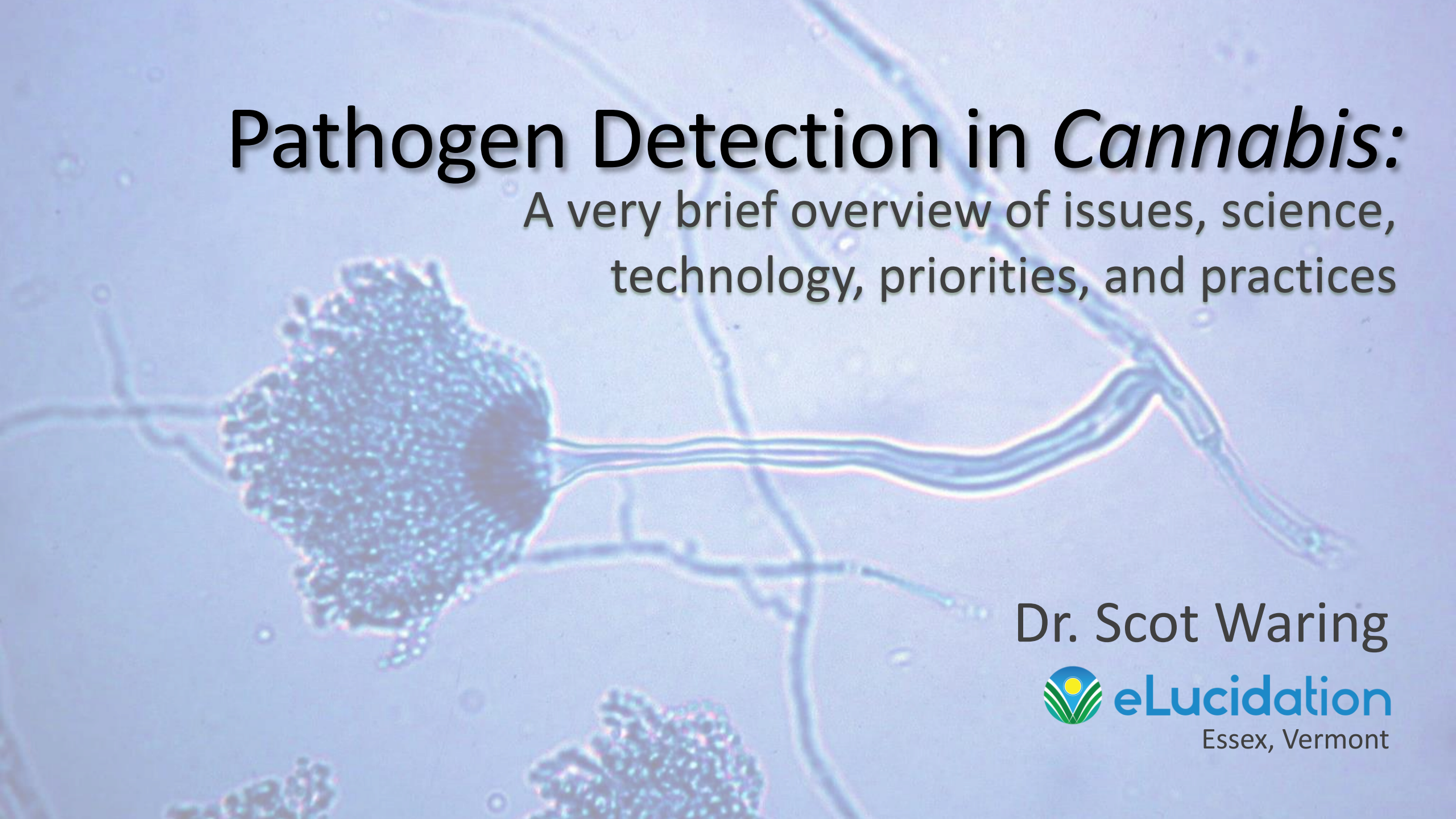


Pathogen Detection in *Cannabis:*

A very brief overview of issues, science, technology, priorities, and practices

Dr. Scot Waring

eLucidation

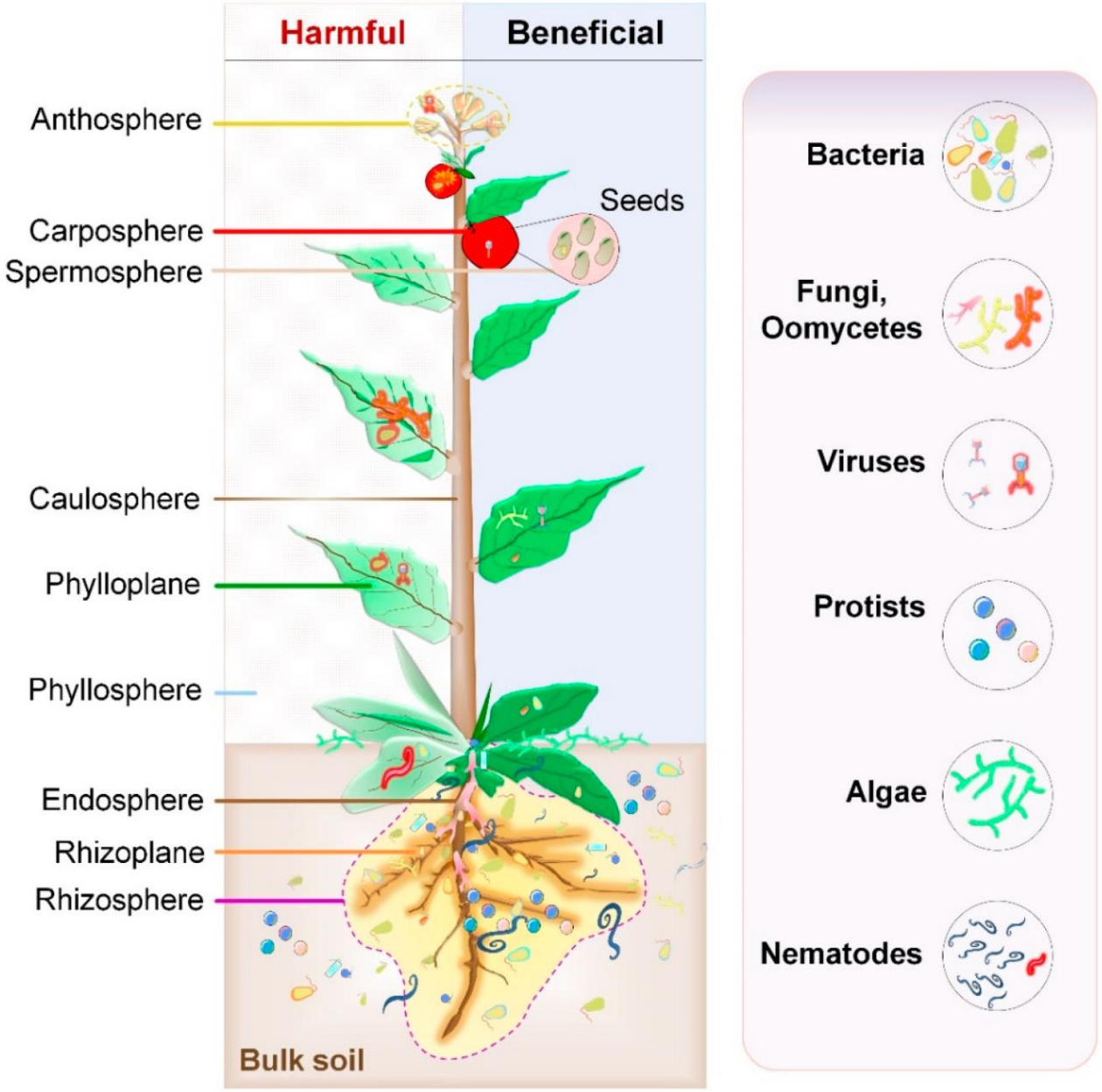
Essex, Vermont

OVERVIEW

All plants and animals have a **microbiome** of organisms that live in and on them.

These microscopic **communities** provide important services to their hosts, like:

- enhancing nutrient availability
- production of defensive chemicals
- occupying “real estate”



OVERVIEW

Pathogens are microorganisms that become overly-abundant and cause diseases**.**

- Bacteria
- Viruses
- Protozoa
- Fungi
  - mycotoxins

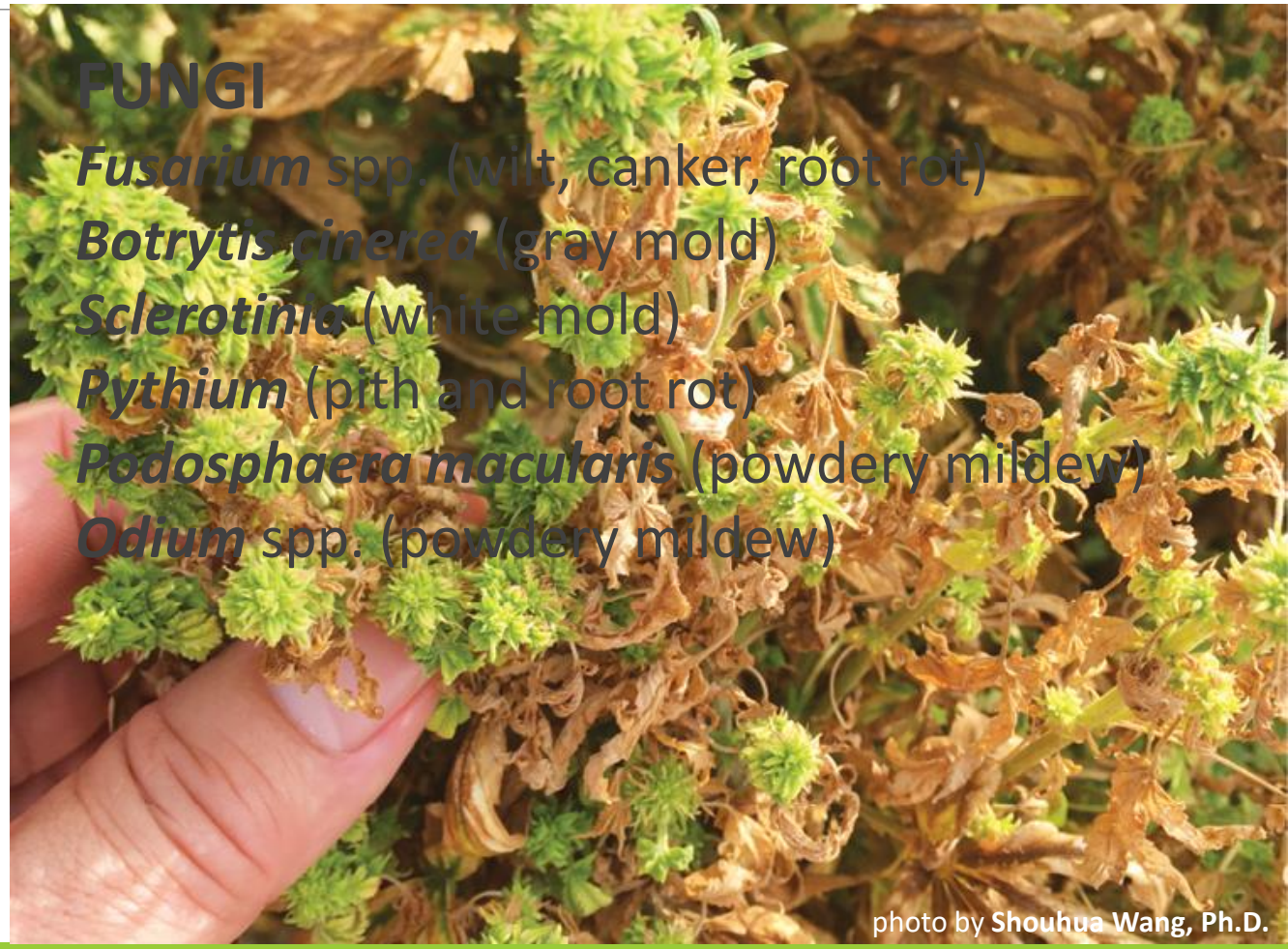
PLANT PATHOGENS

HUMAN PATHOGENS

PLANT PATHOGENS

BACTERIA

Phytoplasma spp.
Pseudomonas spp. (blight)
Agrobacterium spp. (crown gall)
Xanthomonas spp. (leaf spot)



PLANT PATHOGENS

VIRUSES and VIROIDS	TRANSMISSION METHOD(s)
Alfalfa mosaic virus (AMV)	➢ insects, seeds, pollen, tools
Sunn-hemp mosaic virus (SHMV)	
• **tobacco mosaic virus** (TMV)	➢ insects, soil, water, tools
Hemp streak virus (HSV)	➢ likely thrips
Cucumber mosaic virus (CMV)	➢ aphids
Hop Latent Viroid (HpLVd)	➢ aphids

HUMAN PATHOGENS

- Overdose deaths from *Cannabis:* 0
- Most *Cannabis* consumer deaths and hospitalizations linked to **pathogens**.
  (e.g. Stone et *al.* 2019, Gargani et al. 2011)

Microbes in *Cannabis* originate from:

- Human vectors (body, clothing, tools)
- Contaminated water
- Contaminated soil / substrate
- Insect vectors (e.g. Boiocchi et *al.* 2019)
- Airborne particles

image by **Tasha Sturm**
via Twitter

HUMAN PATHOGENS

- Microbes are everywhere.
- “Healthy” human microbiome is comprised of ~10,000 species.
- Beneficial microbes enhance overall health (e.g. *C. diff* infection).
- People are exposed daily to pathogens.
- Immune response protects.
- **Immunocompromised** population is advised to avoid:
  - ✓ raw produce (e.g. *E. coli, Nocardia, Listeria*)
  - ✓ soil (e.g. *Aspergillus, Mucor, Legionella*)
  - ✓ standing water (e.g. *Pseudomonas, Klebsiella, Salmonella*)

Tomblyn et *al.* 2017

HUMAN PATHOGENS *in Cannabis*

Thompson et *al.* (2017) sequenced microbiomes of *Cannabis* flower samples purchased from 20 medical marijuana dispensaries in N. California.

- *Acinetobacter baumannii*
- *Escherichia coli*
- *Klebsiella pneumoniae*
- *Pseudomonas aeruginosa*
- *P. fluorescens*
- *P. putida*
- *Stenotrophomonas maltophilia*
- *Aspergillus fumigatus*
- *Mucor circinelloides*
- *Cryptococcus laurentii* (yeast)
- *Cyclospora cayetanensis* (protozoan)

Immunocompromised individuals are highly vulnerable to infection by these microbes. Healthy individuals are also at-risk of inhaling spores and live microbes.

HUMAN PATHOGENS *in Cannabis*

McKernan et *al.* (2015) examined the sequenced microbiomes of *Cannabis* flower samples from Amsterdam and Massachusetts that contained several pathogenic and **mycotoxin**-producing fungi.

- *Penicillium paxillin*
- *P. citrinum*
- *P. commune*
- *P. chrysogenum*
- *P. corylophilum*
- *Aspergillus terreus*
- *A. niger**
- *A. flavus*
- *A. versicolor*
- *Eurotium repens*
- *Cryptococcus liquefaciens* (yeast)

Subsequently, McKernan et *al.* (2016) found that *Aspergillus* failed to culture on the two most common agar plating systems for fungi. Mycotoxins are difficult to destroy.

HUMAN PATHOGENS

qPCR: New industry standard

- USDA phasing in qPCR for pathogen detection and monitoring in food supply.
- Multiple products now available to meet state microbial testing requirements for medical and adult use *Cannabis.*
- Safer than growing cultures of pathogens.
- Technology now more affordable.

Bio-Rad's PCR Kit a "Fast and Accurate" Way to Detect Aspergillus in Cannabis

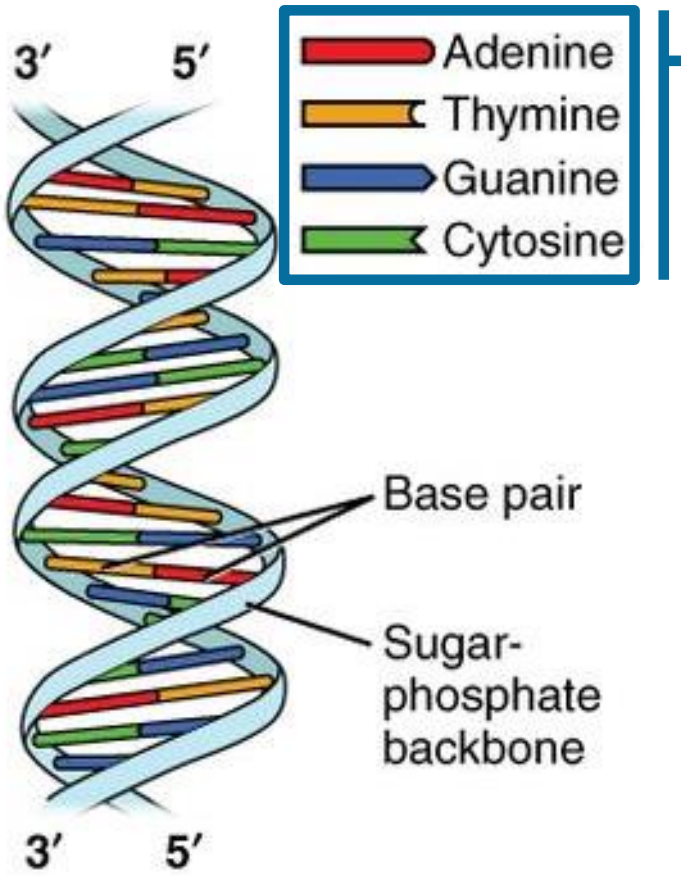
News | Nov 14, 2019 | by Bio-Rad Laboratories

Credit: Bio-Rad Laboratories

Bio-Rad Laboratories, Inc. has announced the launch of the **iQ-Check Aspergillus Real-Time PCR Kit**, a fast and accurate method for detecting four potentially hazardous molds: Aspergillus flavus, A. fumigatus, A. niger, and A. terreus in cannabis and cannabis-infused products.

from analyticalcannabis.com

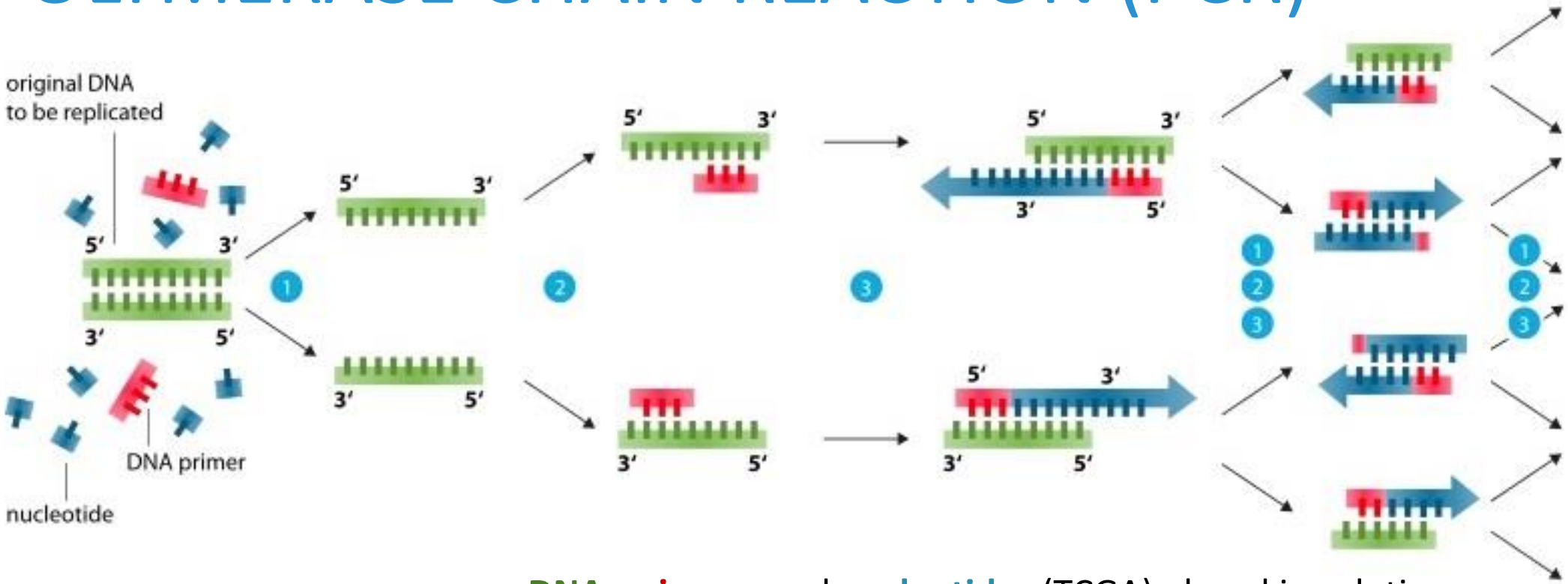
DNA-BASED PATHOGEN DETECTION



PCR (polymerase chain reaction)

- Method developed for making copies of DNA
- Requires enzyme (**polymerase**)
- Free **nucleotides** (T, A, C, G)
- **Primers** attach to target DNA sequence

POLYMERASE CHAIN REACTION (PCR)



1. **Denaturation** at 94-96°C
2. **Annealing** at ~68°C
3. **Elongation** at ca. 72 °C

DNA, **primers**, and **nucleotides** (TCGA) placed in solution.

1. Solution heated, DNA "unzipped" (denatured).
2. Primers adhere to single-stranded DNA.
3. Free-floating nucleotides collected and assembled as polymerase builds complementary strand.

Process repeats many times and amplifies targeted DNA sequences.

DNA-BASED PATHOGEN DETECTION

PCR (polymerase chain reaction)

- Method developed for making copies of DNA
- Requires enzyme (**polymerase**)
- Free **nucleotides** (T, A, C, G)
- **Primers** attach to target DNA sequence

Quantitative polymerase chain reaction **(qPCR)**

- **Flourophores** attached to molecules in process. Light emitted when double strand DNA is made
- Precise wavelength of light linked to species DNA
- Rise and intensity of fluorescence observed over cycles
- Algorithm-processed data identify and calculate abundance

MICROBIAL TESTING

traditional Petri dish (agar) plating

quantitative PCR (qPCR)

- Some microbes culture better than others: some pathogens do not, while many beneficial and benign microbes do.
- Identification of cultures can be difficult and CFUs (spots) are used as proxy for abundance.
- Some mycotoxin-producing fungi do not culture.
- False negatives occur frequently.
- False positives occur occasionally.
- Results in **48** to **72 hours**.

- Primers target DNA segments of known pathogens, while “ignoring” other microbial DNA.
- Can **identify** multiple pathogens and determine their **abundance** with a high level of precision.
- Can detect DNA of mycotoxic fungi (dead or alive).
- False negatives occur very rarely.
- False positives do not occur in modern systems.
- Results available in **2 hours**.

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DNA MCIROARRAYS

Matching DNA sequences

- Requires reference genome/gene (probe)
- Sample DNA is labelled with fluorophores
- Highly complementary (matching) sequences fluoresce more
- Effective at finding differences and similarities in sequences (pathogens)

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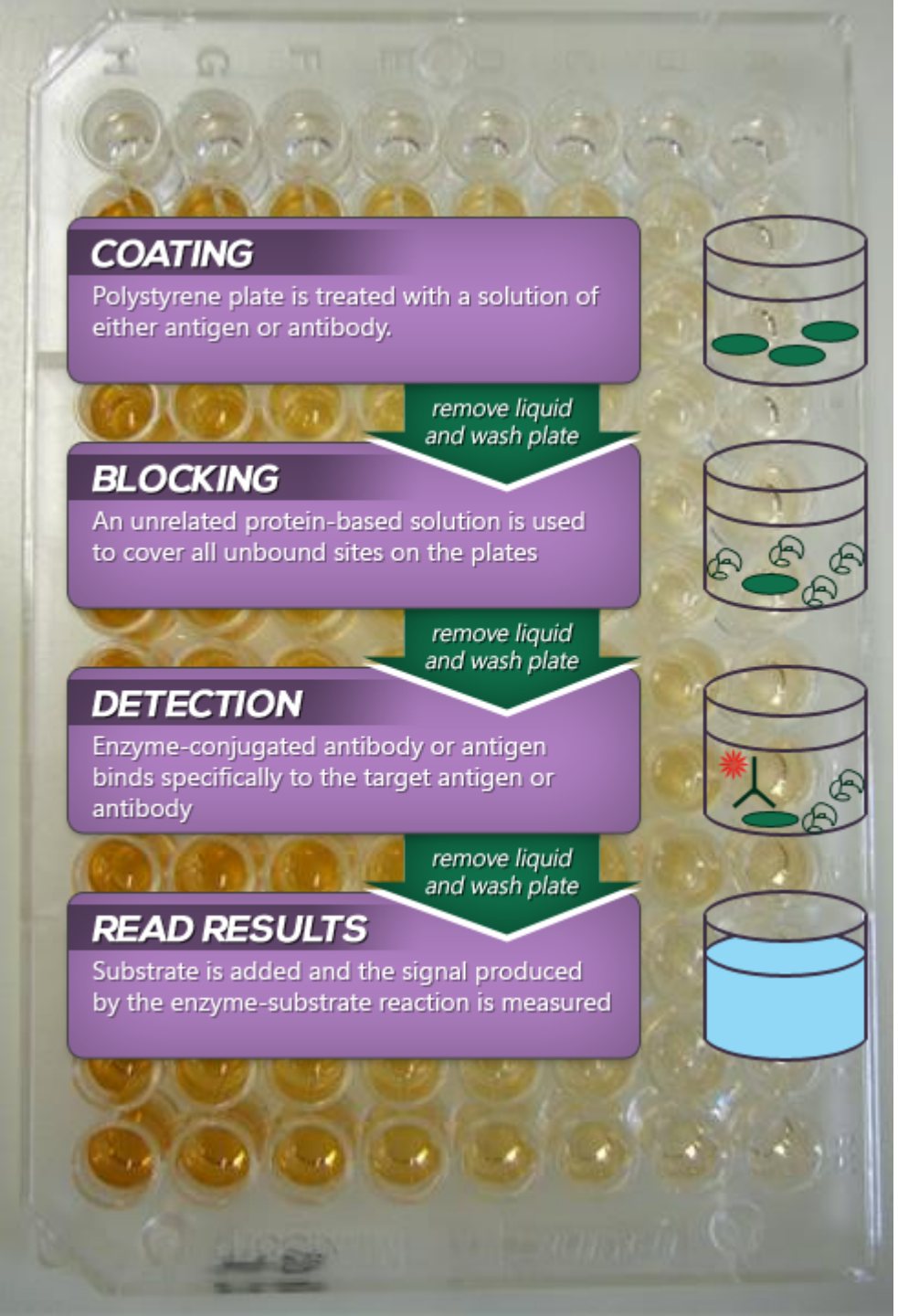

ELISA

ELISA (enzyme-linked immunosorbent assay)

- Enzyme-based immunoassay
- Quantify proteins (incl. antibodies and hormones)
- Able to ID and quantify mycotoxins, viruses, and microscopic arthropods (and their eggs)

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LITERATURE CITED

Boiocchi F, Davies MP, and Hilton AC. 2019. **An examination of flying insects in seven hospitals in the United Kingdom and carriage of bacteria by true flies (Diptera: Calliphoridae, Dolichopodidae, Fanniidae, Muscidae, Phoridae, Psychodidae, Sphaeroceridae).** *Journal of Medical Entomology*, 56(6): 1684–1697.

Gargani Y, Bishop P, and Denning DW. 2011. **Too many mouldy joints - marijuana and chronic pulmonary aspergillosis**. *Mediterranean Journal of Hematology and Infectious Diseases*. 3(1).

Li J, Shi X, Yin W, Wang Y, Shen Z, Ding S and Wang S. 2017. **A multiplex SYBR green real-time PCR assay for the detection of three colistin resistance genes from cultured bacteria, feces, and environment samples**. *Frontiers of Microbiology*. 8:2078.

McKernan K, Spangler J, Helbert Y, Lynch RC, Devitt-Lee A, Zhang L, Orphe W, Warner J, Foss T, Hudalla CJ, Silva M, and Smith DR. 2016. **Metagenomic analysis of medicinal Cannabis samples; pathogenic bacteria, toxigenic fungi, and beneficial microbes grow in culture-based yeast and mold tests** *F1000Research.* 5: 2471.

McKernan K, Spangler J, Zhang L, Tadigotla V, Helbert Y, Foss T, and Smith D. 2015. ***Cannabis* microbiome sequencing reveals several mycotoxic fungi native to dispensary grade *Cannabis* flowers**. *F1000Research*. 4: 1422.

Stone T, Henkle J, and Prakash V. 2019. **Pulmonary mucormycosis associated with medical marijuana use**. *Respiratory Medicine Case Reports*. 26: 176–179.

Thompson GR, Tuscano JM, Dennis M, Singapuri A, Libertini S, Gaudino R, Torres A, Delisle JMP, Gillece JD, Schupp JM; et al. 2016. **A microbiome assessment of medical marijuana**. *Clinical Microbiology and Infection.* 23(4): 269-270.

Tomblyn M, Chiller T, Einsele H, Gress R, Sepkowitz K, Storek J, Wingard JR, Young JA, and Boeckh MJ. 2009. **Guidelines for preventing infectious complications among hematopoietic cell transplantation recipients: a global perspective.** *Journal of the American Society for Blood and Marrow Transplantation*. 15(10): 1143-1238.

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