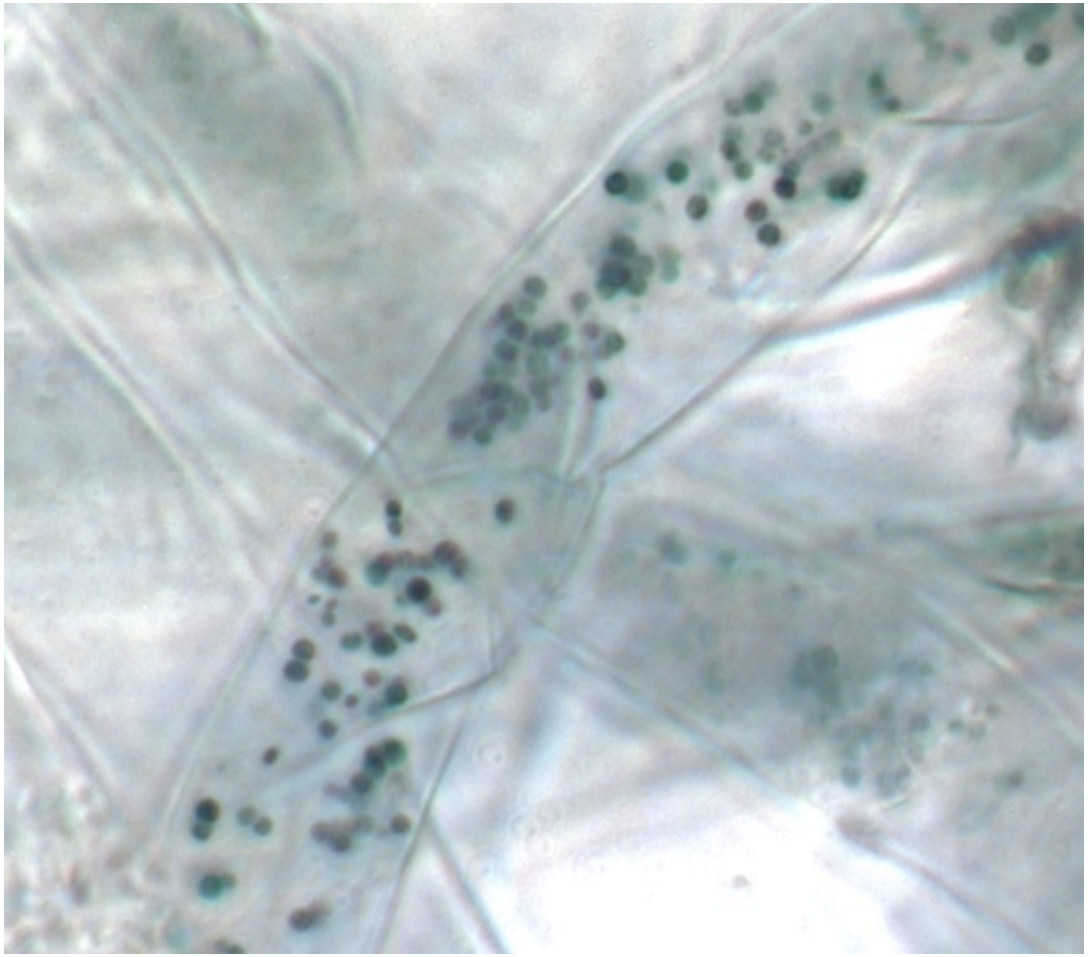
Oxidized bacteria in root hair



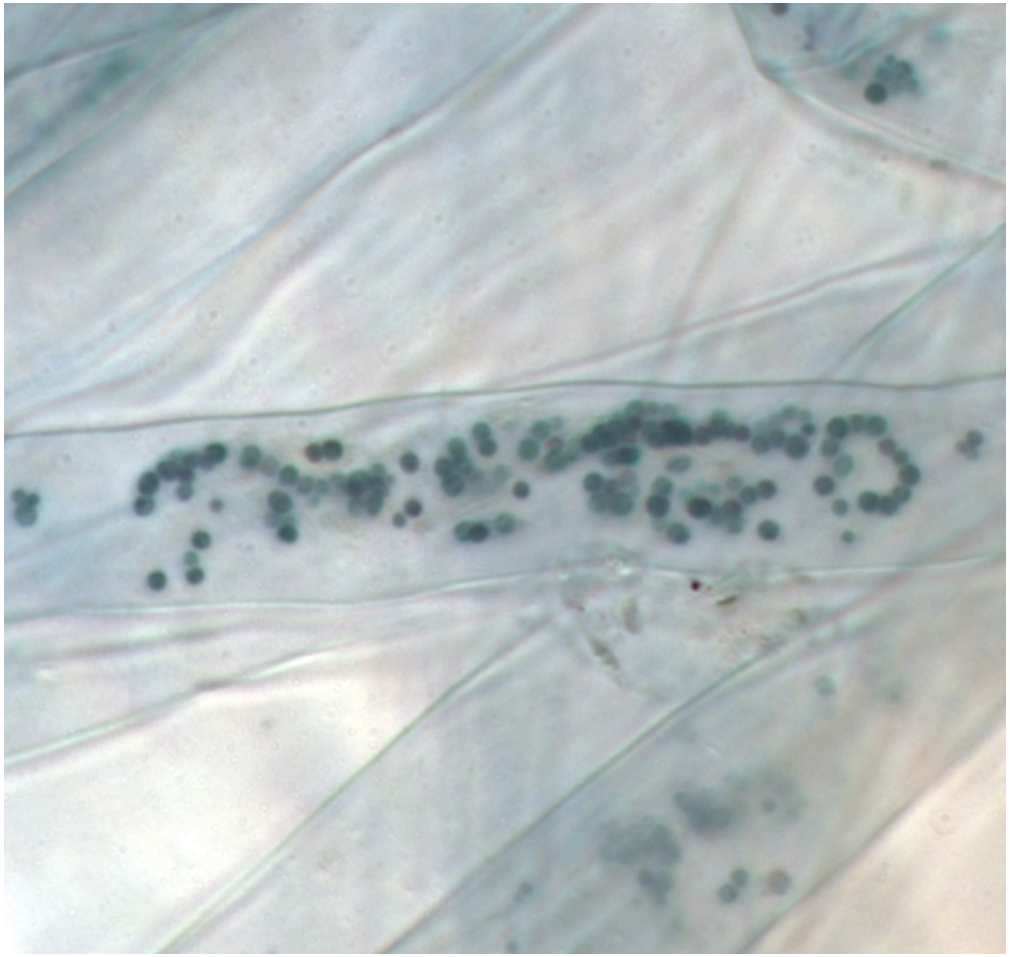
Chains of bacteria in crevasses of root cell plasma membrane



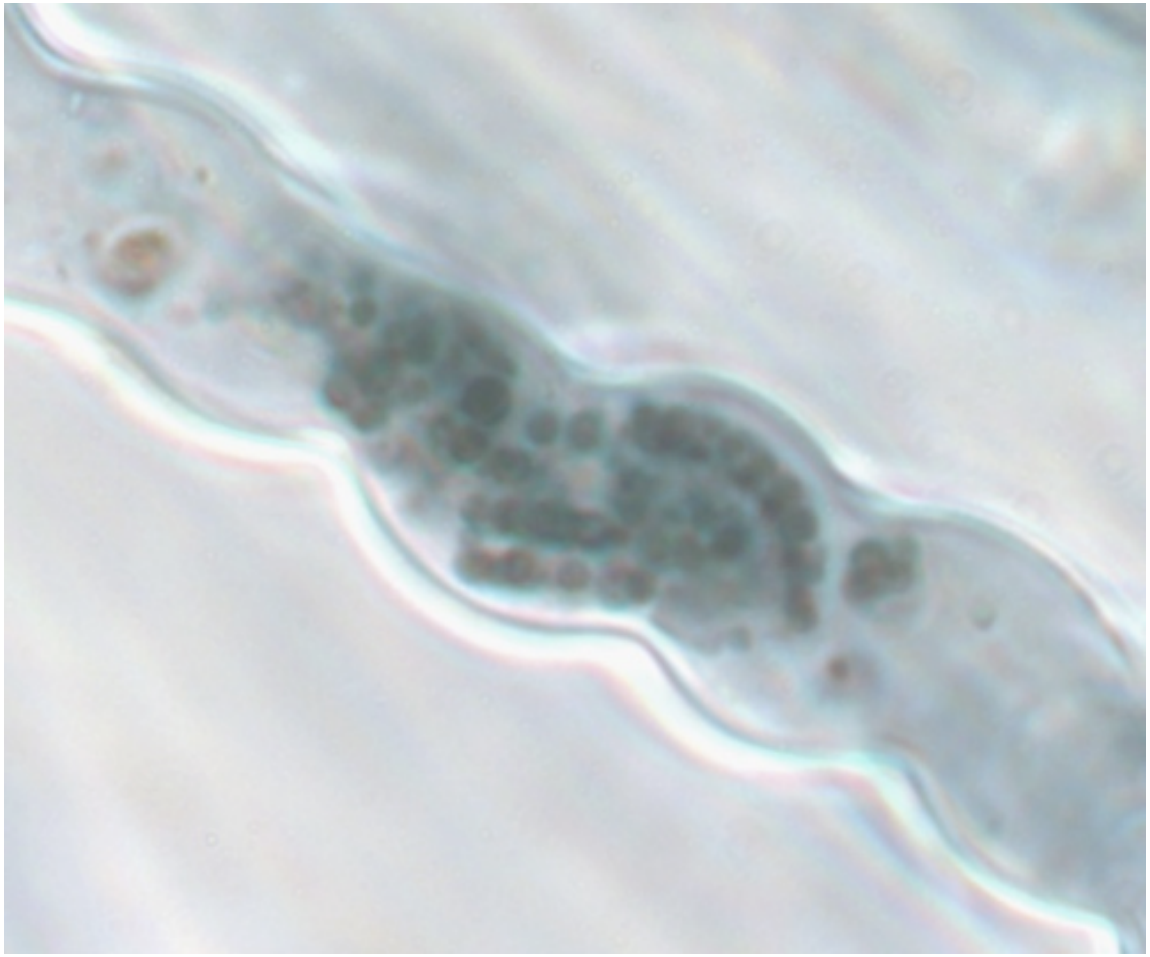
Bacteria in root hairs showing recently divided pairs



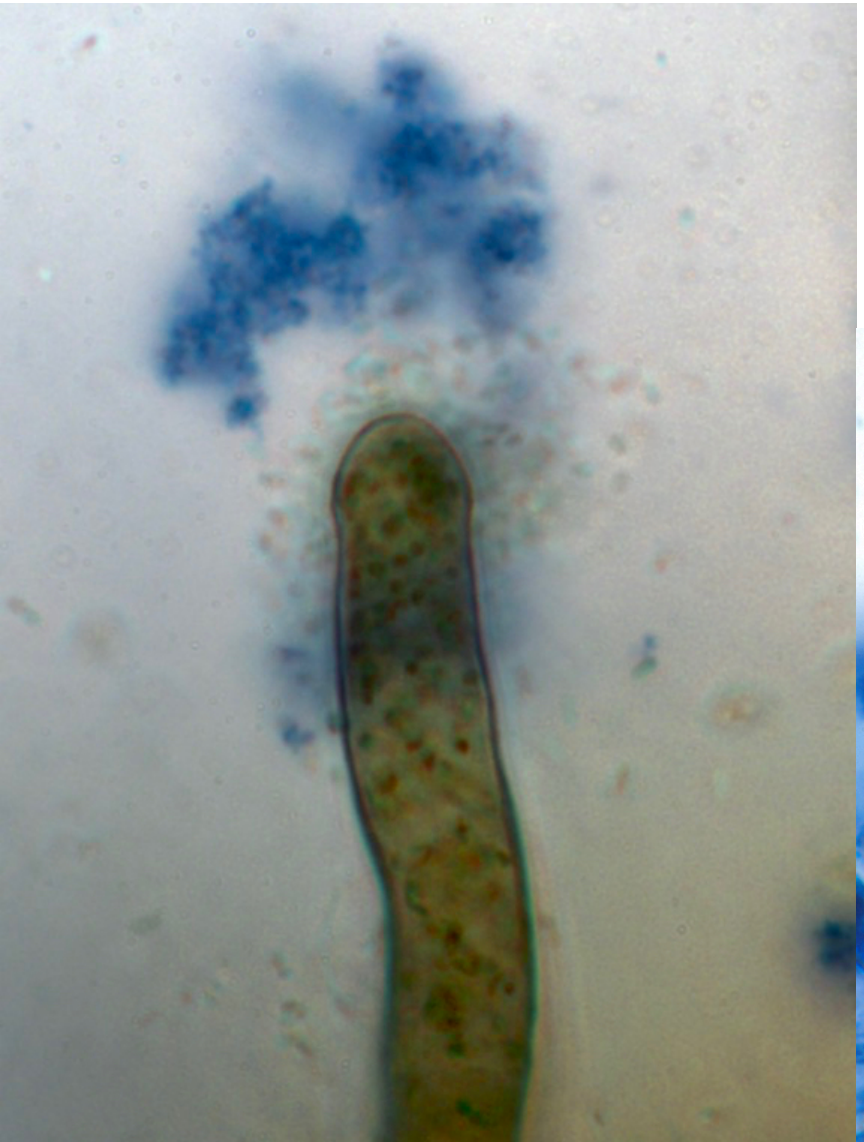
Chains of bacteria in root hairs



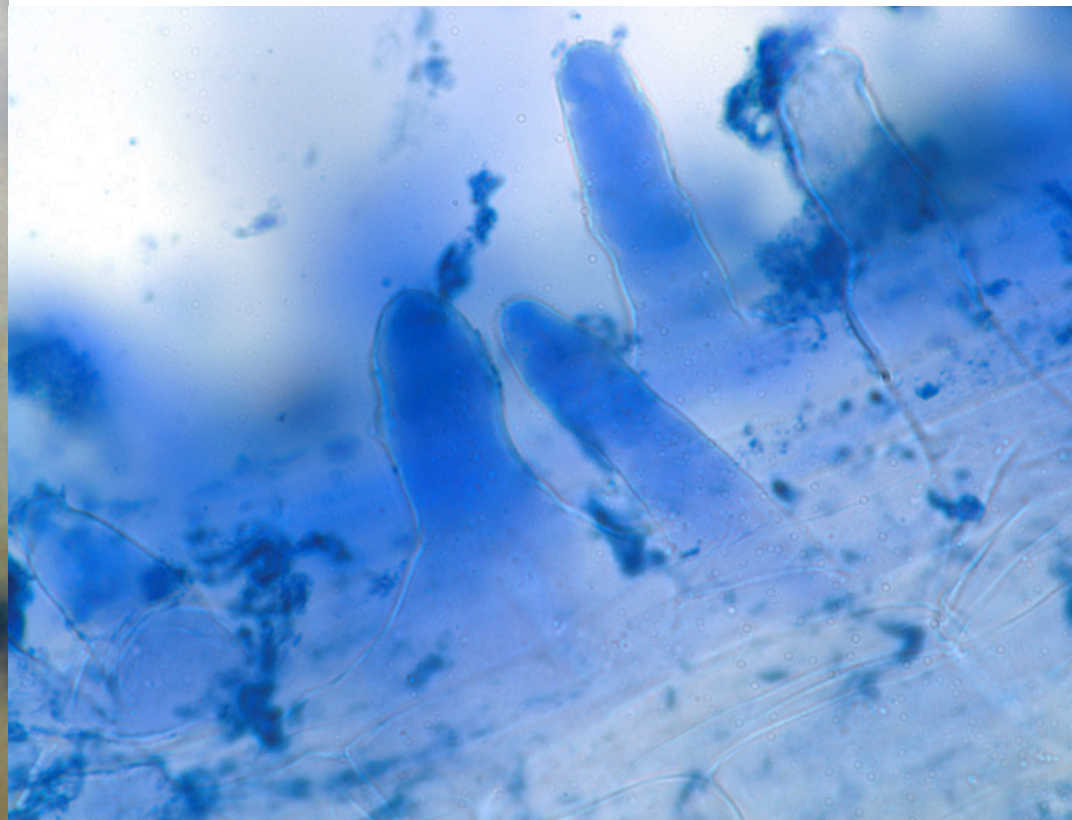
Close-up of bacterial chains in root hair



Amaranthus viridis with endophytic bacteria (left) and without microbes (right)

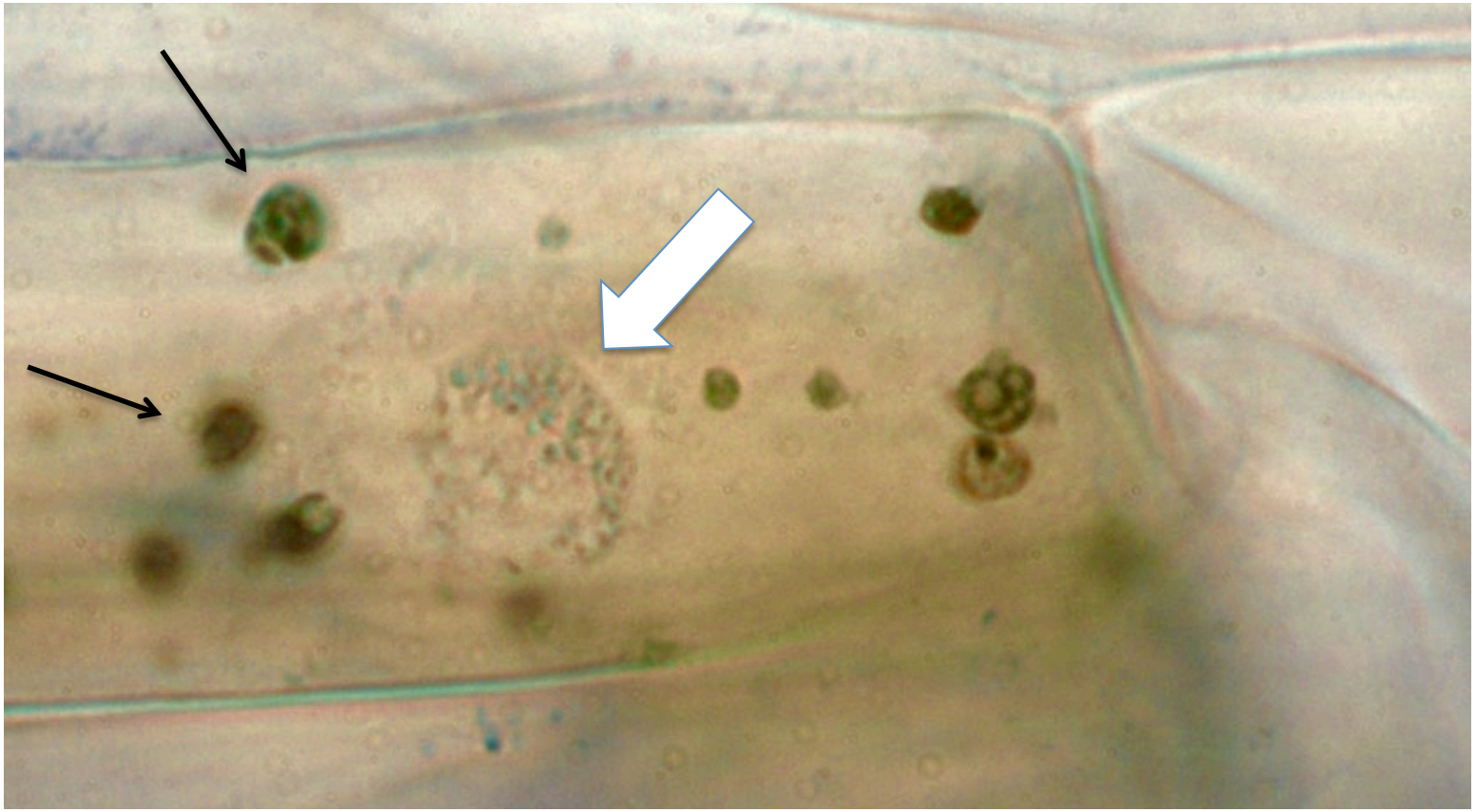


Bacteria are *Curtobacterium* sp. from plant *Froelichia gracilis*.



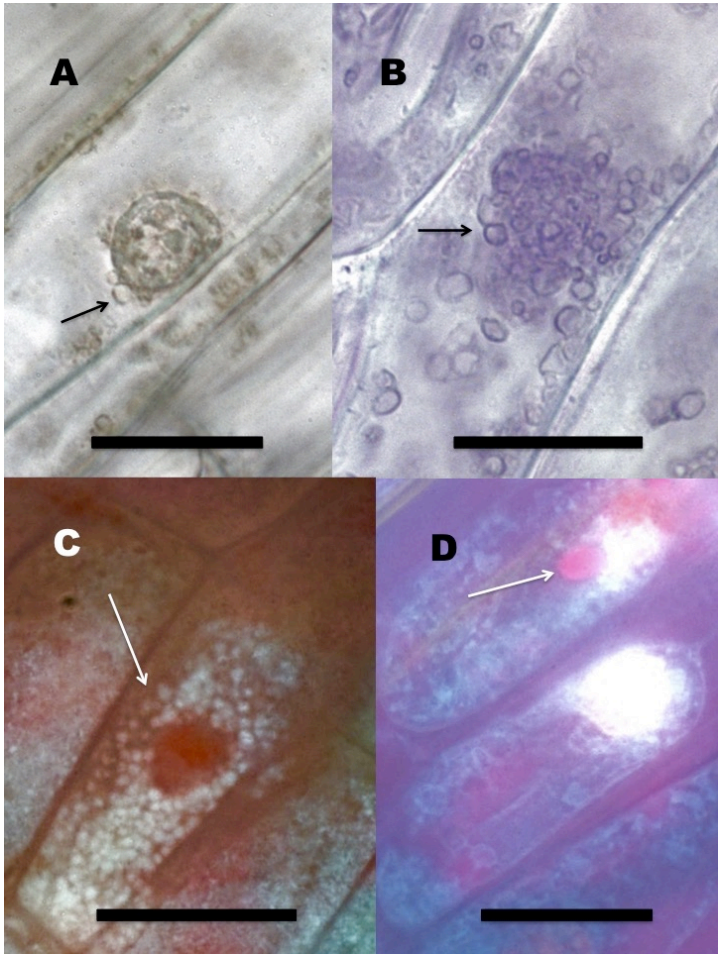
Nuclear Colonization

Phenomenon of nuclear colonization by alpha-Proteobacteria (white arrows).



Bacteria also present in depressions of the root cell plasma membrane (black arrows).

Nuclear colonization may be a way that cytoplasm-colonizing microbes may escape oxidation/degradation.

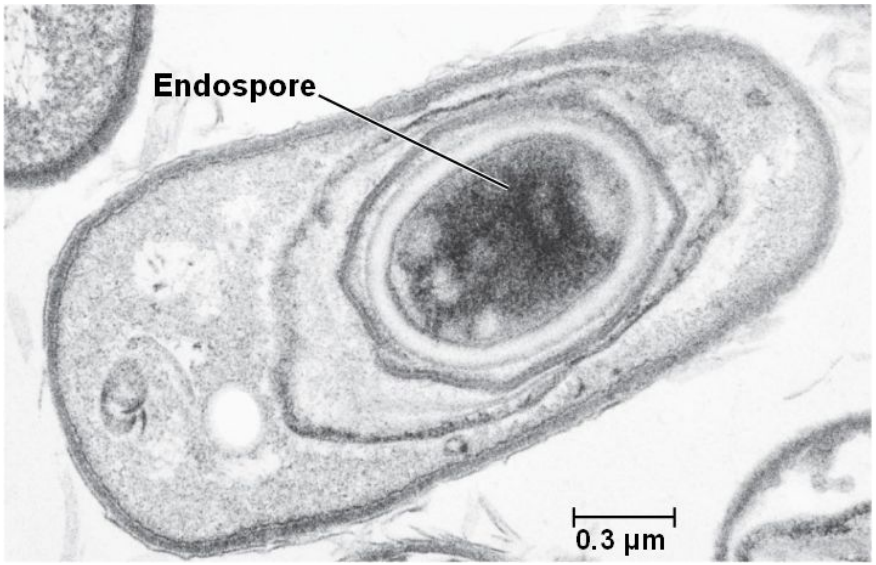


Methylobacterium exiting plant root cell

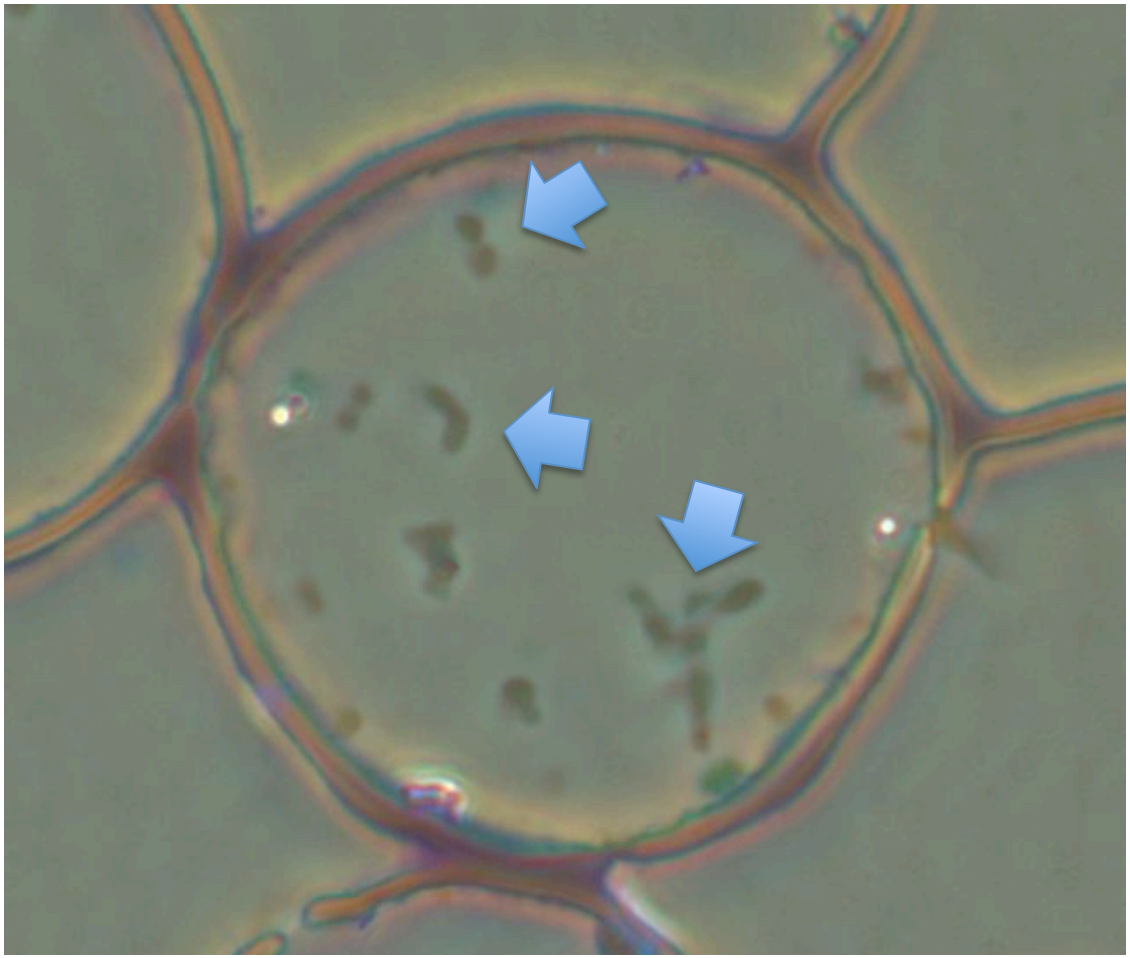
Bacillus L-forms and Endospores within Plant Cells: A Study of Red Yucca (Agavaceae) and *Bacillus* Endophyte

Bacillus endophytes of red Yucca (Agavaceae)

- Predominant endophyte is *Bacillus tequilensis*
- *Bacillus* shows intracellular development
- *Bacillus* shows endospore formation

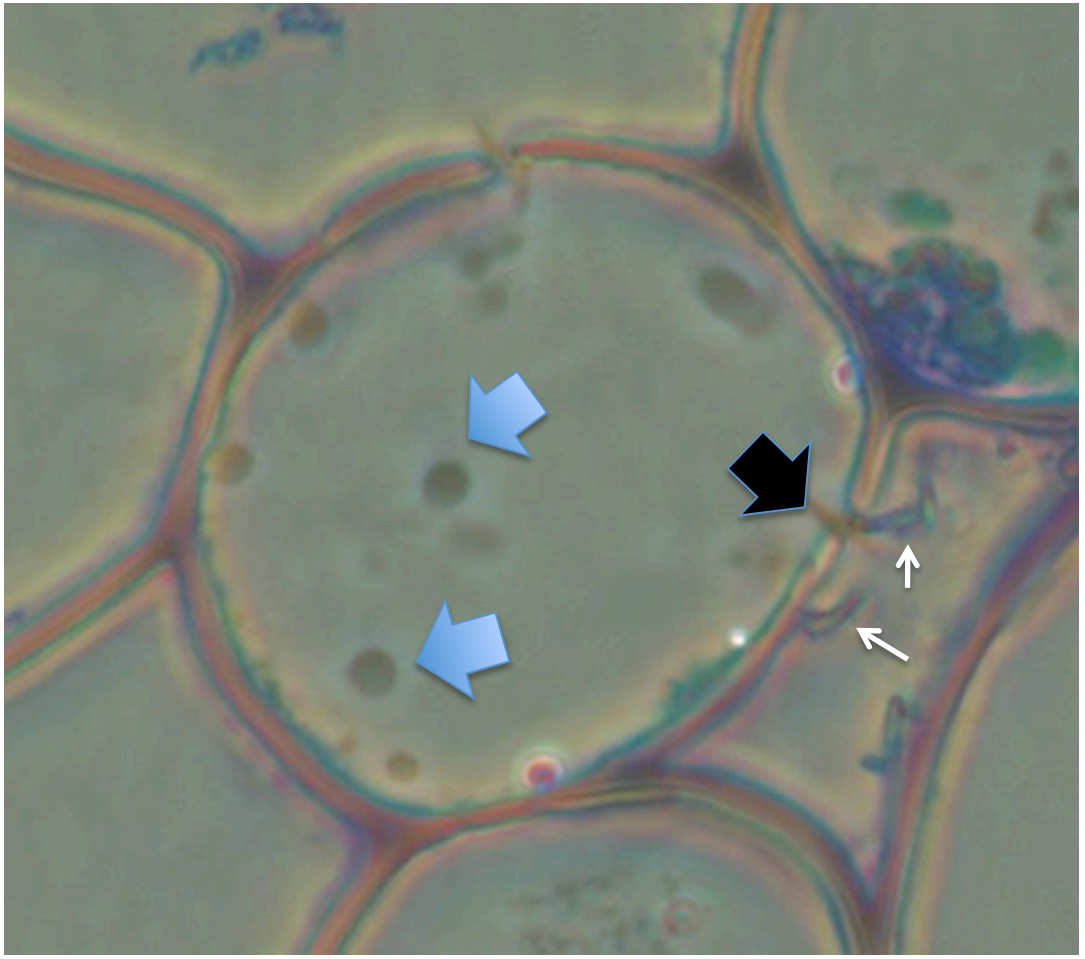


Bacillus L-forms (arrows) in mesophyll cell of red Yucca.



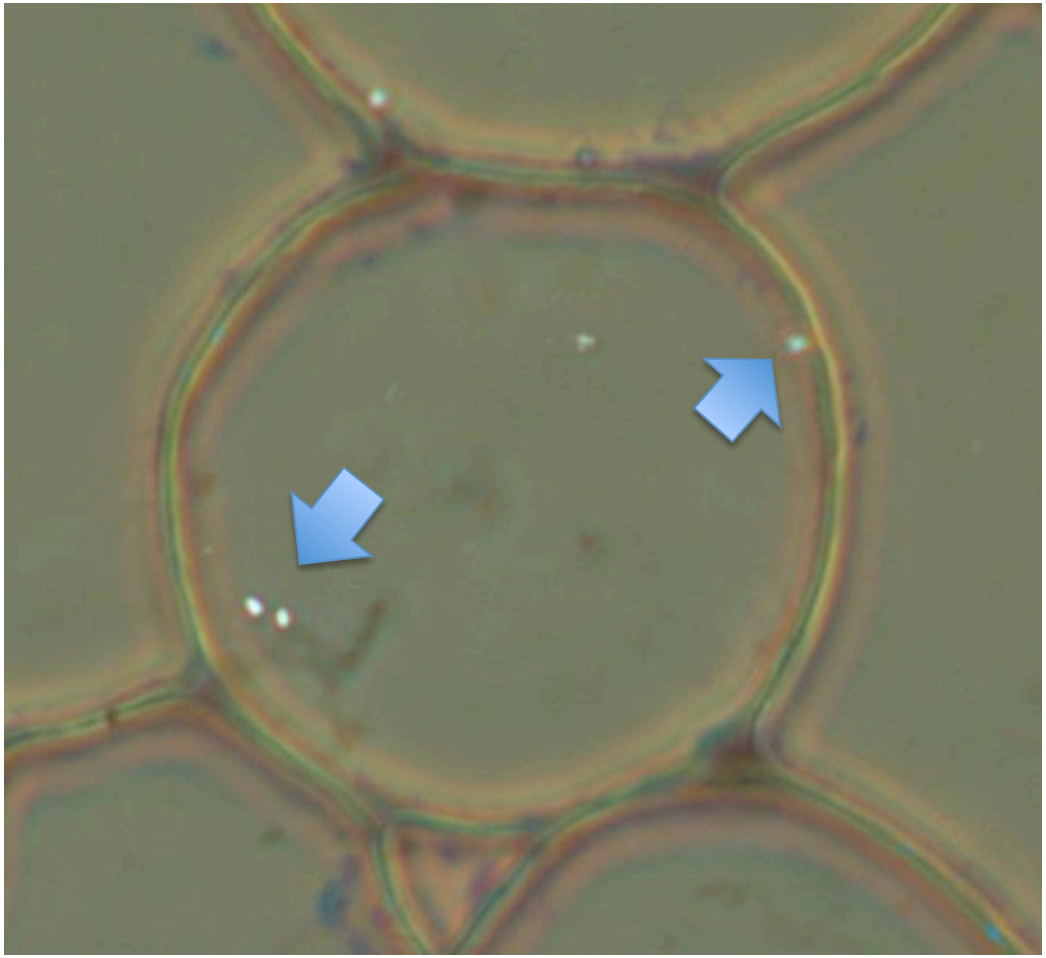
Note the irregular shapes and sizes of L-form cells. Stained with malachite green followed by toluidine blue.

Bacillus L-forms (blue arrows) in mesophyll cell of red Yucca.

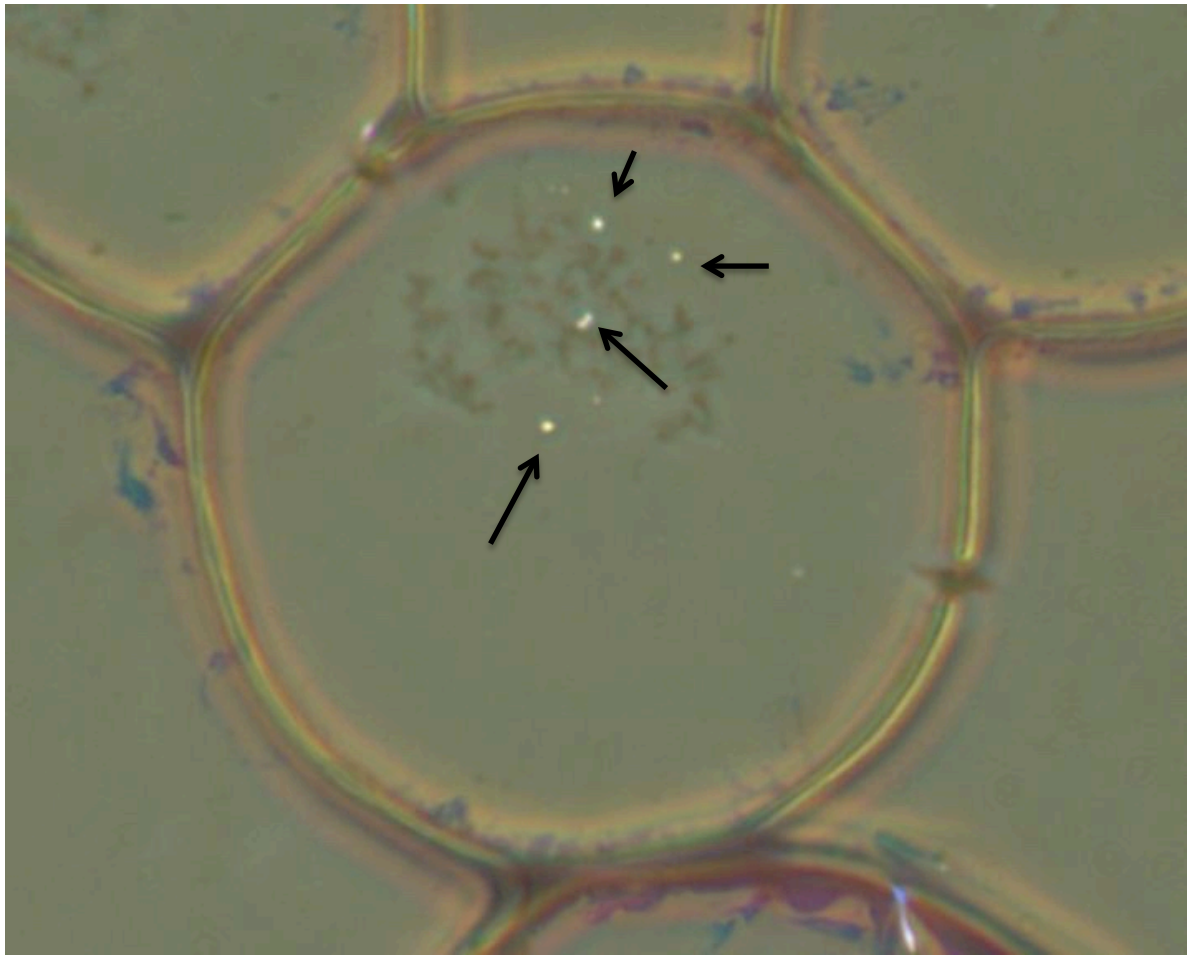


Bacteria appear to enter cells through pores (black arrow) in the plant cell walls. Bacterial rods (white arrows) are observable near the pore in intercellular space just outside cell.

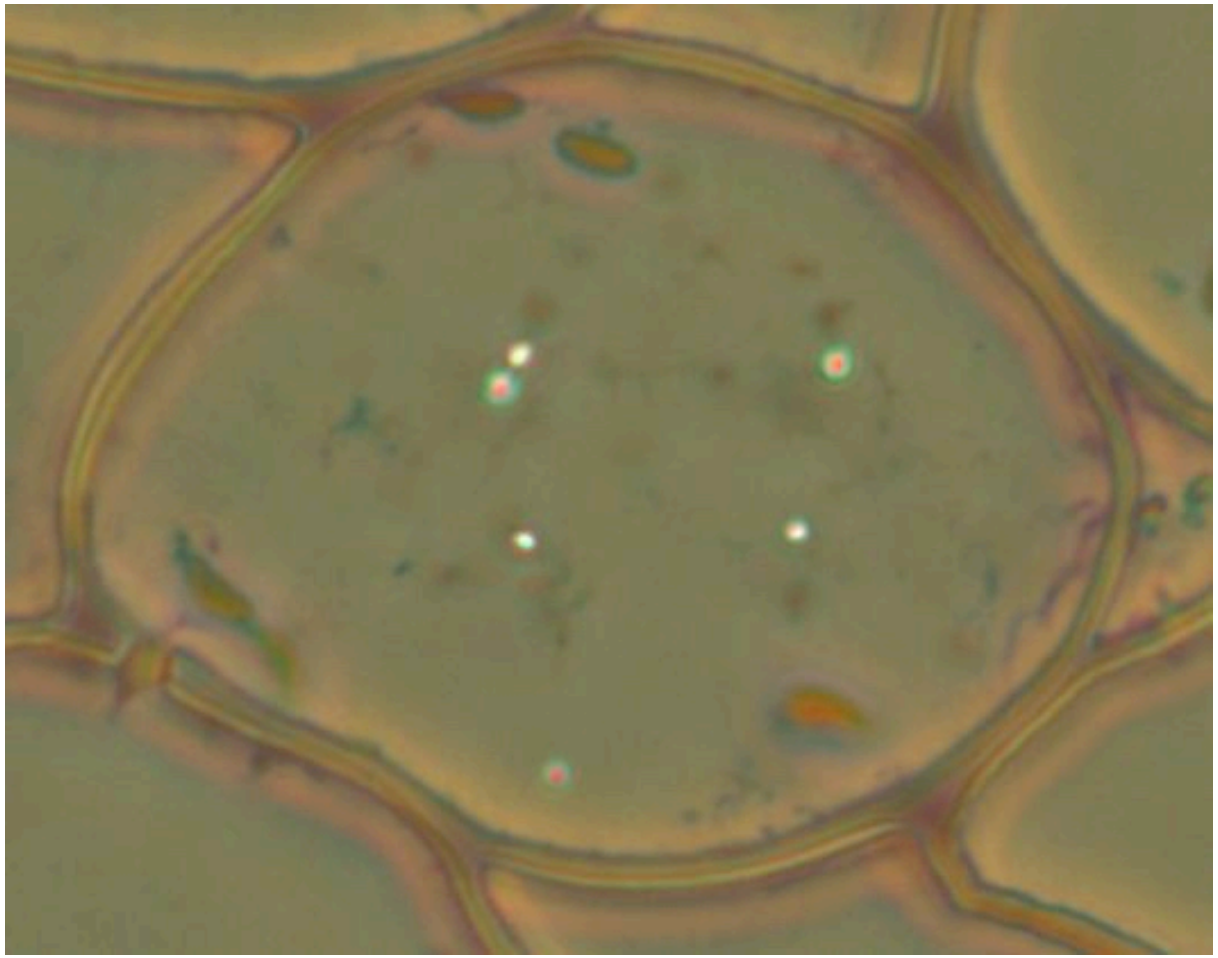
Bacterial L-forms produce refractive endospores (arrows) within mesophyll cells.



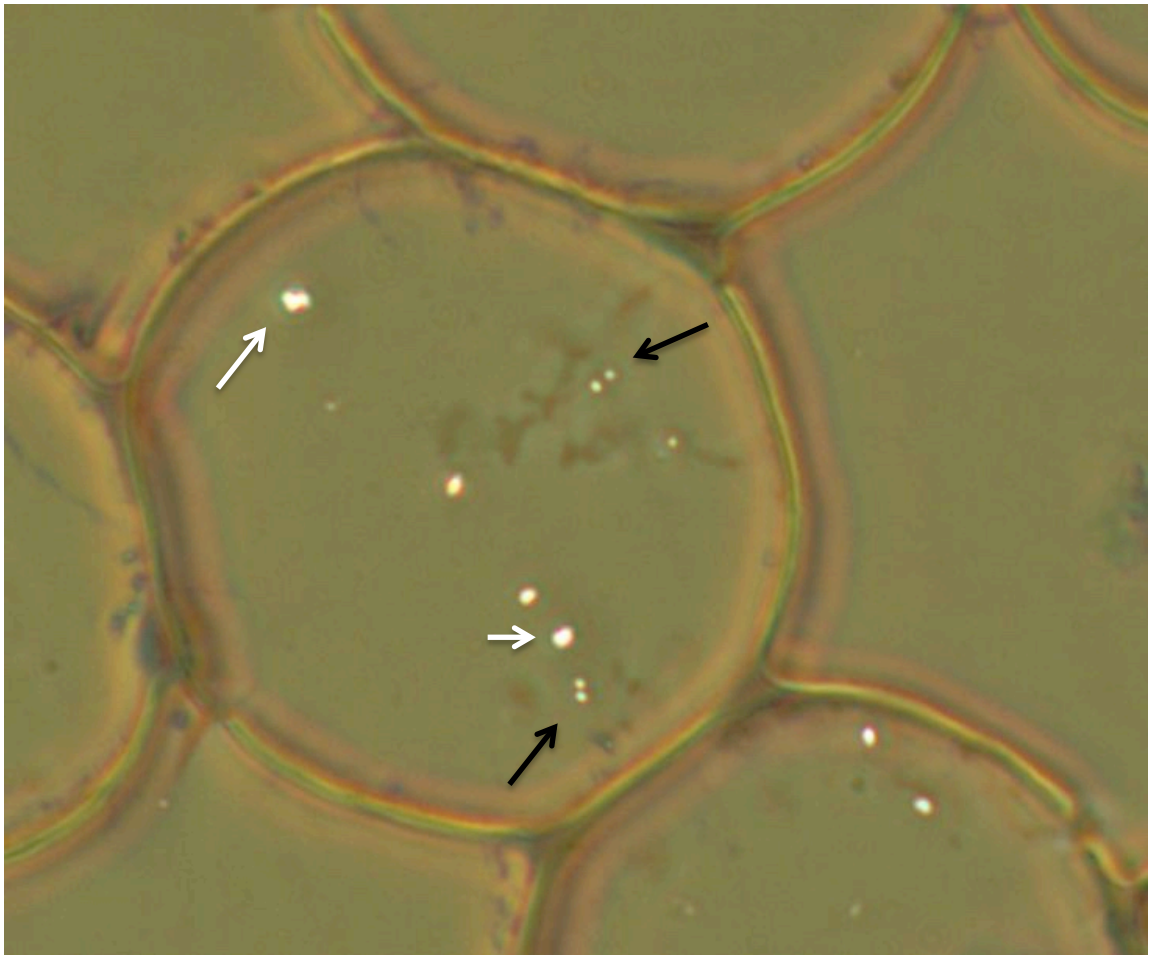
Endospores (arrows) forming in disintegrating intracellular L-forms.



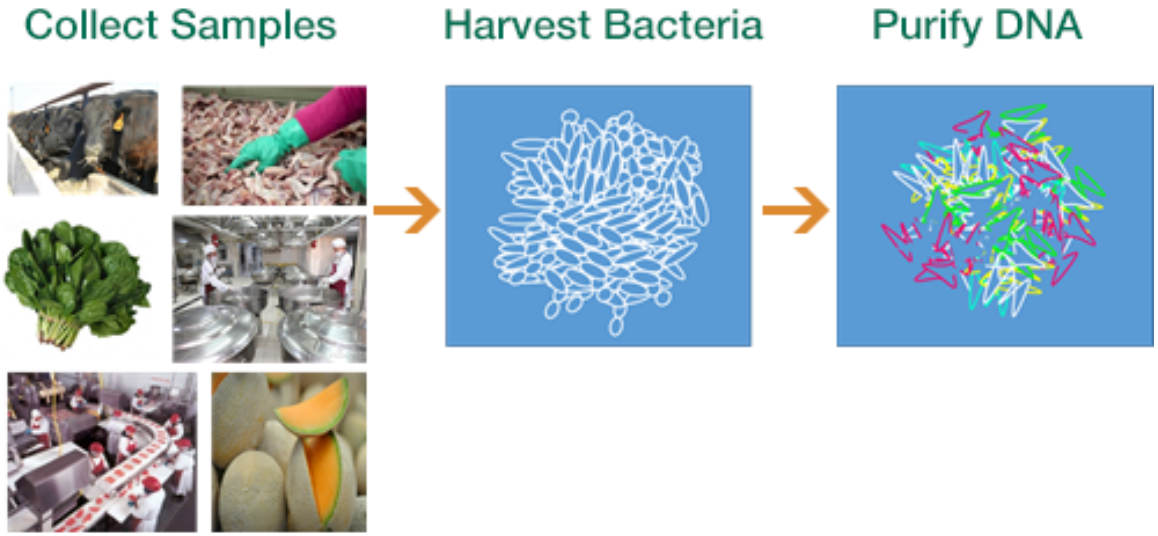
More endospores forming in mesophyll cells



Pairs of endospores (black arrows) forming in disintegrating L-forms. Endospores may also be clustered (white arrows).



Non-culture methods do not detect endospores because they are resistant to DNA extraction!



- Endospores germinate and become vegetative in meristematic tissues of the plant (like root or shoot tips, new buds, branch roots, etc…)

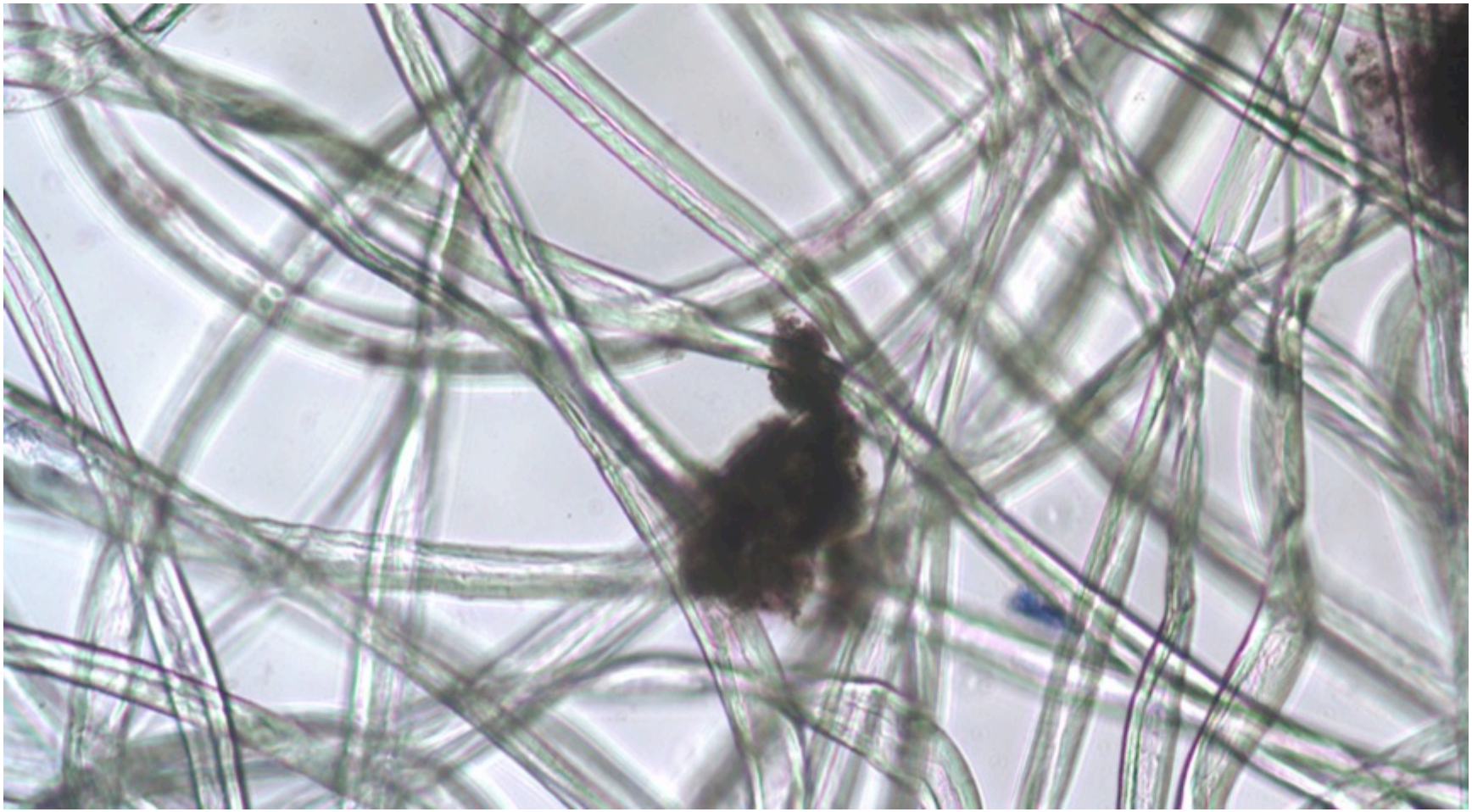
Part 2. Isolation of endophytes.

- Culture methods for fungi
- Culture methods for bacteria

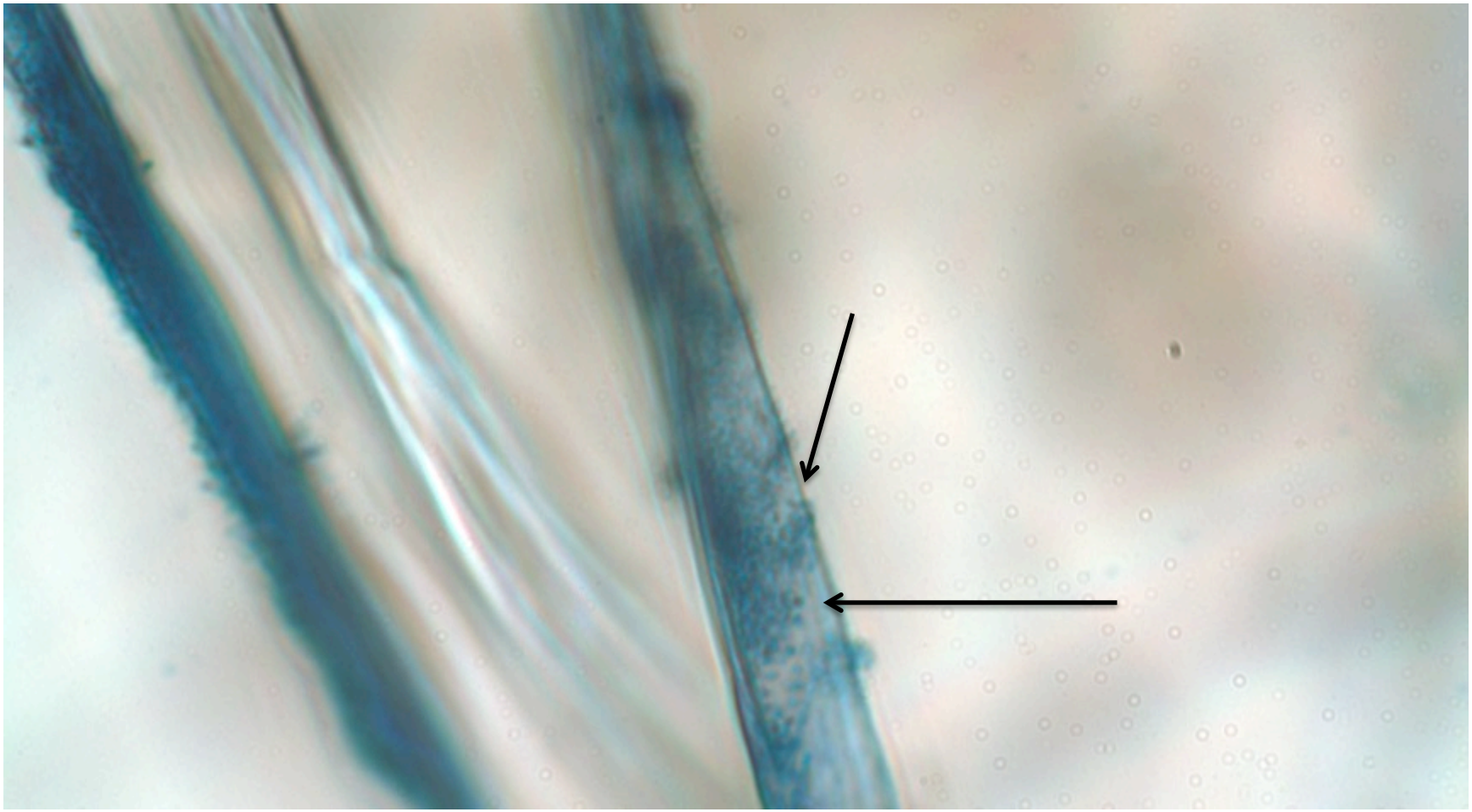
Culture methods for seeds

- Seeds may carry endophytes on surfaces that colonize the seedlings after emergence
- Example 1: and cotton (family Malvaceae)
- Example 2: sotol (family Agavaceae)

Microbes borne on cotton fibers

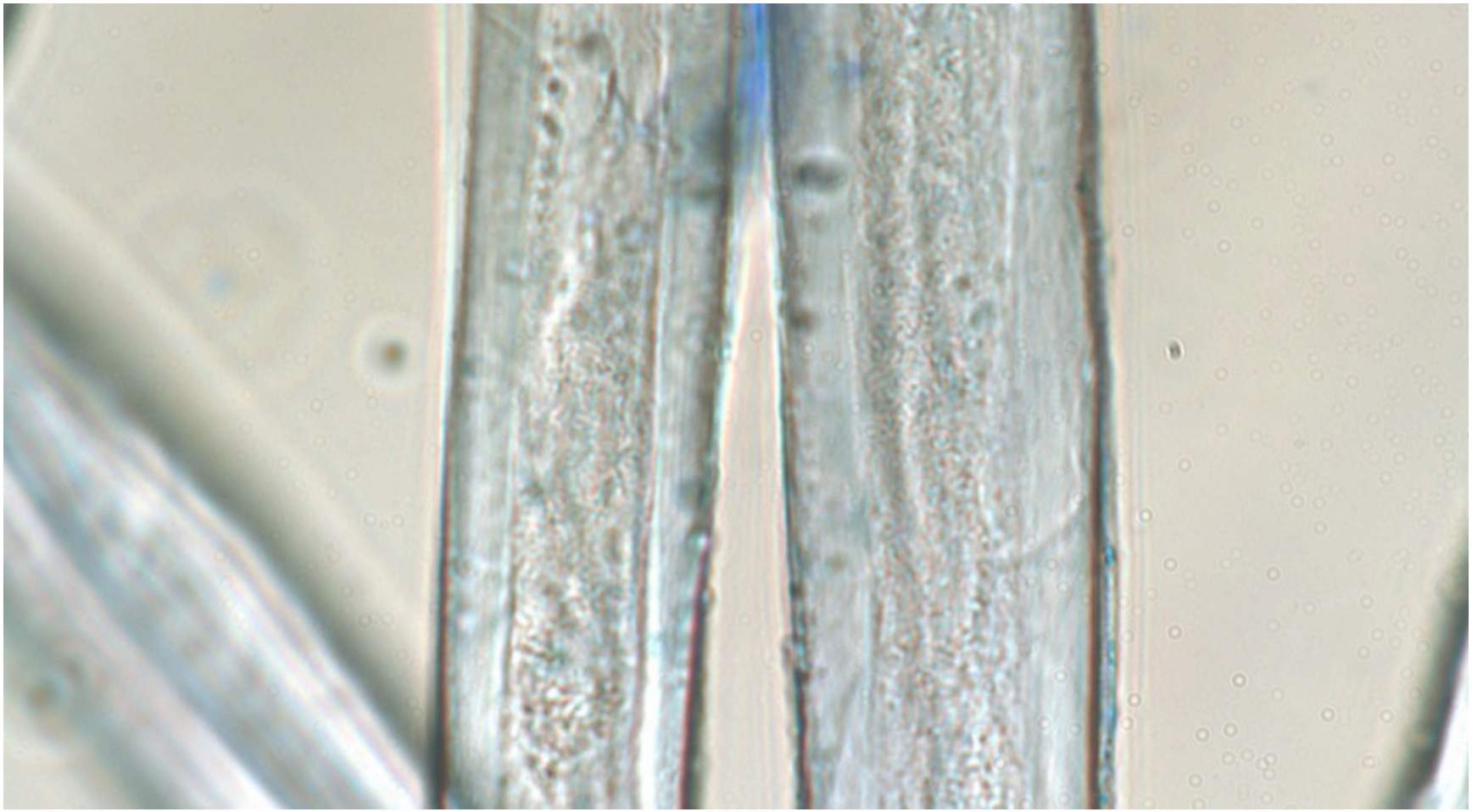


Bacteria (arrows) on cotton fibers



Stained with aniline blue.

Bacterial biofilms on cotton fibers. Note: granulose appearance in fibers.

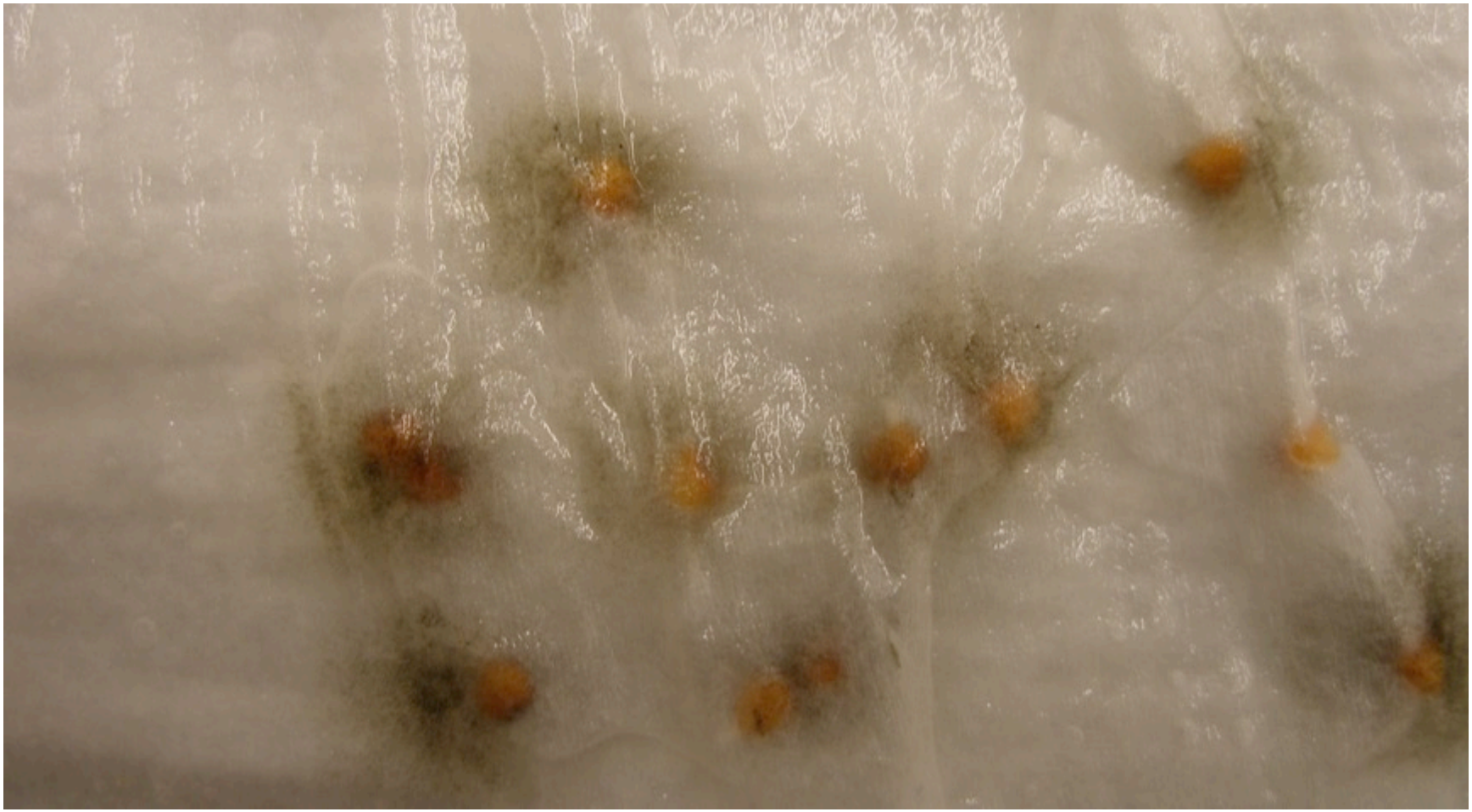


Cotton seeds are soaked in concentrate acids (sulfuric or nitric acids) to remove all the seed fibers.

- Acid treatment of seeds makes seeds smoother and improves planting of seeds using planting machines.
- Acid treatment also removes the microbes that are vectored on the hairs and the seed surface.
- Removal of seed surface bacteria leaves seedlings without protective endophytes.

Dasylirion wheeleri-sotol

Sotol seeds are covered by fungus (*Alternaria* sp.) and associated bacteria (*Klebsiella* sp.) that colonize seedlings after germination.



Fungus colonizes seedling after germination and becomes systemic in plant.

For isolation of seed surface-vectored microbes:

- Water washes from surface—then dilution plating may capture most microbes

- Minimal surface disinfection (<5 mins in 4% NaOCl) may remove many microbes and allow isolation of only the most resistant seed surface and internal microbes.

- Rigorous surface disinfection (>40 mins in 4% NaOCl) will remove most or all surface microbes—and permit isolation of only microbes vectored within seeds.

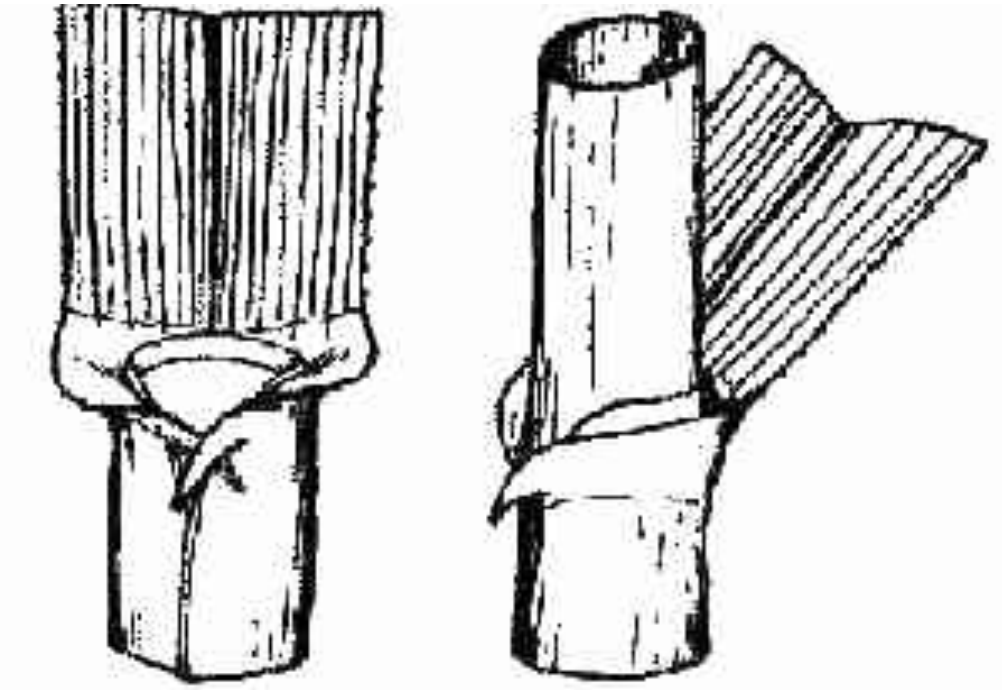
Suggested media

- For fungi use: potato dextrose agar (PDA) or malt extract agar (MEA)—add antibiotics to eliminate bacteria.

- For bacteria use: 10% tripticase soy agar (TSA) or 1% yeast extract + 1% sucrose + 1.2% agar (YESA)

- For isolation of fungi with associated bacteria—try PDA or MEA without addition of antibiotics.

Isolation of endophytes in stems, leaves or roots



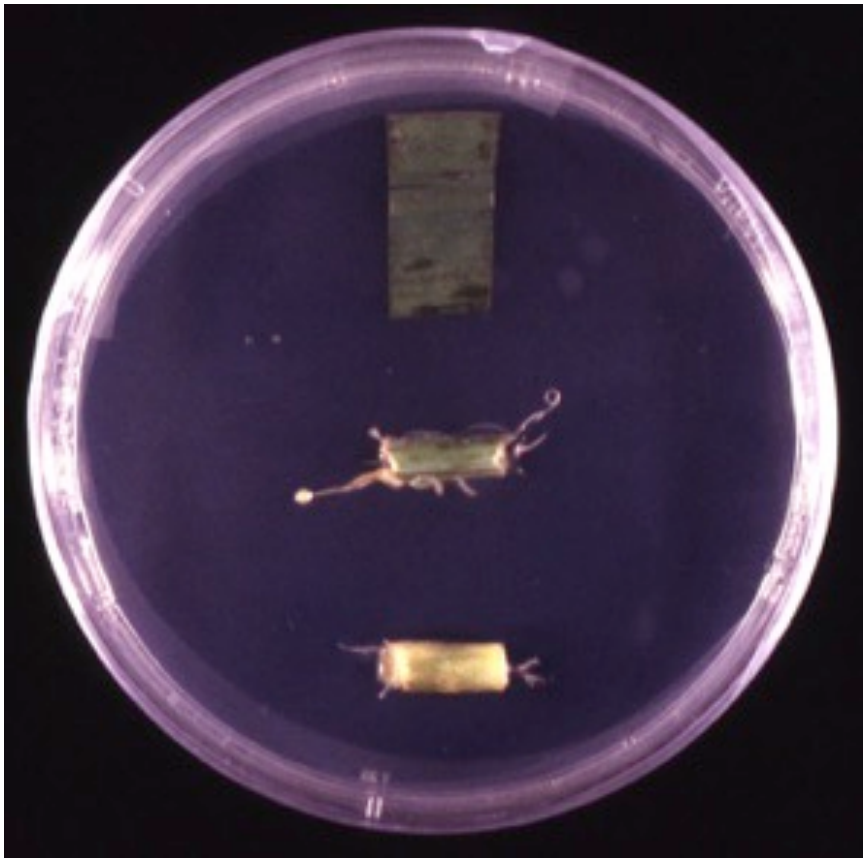
Leaves or stems (tissue pieces method):

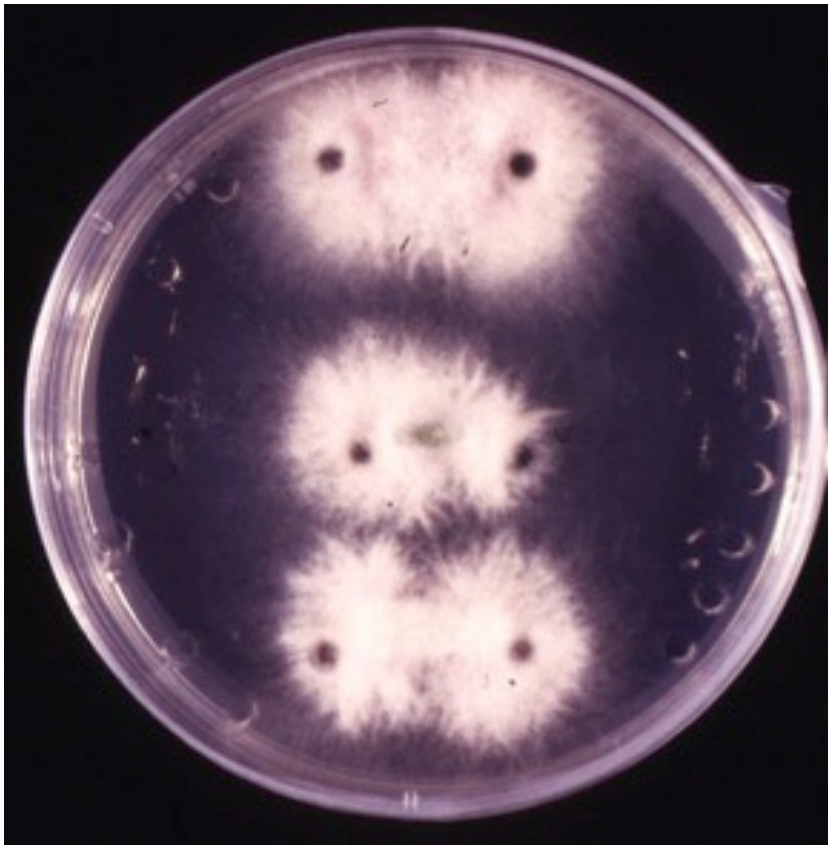
1. Cut into pieces 1-2 mm.
2. Disinfect in 4% NaOCl for 3-5 minutes.
3. Wash in sterile H20 several times until no chlorine smell remains.
4. Plate onto PDA with antibiotics for fungi or without antibiotics for fungi with bacteria.

Leaves, stems or roots (tissue trituration method):

1. Cut tissues into pieces as above.
2. Sterilize and wash as above.
3. Macerate in a sterilized mortar with pestle.
4. Mix triturate with sterile water.
5. Spread triturate solution onto agar medium surface—and pour off excess liquid.

- Recovery from surface-sterilized plant material
- Microscopically demonstrated to reside within plant tissue





For isolation of difficult to culture bacteria from seedling tissues use the **Maria Molina Method***:

- Sterilize seeds thoroughly (40+ min).
- Germinate seeds on sterile agarose (0.7% agarose without any nutrients added).
- Triturate a seedling in sterile mortar and pestle.
- Put triturate into a flask containing 100mls of 1% yeast extract and 1% sucrose.
- Agitate medium with triturate for 2-3 days at room temperature.
- Streak liquid medium onto solid YES agar, PDA or MEA to separate out bacteria.

*Some 'non-culturable' bacteria may be cultured using this method.

Why Maria Molina Method?

- Many bacterial endophytes are in a symbiotic mode of growth within plant cells.
- Grinding the tissue breaks the symbiosis with the plant.
- Putting triturate in liquid culture medium dilutes inhibitors from plant—and puts bacteria in a high nutrient, reduced oxygen, environment to favor non-symbiotic growth.

Heat-Treatment Method for isolation of *Bacillus* spp. from plant tissues:

- Cut tissues into pieces 1-3 mm in size.
- Surface disinfect for 3-5 mins in 4% NaOCl).
- Rinse in sterile water until smell of chlorine is gone.
- Put tissue pieces on moistened filter paper--then put tissues in oven at at 50-60 degrees centigrade for 8-10 hours. (Alternate method: 80C hot water for 20 mins)
- Plate tissue pieces on 10% tripticase soy agar (TSA).

Heat treatments to reactivate endospores in plant tissues

MICROSCOPY RESEARCH AND TECHNIQUE 00:00–00 (2014)

Vol. 77: 874-85. doi: 10.1002/jemt.22410

Microscopy Research and Technique: *Occurrence of* Bacillus amyloliquefaciens *as a Systemic Endophyte of Vanilla Orchids*

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KEY WORDS lipopeptides; plant disease protection; defensive mutualism; endospores

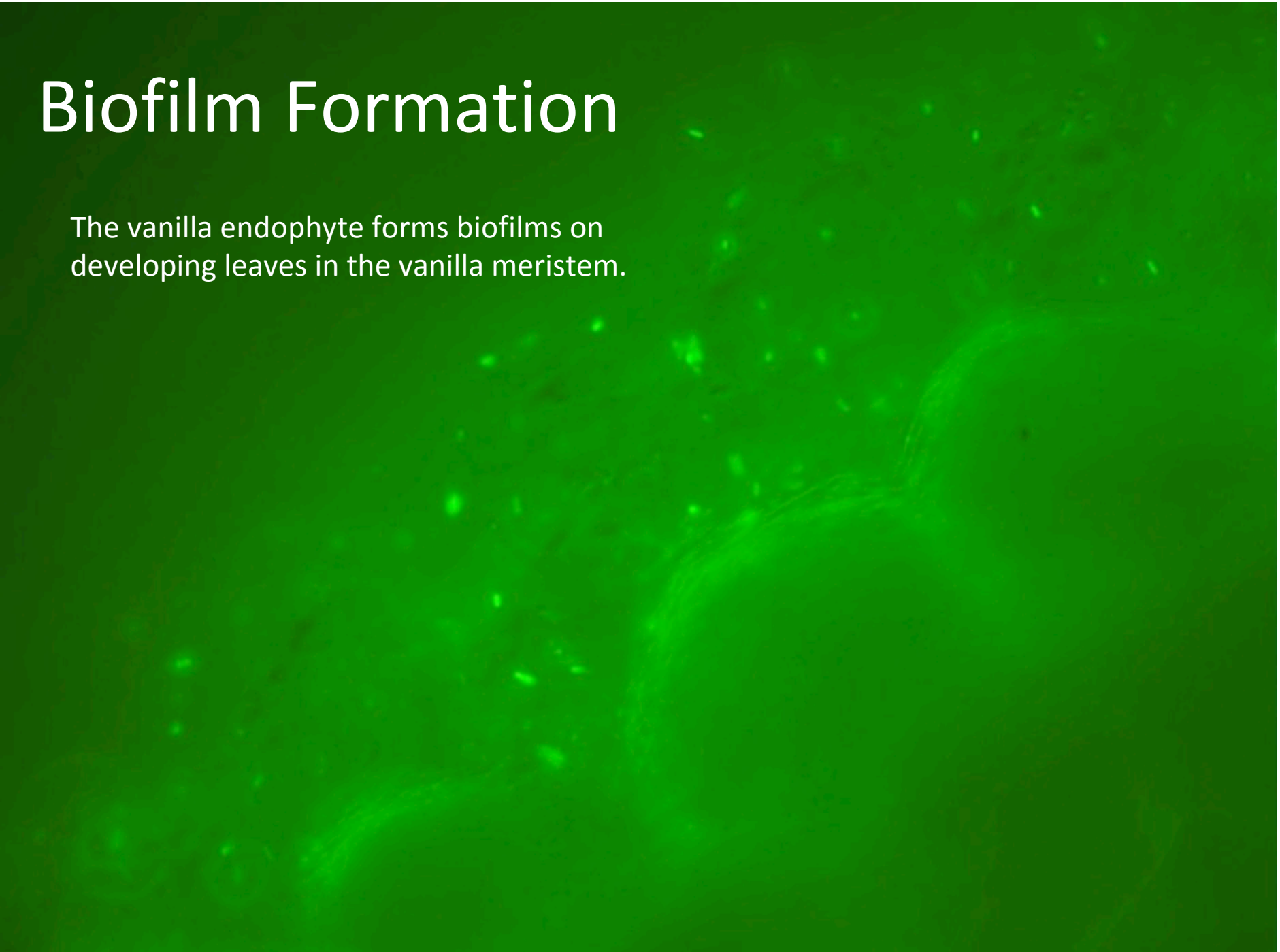
ABSTRACT We report the occurrence of *Bacillus amyloliquefaciens* in vanilla orchids (*Vanilla phaeantha*) and cultivated hybrid vanilla (*V. planifolia* × *V. pompona*) as a systemic bacterial endophyte. We determined with light microscopy and isolations that tissues of *V. phaeantha* and the cultivated hybrid were infected by a bacterial endophyte and that shoot meristems and stomatal areas of stems and leaves were densely colonized. We identified the endophyte as *B. amyloliquefaciens* using DNA sequence data. Since additional endophyte-free plants and seed of this orchid were not available, additional studies were performed on surrogate hosts *Amaranthus caudatus*, *Ipomoea tricolor*, and *I. purpurea*. Plants of *A. caudatus* inoculated with *B. amyloliquefaciens* demonstrated intracellular colonization of guard cells and other epidermal cells, confirming the pattern observed in the orchids. Isolations and histological studies suggest that the bacterium may penetrate deeply into developing plant tissues in shoot meristems, forming endospores in maturing tissues. *B. amyloliquefaciens* produced fungal inhibitors in culture. In controlled experiments using morning glory seedlings we showed that the bacterium promoted seedling growth and reduced seedling necrosis due to pathogens. We detected the gene for phosphopantetheinyl transferase (*sfp*), an enzyme in the pathway for production of antifungal lipopeptides, and purified the lipopeptide "surfactin" from cultures of the bacterium. We hypothesize that *B. amyloliquefaciens* is a robust endophyte and defensive mutualist of vanilla orchids. Whether the symbiosis between this bacterium and its hosts can be managed to protect vanilla crops from diseases is a question that should be evaluated in future research. *Microsc. Res. Tech. 00:000–000, 2014.*

Vegetative bacteria are located in shoot tip tissues of *Vanilla phaeantha*

A = Shoot tip showing liquid (arrow) emerging from tip.

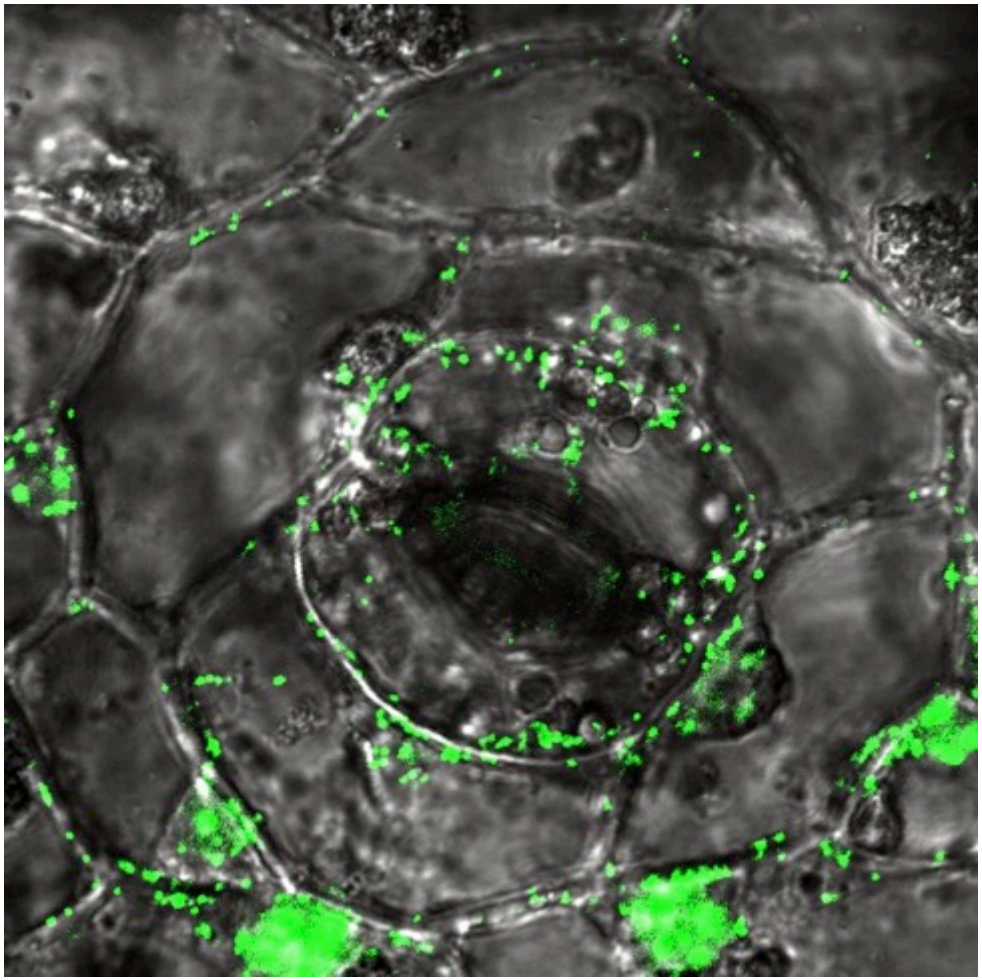
Biofilm Formation

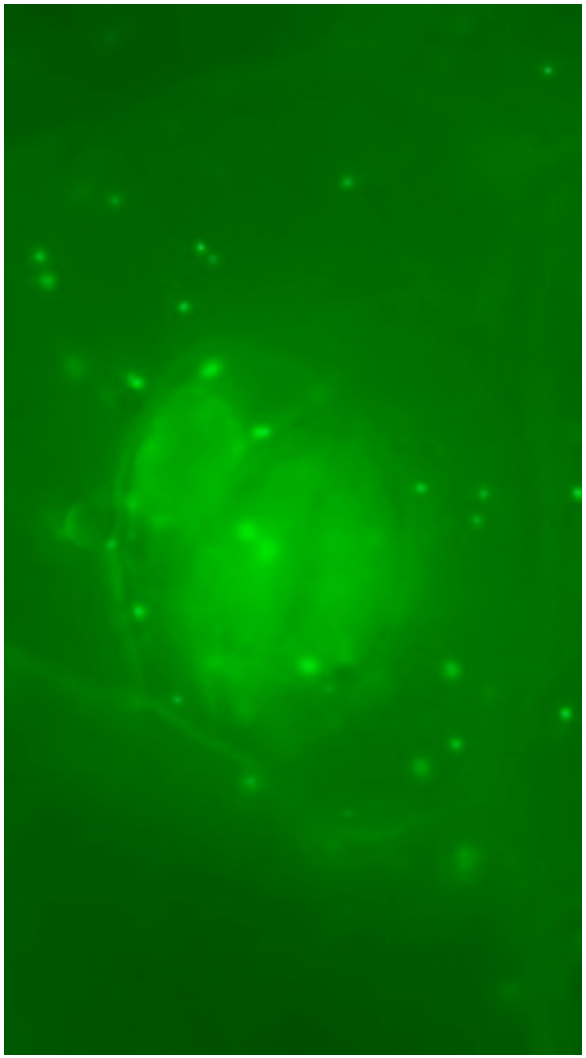
The vanilla endophyte forms biofilms on developing leaves in the vanilla meristem.



The bacterium also internally colonizes epidermal cells and tissues of vanilla leaves (left) and surrogate host (*Ipomoea* sp.; right) seedling leaves.

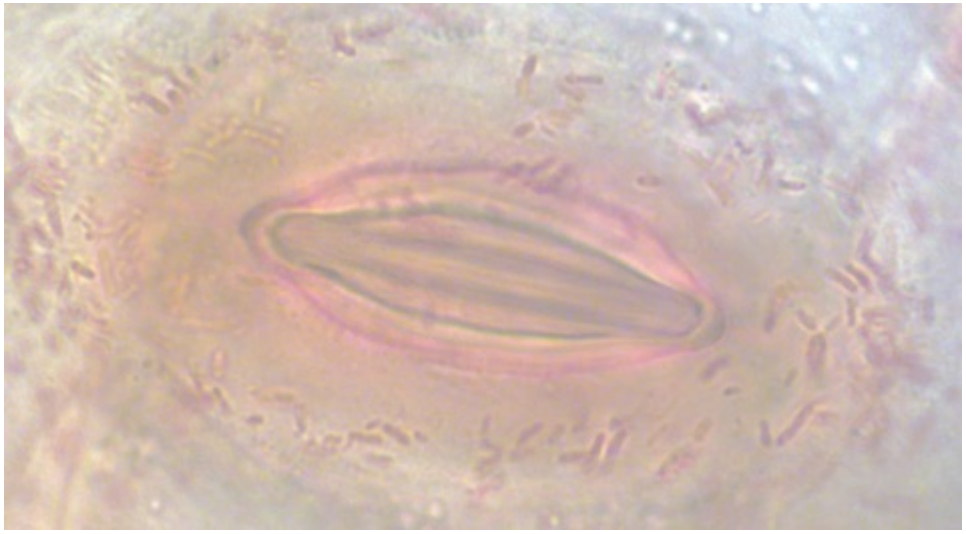
Confocal microscope image showing *Bacillus* (green) Colonizing epidermal cells of vanilla leaves around meristem (Stained with Syto-13 nuclear stain)





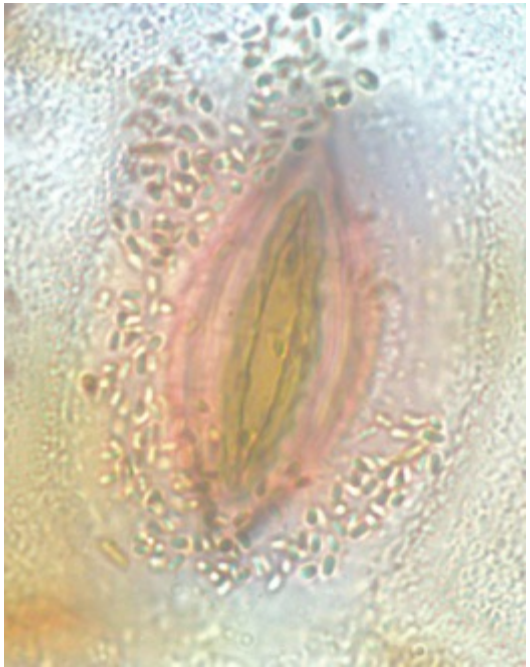
Florescence image showing Bacillus internally colonizing epidermal cells of *Ipomoea* seedlings (stained with Syto-13 nuclear stain).

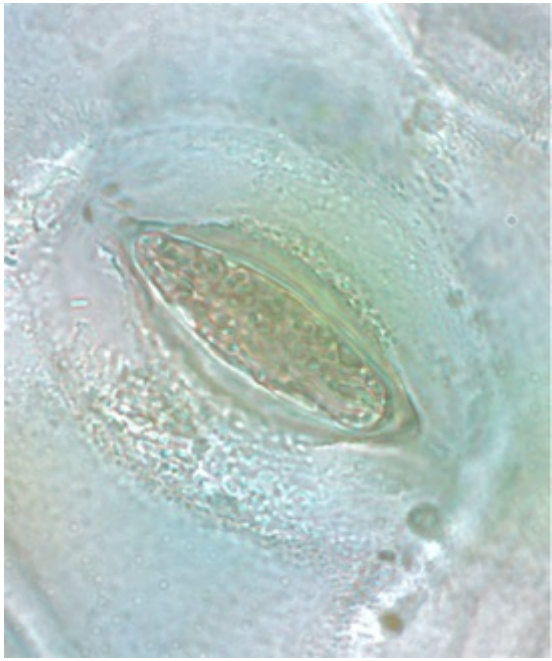
Why heat treatment? As plant tissues mature Bacillus endophytes produce endospores that become embedded in plant tissues.



Below: vanilla plant stomata with endospores embedded in cuticle around Stomata and within stomal cavity.

Above: vanilla plant stomate with vegetative bacterial cells (rods) around it.

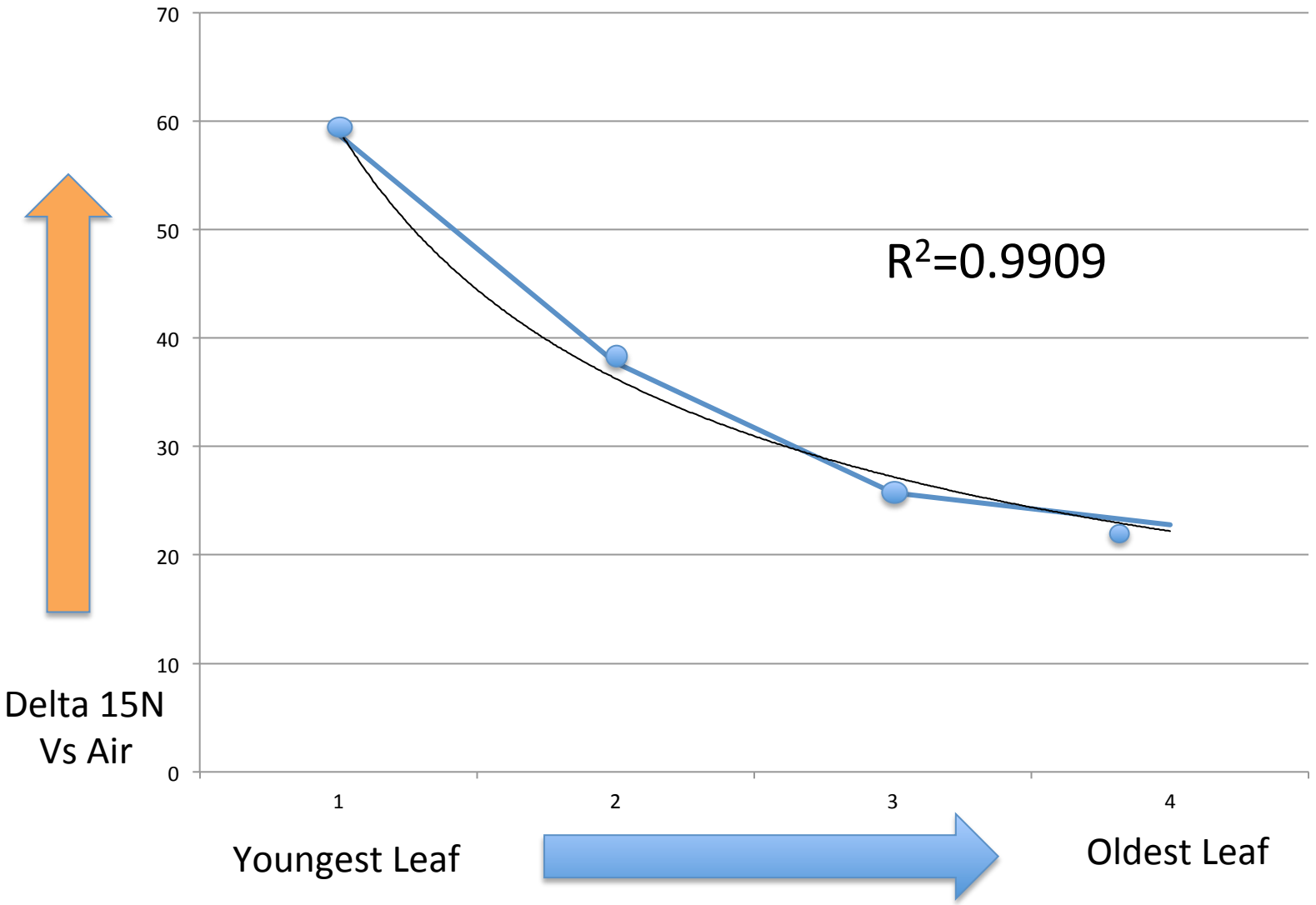




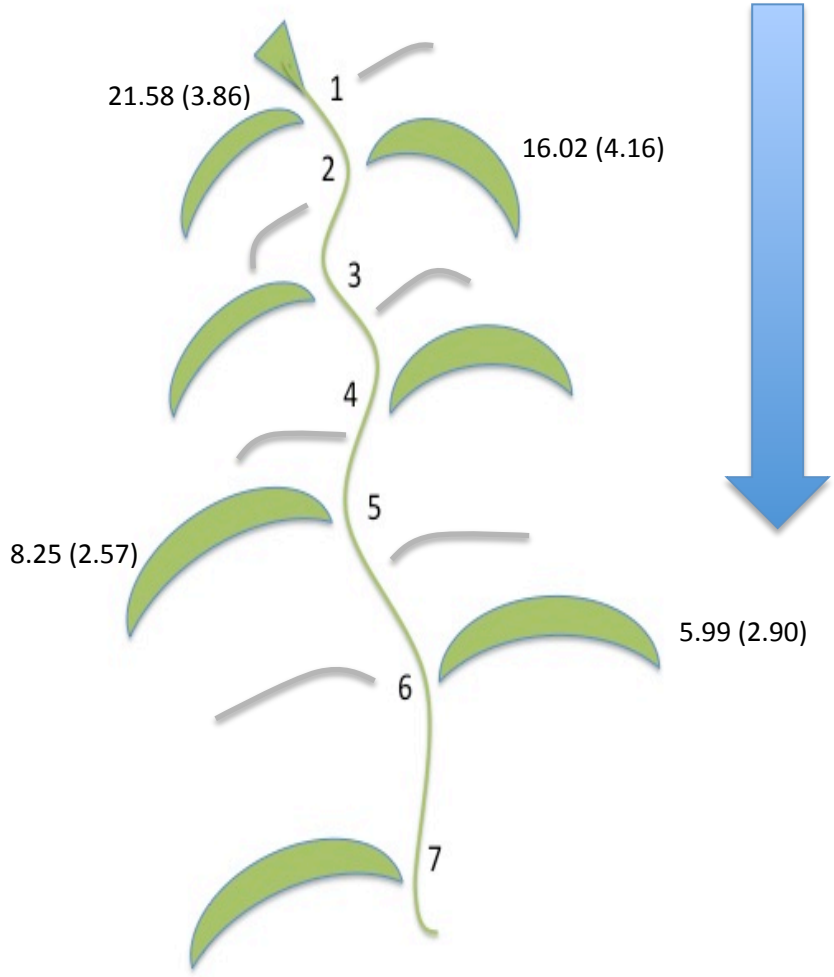
Vanilla Orchid $15N_2$ Experiment

33 mls $15N_2$ injected into 5 liter chamber over 7 day period.

Nitrogen assimilation data on the youngest 4 leaves of vanilla orchids in a 15N2 assimilation experiment showing highest assimilation in the youngest leaves (n=8).



Nitrogen Assimilation Gradient



Vegetative cells abundant (High N gas assimilation)

Endospores abundant (Reduced N gas assimilation)

Mass Spec analysis of leaves shows that 15N is incorporated into young growing tissues where bacteria are in the vegetative phase of growth. In older plant tissues vegetative cells produce endospores and N-assimilation stops.

Suggested heat treatment options to break endospore dormancy (from survey of scientists):

- 65C for 45 mins
- Boil for 30 mins
- 50C for 30-50 mins
- 80C for 20 mins
- Autoclave tissues

The heat treatment breaks dormancy of endospores and kills competitor bacteria in the plant tissues.

Bacillus amyloliquefaciens an endophyte widespread in many plant species.

Part 3:

A. Proof of endophytism

B. Evaluation of the functions of endophytes (experimentation)

Proof of endophytism

Apply Modified Koch's Postulates:

1. Visualize endophyte in healthy plant (if possible).
2. Remove endophyte from plant by disinfection or other treatments.
3. Visually or by isolation prove that endophyte in no longer present in plant.
4. Re-infect plant with endophyte
5. Visualize endophyte in healthy plant after re-inoculation.

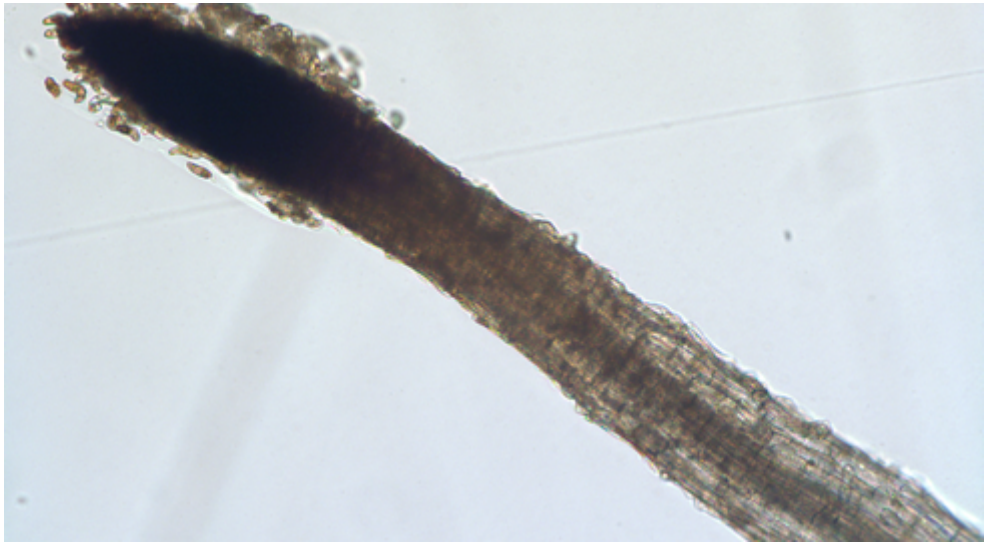
Example: *Cynodon dactylon* (Bermuda grass)

- Bacterial endophyte (*Pantoea agglomerans*) carried on seed coverings (hulls).
- Bacteria colonize the seedling and become intracellular when seed germinates.
- Removal of hull and rigorous disinfection (40 mins in 4% NaOCl) of seed permits removal of bacteria from seedlings.
- Inoculation of axenic seeds with endophytes and its visualization in seedling roots is proof of endophytism.

Roots of Bermuda grass seedling without Endophytic bacteria.

No root hairs and no
internal reactive oxygen staining.

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

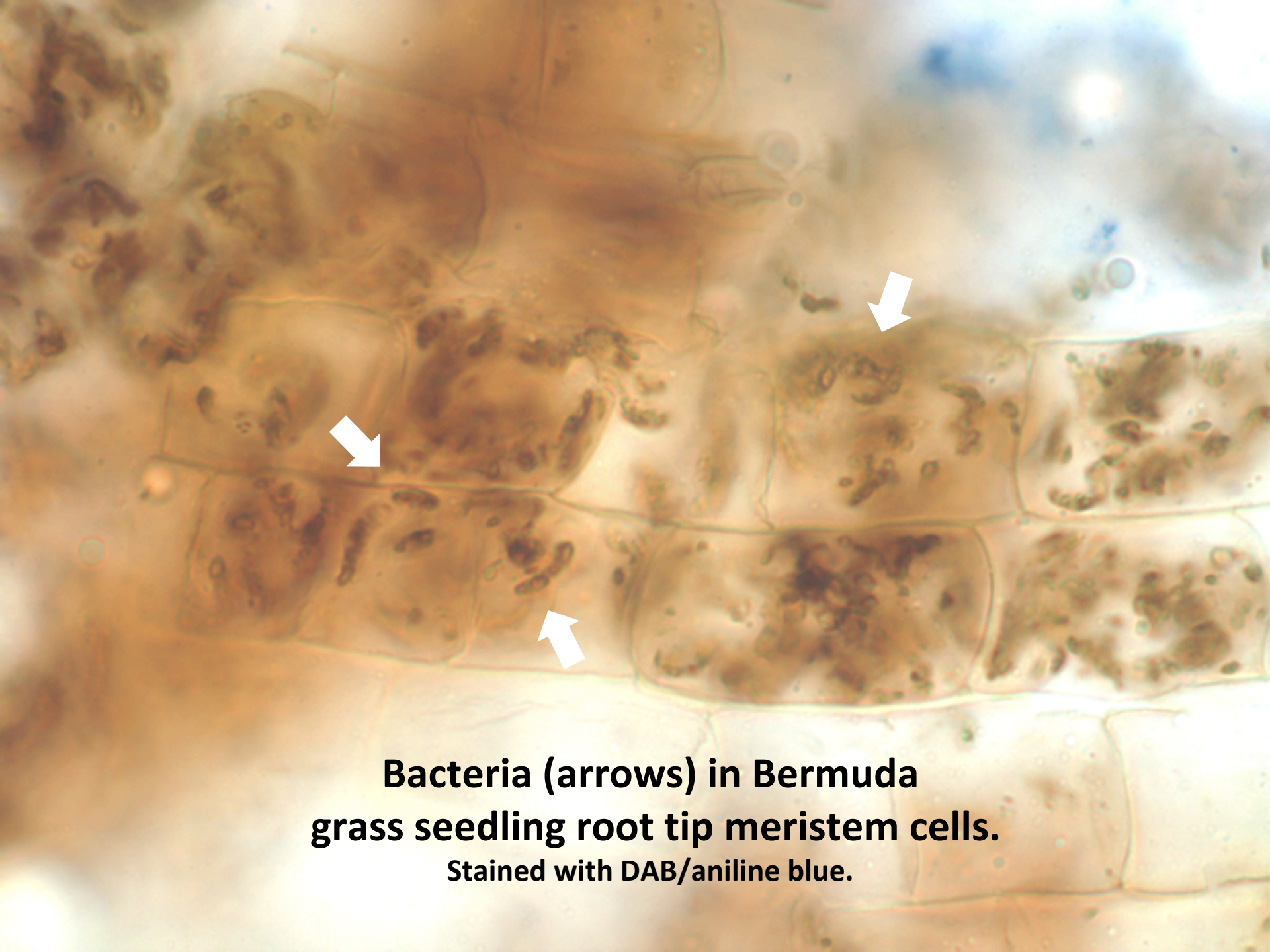


Root tip

More developed region of seedling root

Bermuda grass root containing bacterial endophyte

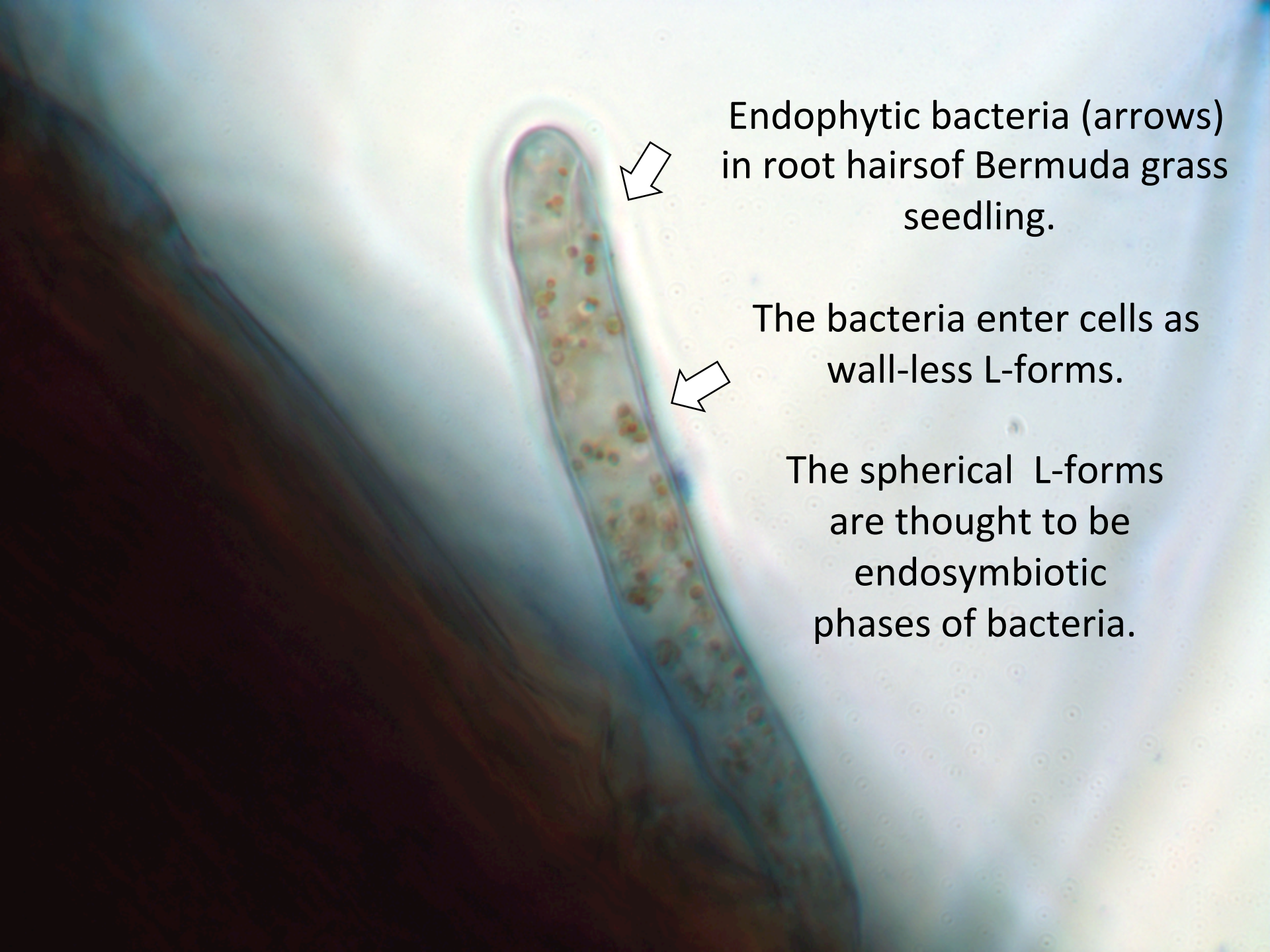
Note presence of root hairs.

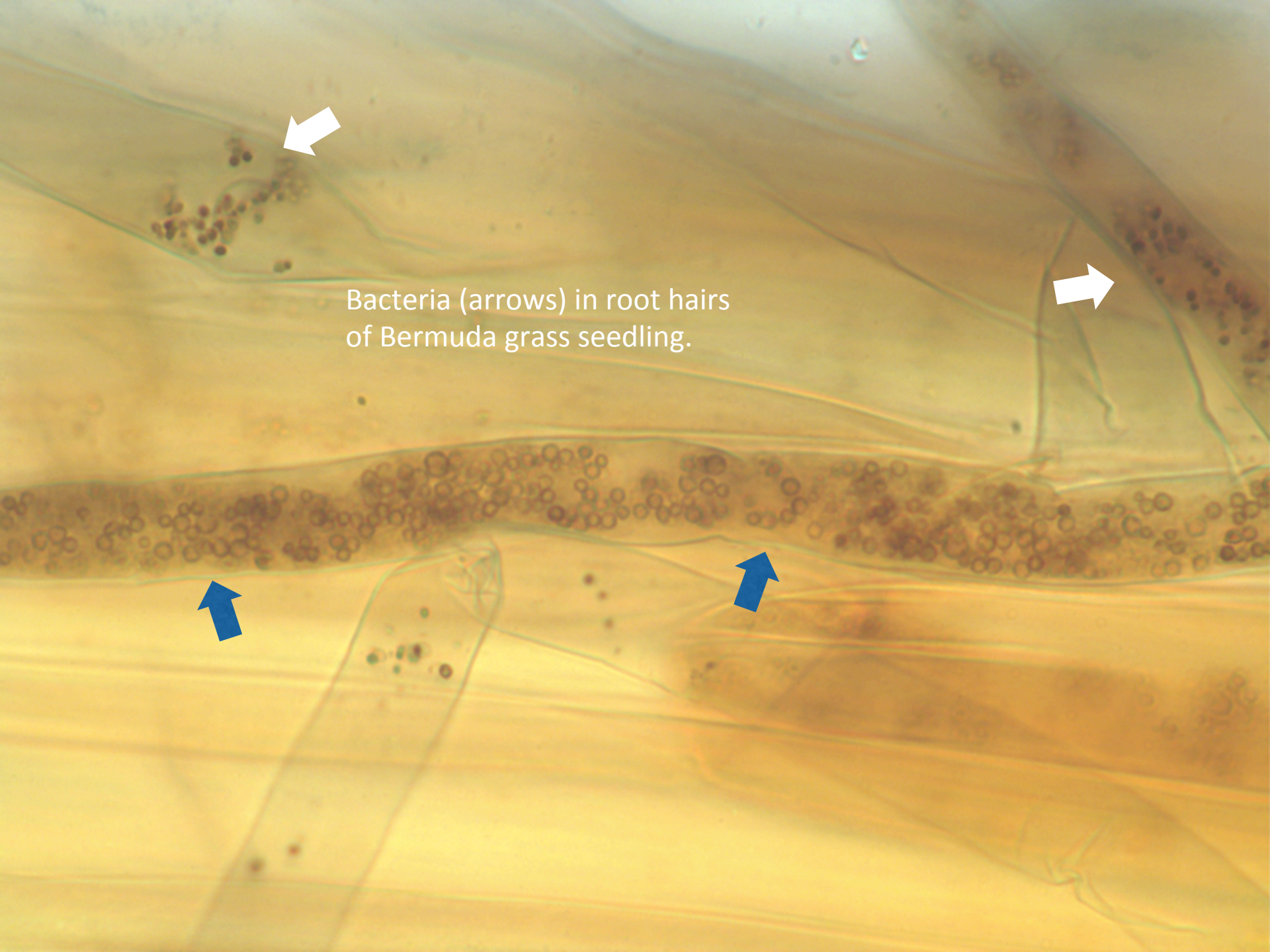


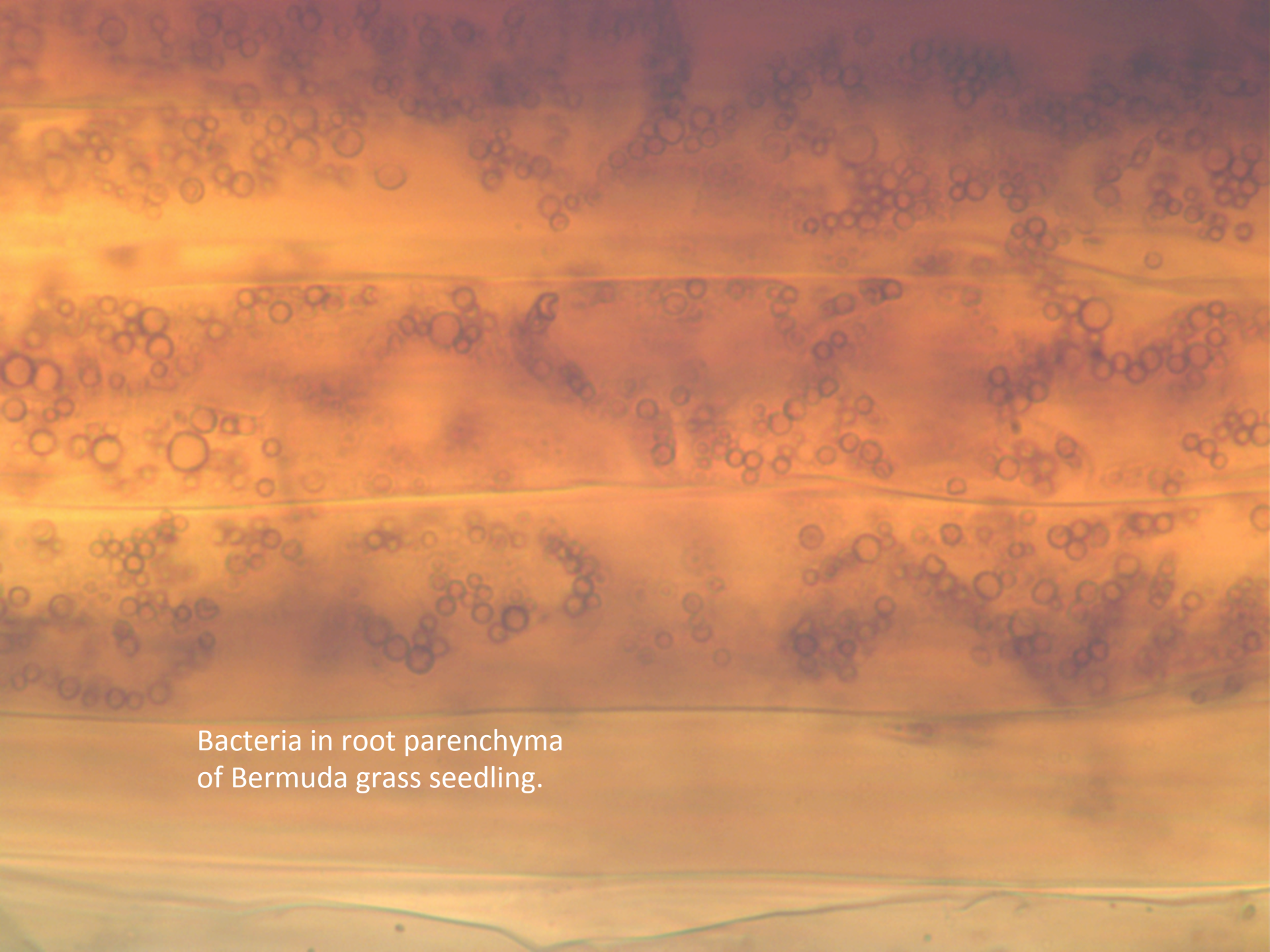
Bacteria (arrows) in Bermuda grass seedling root tip meristem cells.

Stained with DAB/aniline blue.


Bermuda grass seedling root containing endophyte.
All brown spots in roots are Intracellular bacteria.


Endophytic bacteria (arrows) in root hairsof Bermuda grass seedling.
The bacteria enter cells as wall-less L-forms.
The spherical L-forms are thought to be endosymbiotic phases of bacteria.

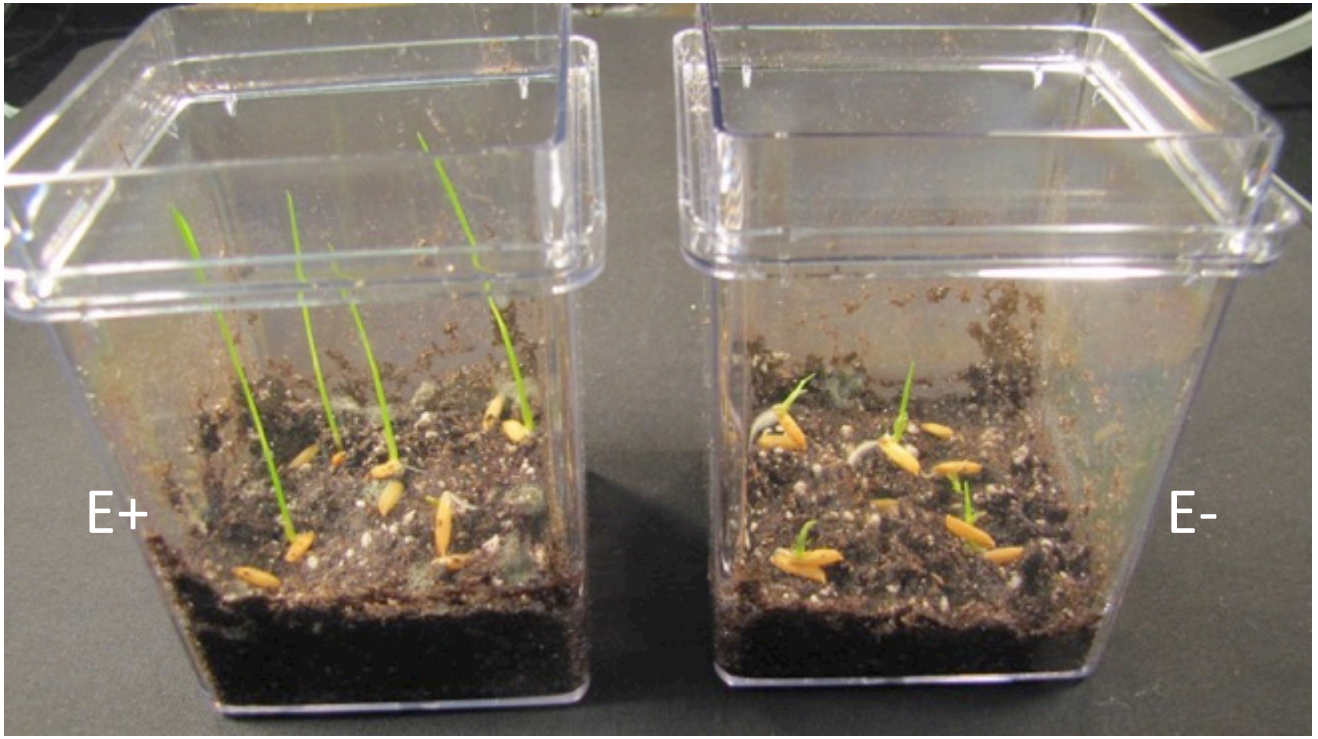

Bacteria (arrows) in root hairs
of Bermuda grass seedling.



Bacteria in root parenchyma
of Bermuda grass seedling.

Surrogate Host Test for Endophytism

If endophytes cannot be removed for the host (or the host cannot be cultivated)—plants that can be cleaned of endophytes can be used to test for endophytism. Below rice was used to test bacteria isolated from reed grass (that was difficult to cultivate in the laboratory).



Methods to clean seeds of endophytes:

- Rigorous disinfection (30-40 mins) with 4% NaOCl.
- Soaking for 24 hours in antibiotic (100-300 mg/L streptomycin sulfate (rinse thoroughly afterward))
- If endophyte is fungal—fungicides may be used to clean seeds.
- If endophytes vector within seeds it may be very difficult to remove the endophytes.

Evaluating functions of endophytes

- Modulation of plant development
- Alter gene expression in plants
- Increase tolerance to abiotic stresses
- Improve tolerance to biotic stresses
- Improve nutrient acquisition in plants
- Adapt plants to specific environments

Modulation of plant development:

Some endophytes:

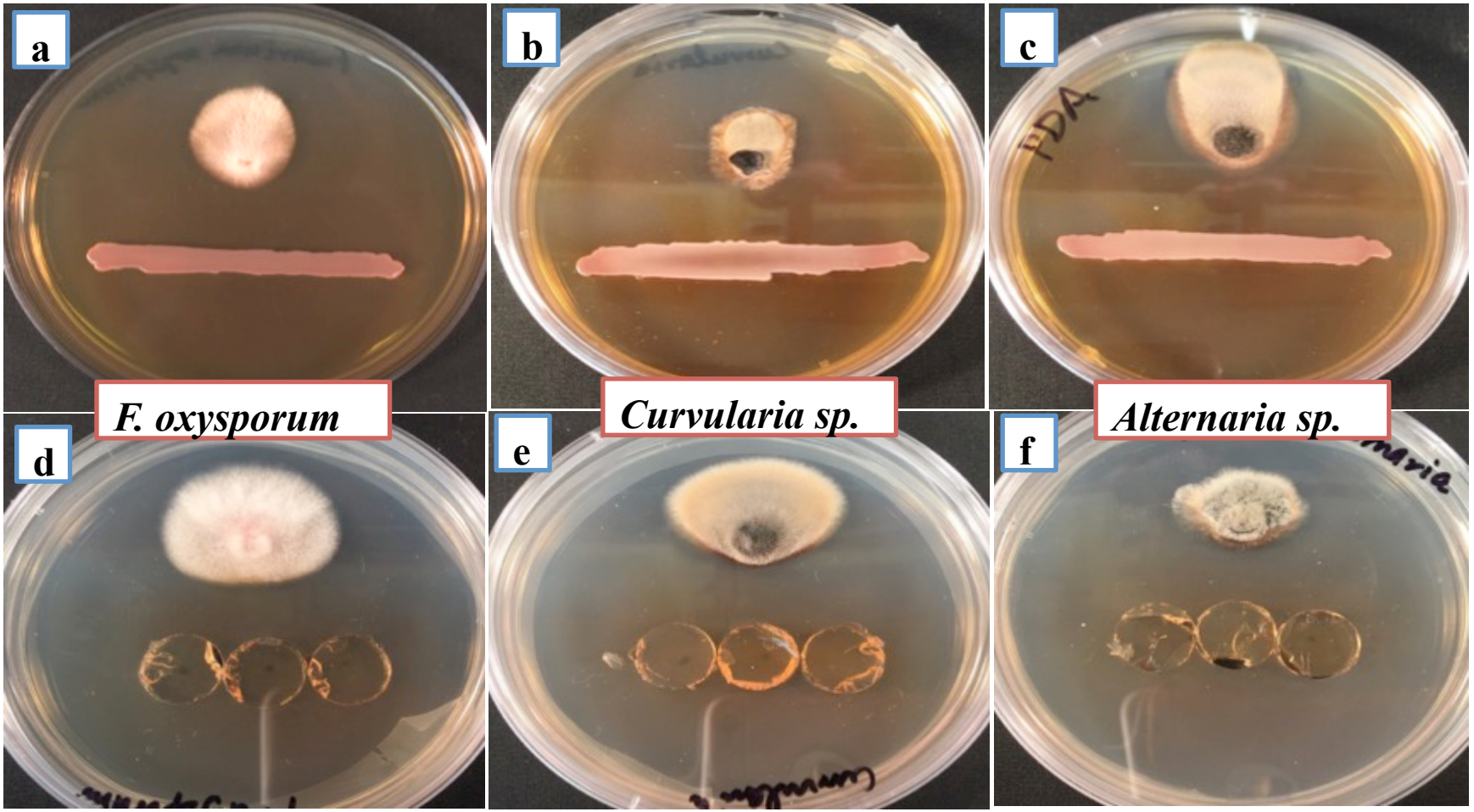
1. Trigger gravitropic response in seedling roots (triggering roots to grow downward).
2. Stimulate root hair development.
3. Increase the growth of roots and shoots in seedlings.
4. Increase branching in roots.

Defensive functions:

Experiments for testing defensive functions:

1. Examine plants with and without endophytes.
2. Expose plants to pathogens.
3. Assess disease in Endophyte-inoculated and endophyte-free plants over time.

Co-Culture Experiments to Pre-screen Microbes



Endophytes for plant defense: English ivy experiment

English ivy plants harbor a bacterial endophyte (*Bacillus amyloliquefaciens*) in all populations we have examined.

Kurt Kowalski

Endophyte containing

Endophyte free

E+ and E- plants treated with the pathogen *Alternaria alternata*. The endophyte free plants showed significant spotting and necrosis caused by the pathogen *Alternaria alternata*. Endophyte infected plants were free of disease symptoms.

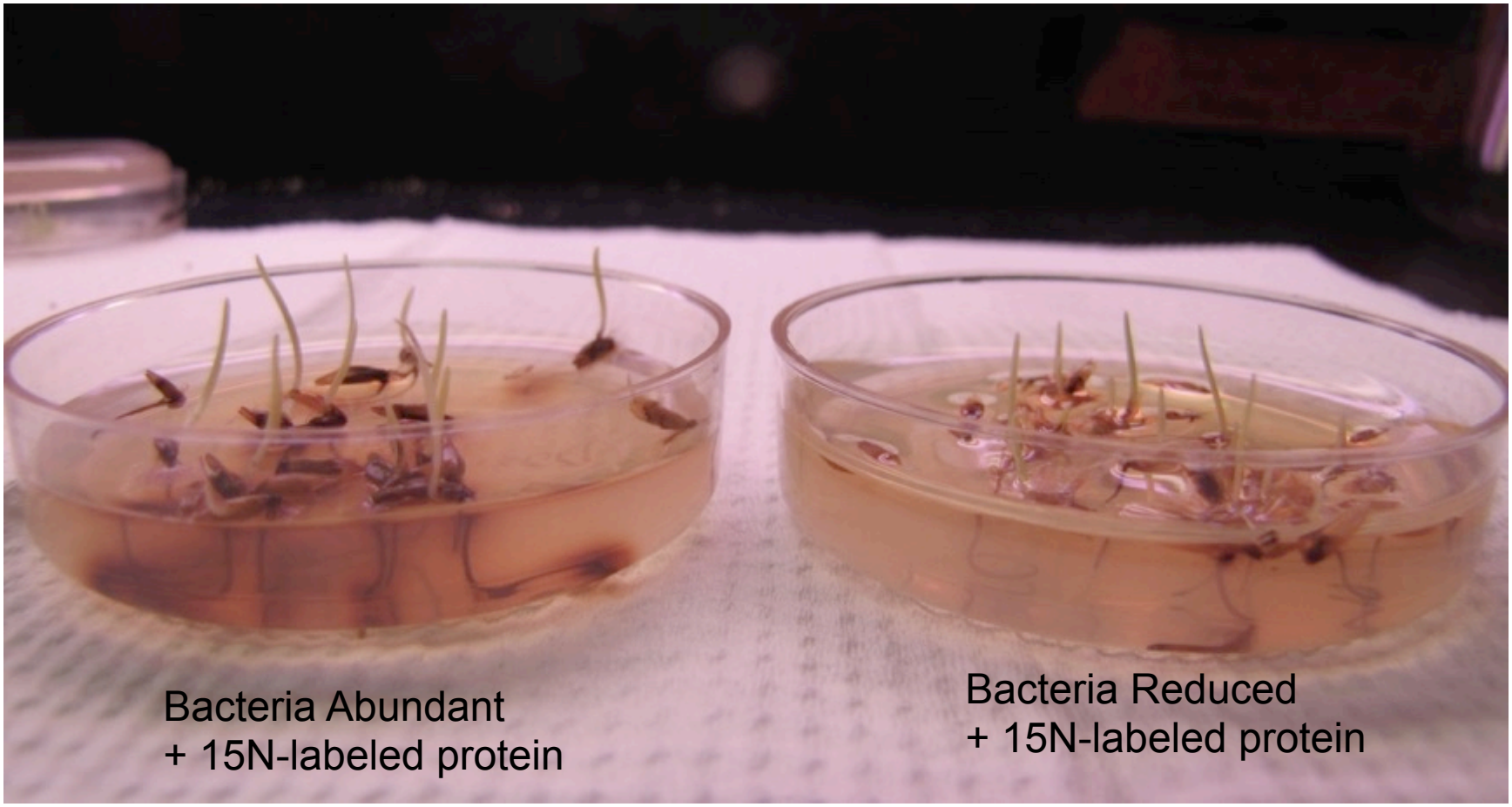
Nutrient Absorption Functions:

1. Conduct isotope tracking experiments
2. Evaluate increased growth of endophytes
3. Measure concentrations of nutrients in plants with and without endophytes.

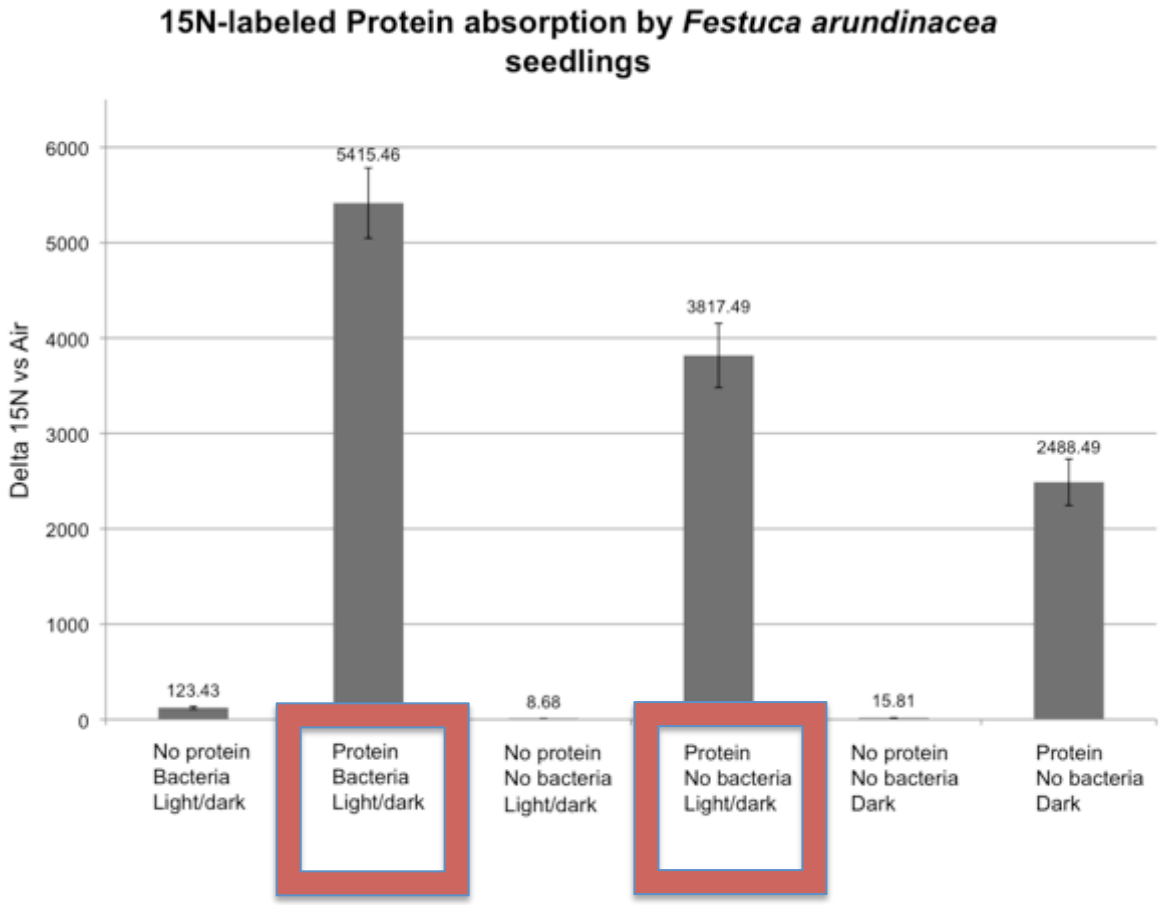
15N-labeled protein absorption experiment:

- *Bacillus amyloliquefaciens* grown in 15N-labeled glycine medium
- Total proteins extract from bacterial cells and freeze dried
- Proteins mixed with egg albumin at ration of 1:5
- Proteins (0.05%) incorporated into 0.7% agarose
- Tall fescue seeds with and without bacteria were germinated on the labeled protein media
- Seedling shoots analyzed for incorporation of 15N

15N tracking experiment: 15-N labeled protein incorporated into agar.



15N-labeled protein absorption experiment: seed disseminated microbes increase labeled protein acquisition by seedlings



The world of endophyte research:

What are people doing?

Some Fungal Endophyte Investigators

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Ecology

Rusty Rodriguez
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Endophyte taxonomy
& chemistry

Gary Strobel
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Endophytic chemistry/
Medicines

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Bacterial endophytes

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Intracellular bacteria

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James Hutton Institute, UK
Bacterial endophytes

Ann Hirsch
UCLA
Root endophytes

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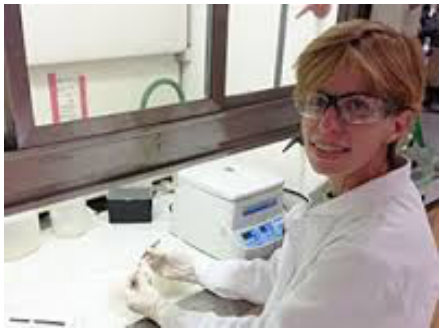

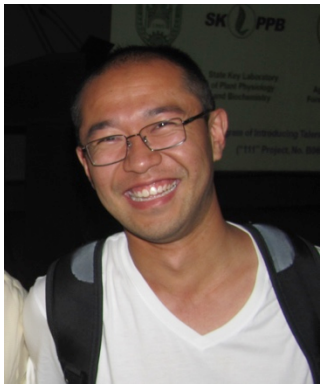

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