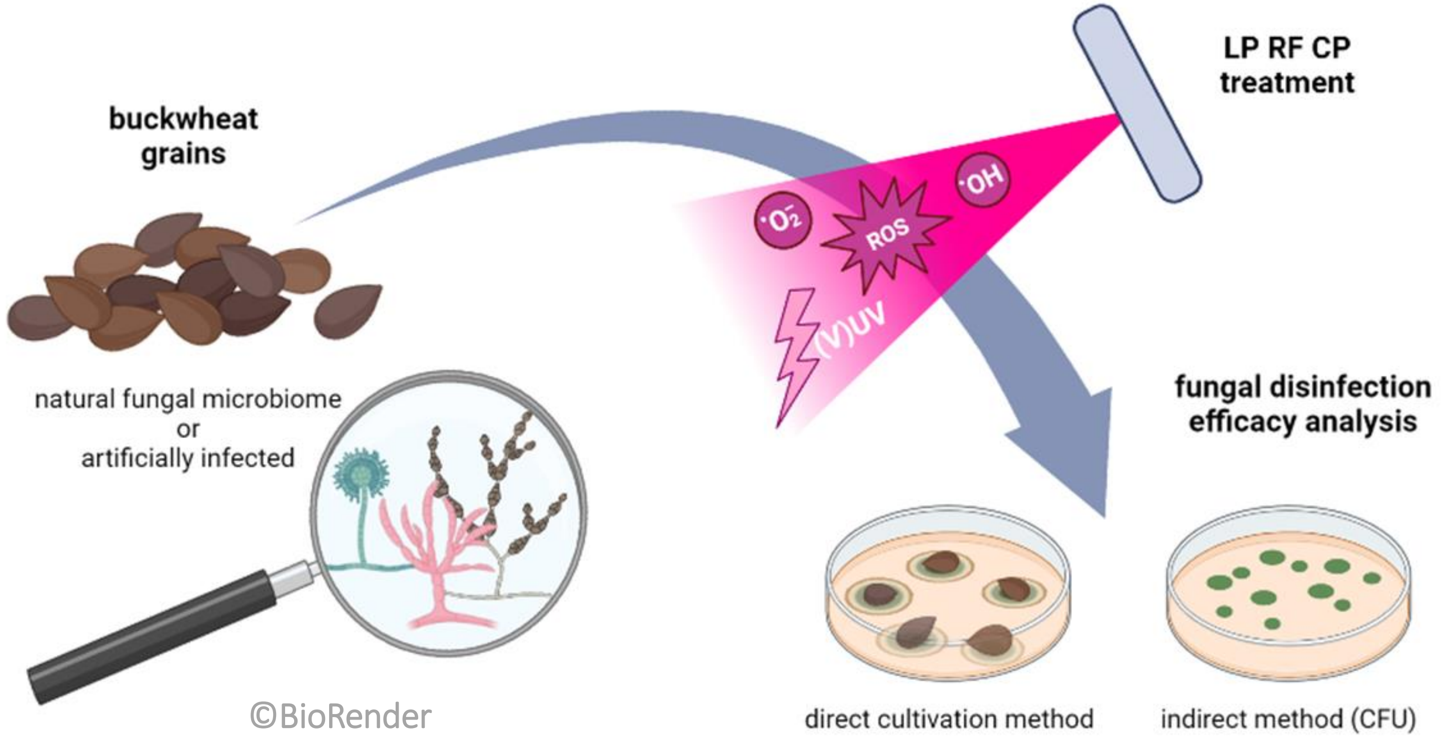
Cold Plasma for Fungal Decontamination of Buckwheat Seeds



©BioRender

UNIVERSITY OF LJUBLJANA
Biotechnical Faculty

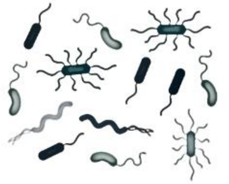
Jure Mravlje, PhD

Department of Biology, Biotechnical Faculty, University of Ljubljana, Slovenia

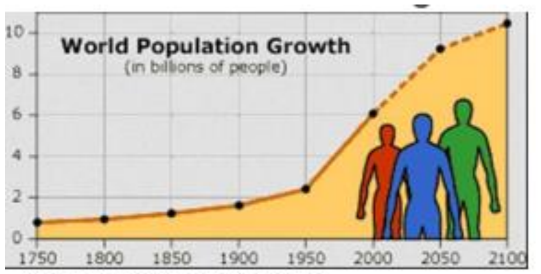
jure.mravlje@bf.uni-lj.si

CA19110
Plasma applications for smart and sustainable agriculture

I. Plasma for Decontamination of MO



- **Why CP for decontamination of seeds?**
  - ↑ world population ~**10 mrd by 2050** *(FAO, UN)*
  - Estimation: ↑ ***70% agricultural production***
    - *Negative impacts on environment*
    - Amount of arable land = limited and declining
  - **↓ use of pesticides and artificial fertilizers**
    - *- effects on human health (and environment)*: *Hodgkinson's disease, lymphoma, Parkinson's disease, endocrine disorders, respiratory in reproductive disorders, cancer…*
  - Key challenge in 21st century: ↑**efficiency,** ↓**losses**
  - *Ecological and sustainable methods for decontamination!*

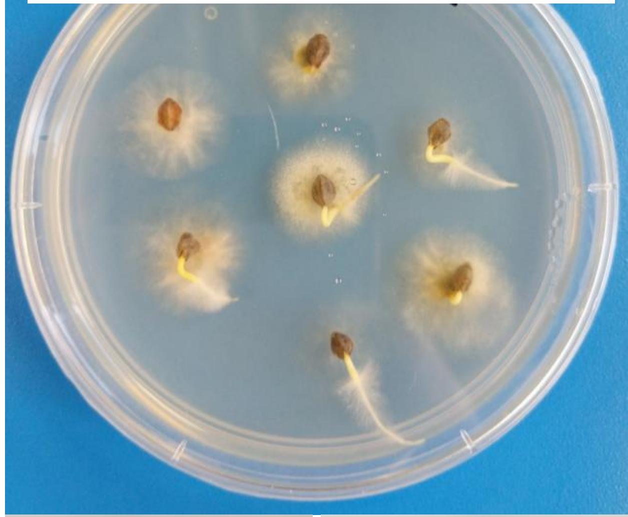


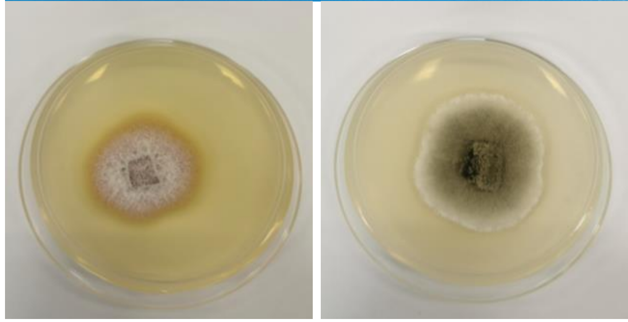
© http://evolution.berkeley.edu

I. Plasma for Decontamination of MO

- **Fungi = Nr. 1 concern in plant production (responsible for more than 70% of all plant diseases worldwide)**
  - *Cereal grains = the most important global food source*
  - ***Fungal infections of grains = ↑ problem & ↑ negative effect on food quality & safety!***
  - Contamintaion:
    - on field (“**field fungi**”) or
    - during storage (“**storage fungi**”) (Christensen, 1957)
  - **Secondary metabolites – mycotoxins:** harmful for health of human and animals (acute toxicity, genotoxic, mutagens, cancer…)
    - *Alternaria, Fusarium, Penicillium, Aspergillus*
  - Contaminated seed material = potential primary source of plant diseases
  - **Preventing fungal infections on seeds/grains = key for providing global food security!!**

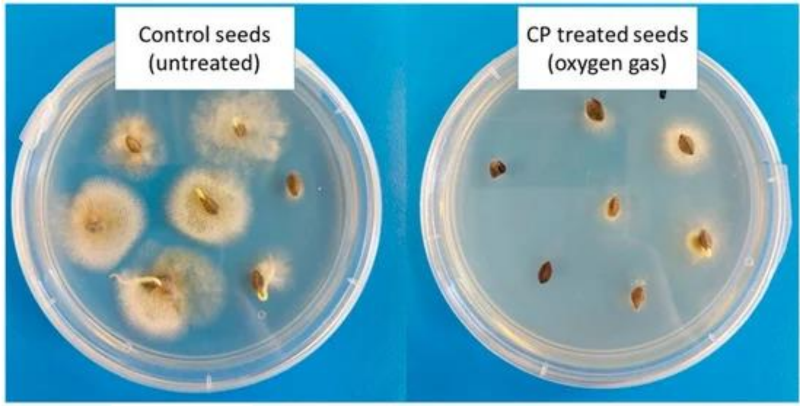
Grains from ecological production after 5 days of cultivation on PDA

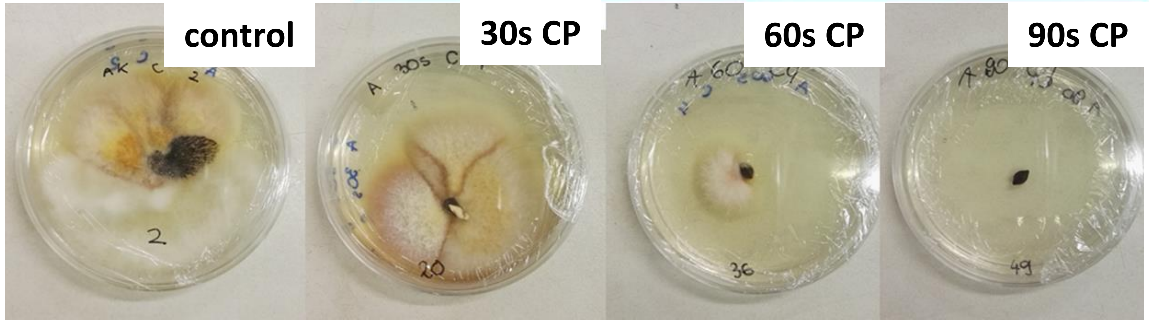


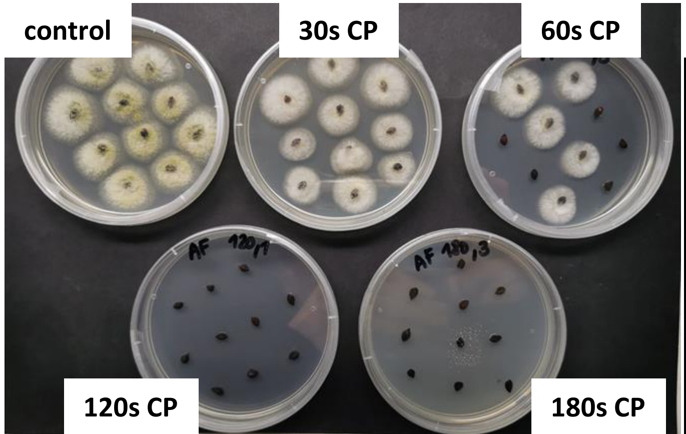


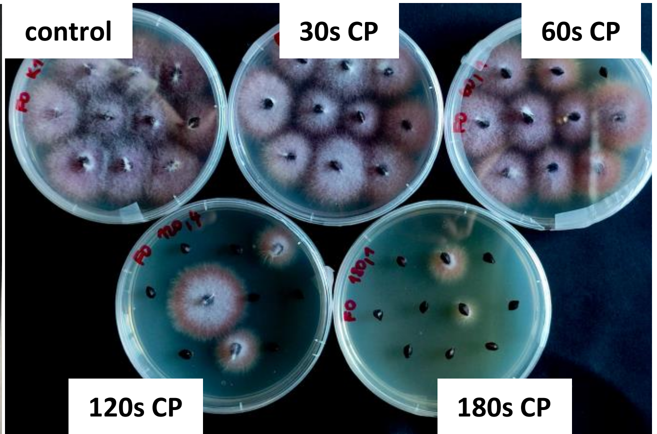
I. Plasma for Decontamination of MO

- **Basics of CP Decontamination of Seeds**
  - **Native fungal microbiota**
    (natural fungal communities)
  - **Artificially contaminated seeds**
    - Effect on selected fungal species/isolates









BF UNIVERSITY OF LJUBLJANA
Biotechnical Faculty

II. Experiments with CP on Buckwheat

- <u>**CP Decontamination of Buckwheat grains**</u>
- ***Buckwheat – alternative crop*** (“pseudocereal”)
  - Low demands → organical farming (“eco-friendly”)
  - No gluten, ↑ nutritional value (proteins, polyphenols)
  - Traditional, local, “functional” food
    - **Common buckwheat** (*Fagopyrum esculentum* Moench)
    - **Tartary buckwheat** (*Fagopyrum tataricum* Gaertn.)
  - History:
    - First mentions in 15th century
    - For food (flour, porridge): **Janez Vajkard Valvasor –** famous book **Slava vojvodine Kranjske** (1689)
    - 1816 (“year without summer” – volcano eruption): year of hunger in Slovenia (whole Europe) → **Žiga Zois** introduces grain of Tartary buckwheat (from Czechia)

UNIVERSITY OF LJUBLJANA
Biotechnical Faculty

II. Experiments with CP on Buckwheat

- **<u>Experiments</u>**

C/T Buckwheat seeds

CP treatment

LP RF CP (1)

- Various parameters: various setups, exposure types, different gases…

(2) Effect on natural fungal microbiota

Effect on germination

Isolation (PDA)

Morphotypes

Molecular identification

- DNA isolation
- PCR
- Gel electrophoresis
- Sequence cleaning
- Sequencing → bioinformatical analysis (MEGA, BLAST)
- Fungal bank + NCBI b

Selection of key fungi*

(3) Artificially infected

- Most frequent + most harmful pathogenic/endophytic fungi *(Alternaria, Aspergillus, Fusarium, Epicoccum…)*

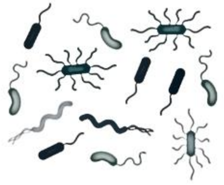
CP treatment

Comparison of 2 methods for evaluation of decontamination

CFU method

(4) % infection

II. Experiments with CP on Buckwheat



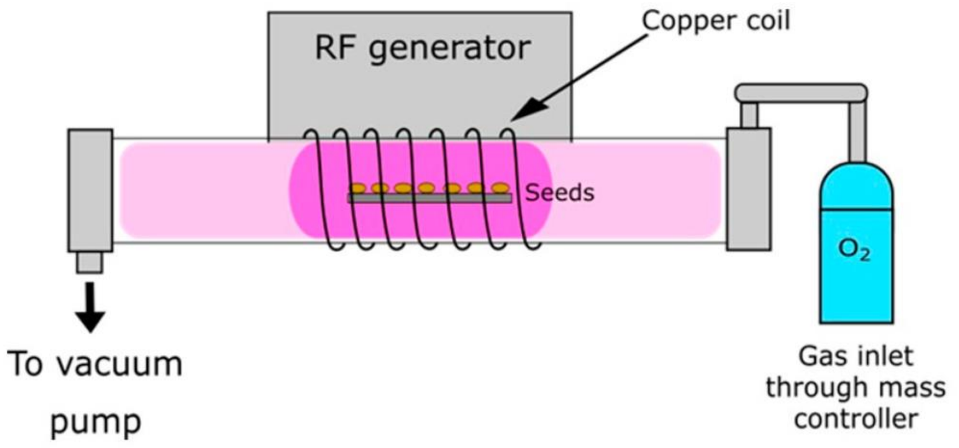
- **CP reactors**
  - Powerful RF oxygen plasma (reduced pressure)

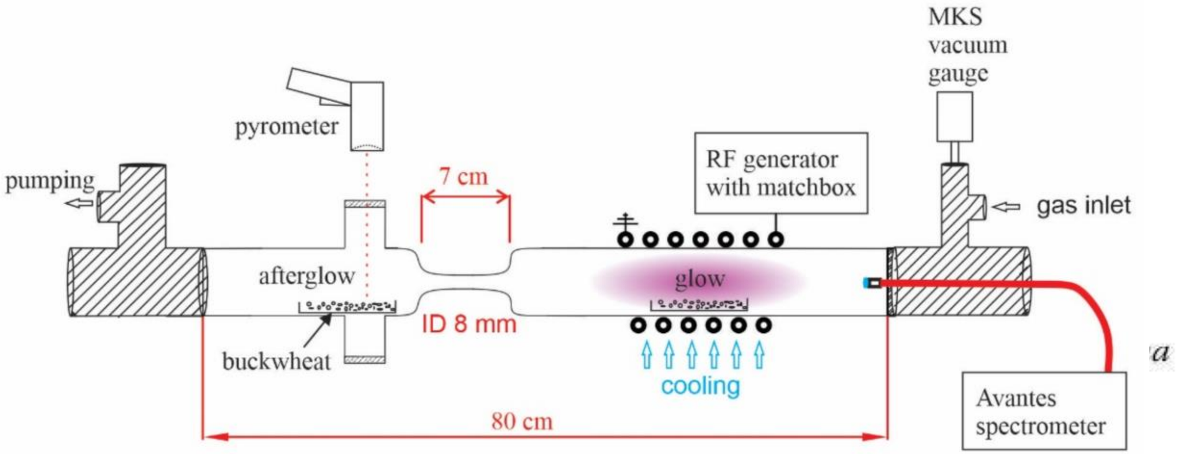
A custom-made large-scale (tube length ~2 m, inner diameter ~0.2 m)
Working pressure ~50 Pa, RF gen. 27.12 MHz, power 1500 W (PD = 30 W/L), flow rate 202 sccm
99,99% oxygen gas

Mravlje et al. (2021) Plants. (DOI: 10.3390/plants10050851)

A custom-made small-scale (tube length ~80 cm, inner diameter ~4 cm)
Working pressure ~50 Pa, RF gen. 13.56 MHz, power 1500 W (PD = **7000** W/L), flow rate 60 sccm
99,99% oxygen gas

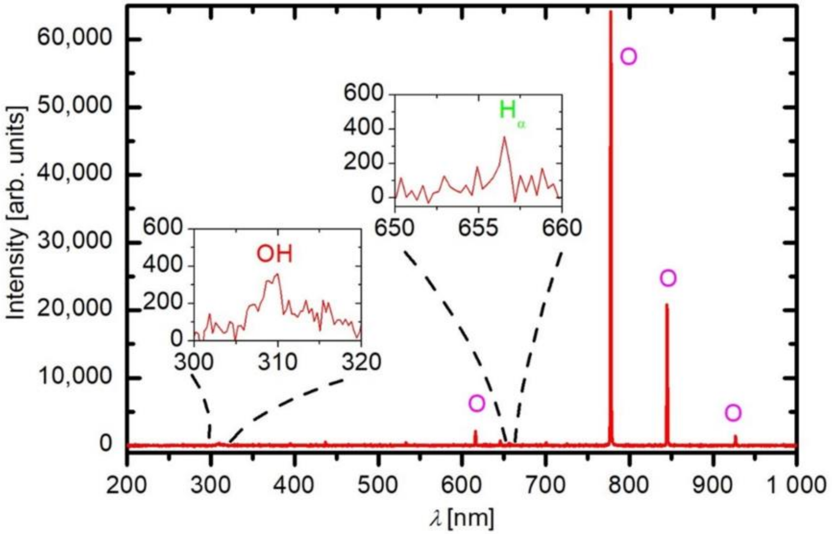
Mravlje et al. (2022) Plants. (DOI: 10.3390/plants11101366)

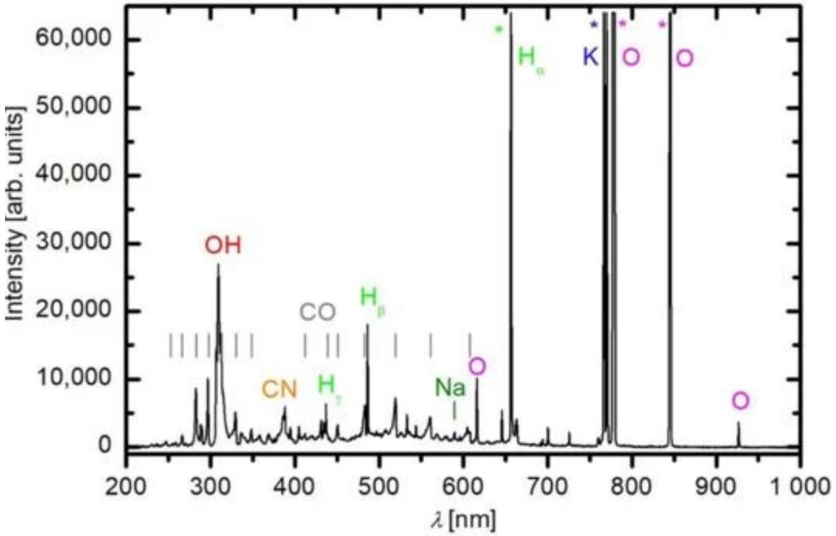


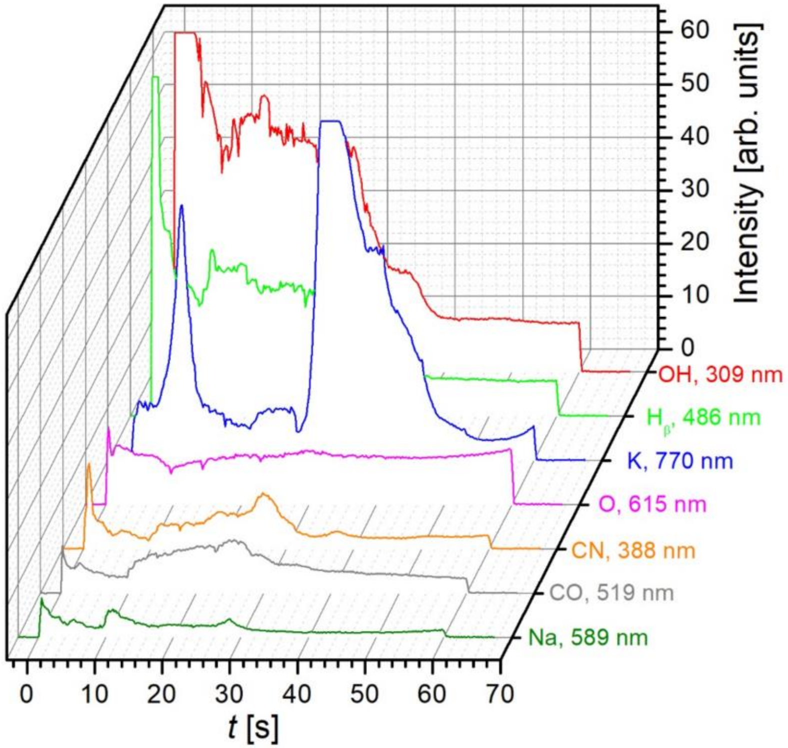


II. Experiments with CP on Buckwheat

- **CP reactors**
  - Typical OES





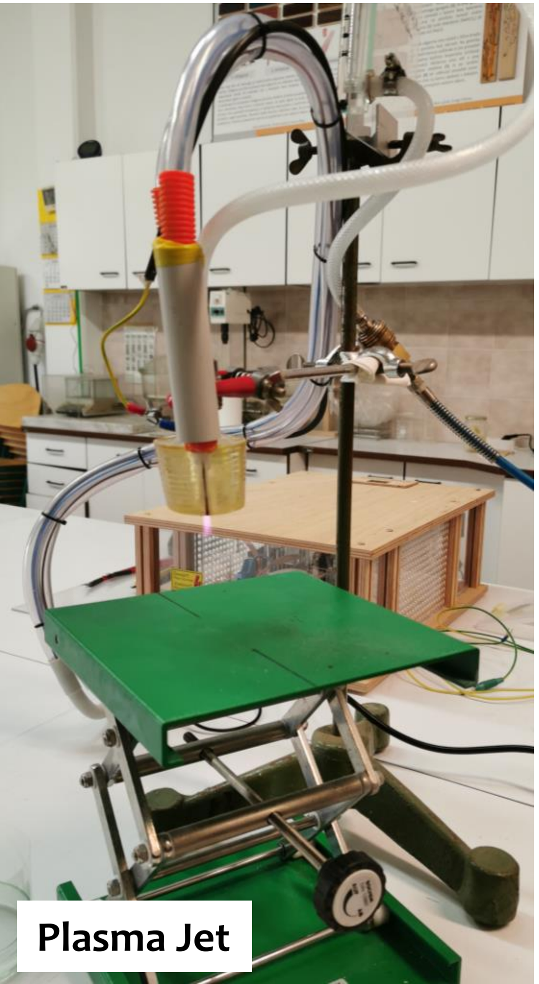


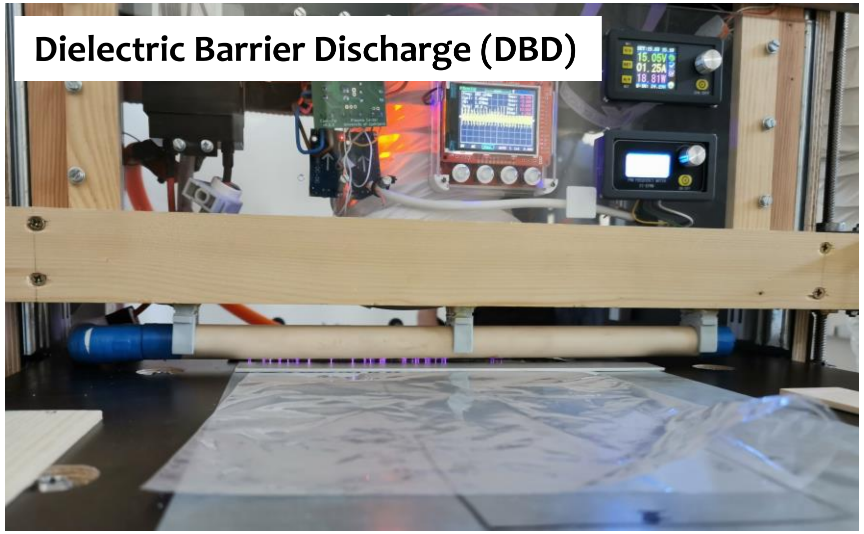
Mravlje et al. (2022) Plants. (DOI: 10.3390/plants11101366)

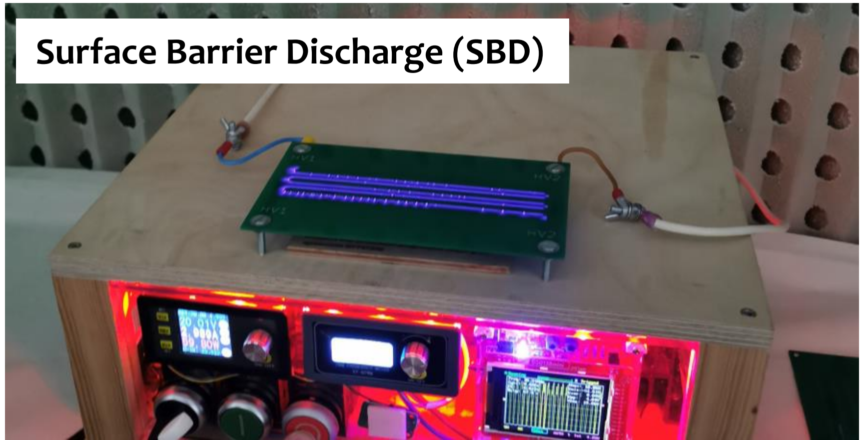
II. Experiments with CP on Buckwheat

AP plasmas

Biotechnical faculty, University of Ljubljana (Dept. of wood science)

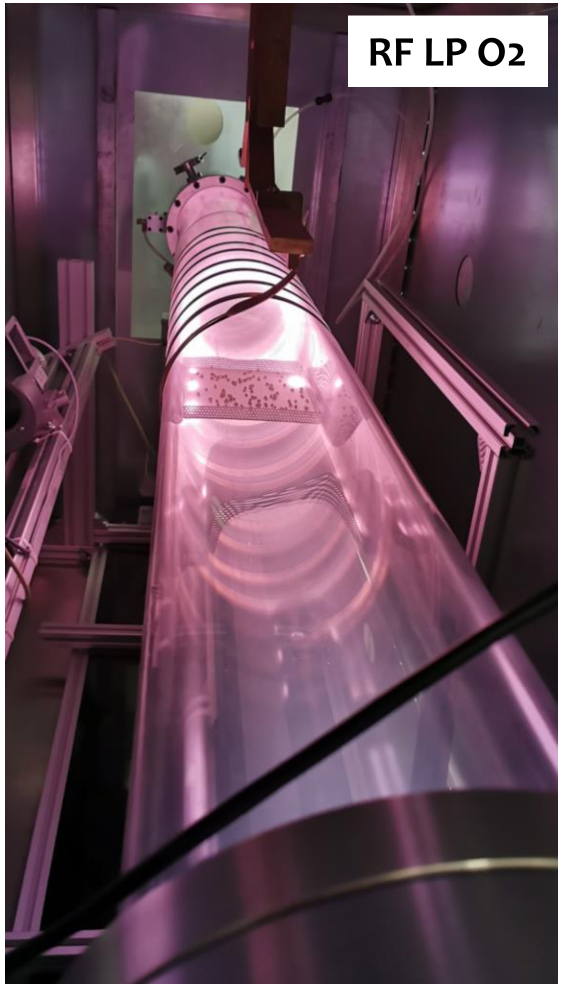


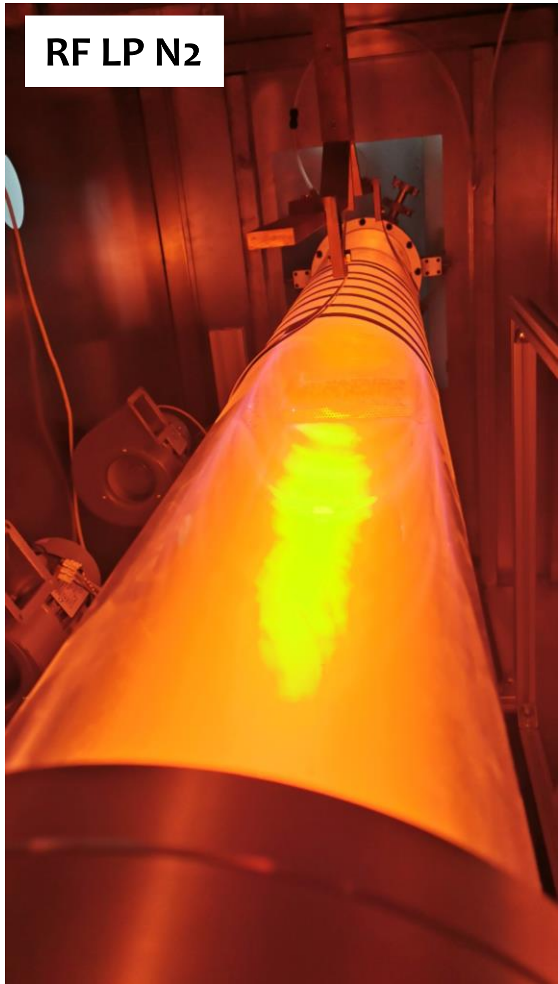




LP plasmas

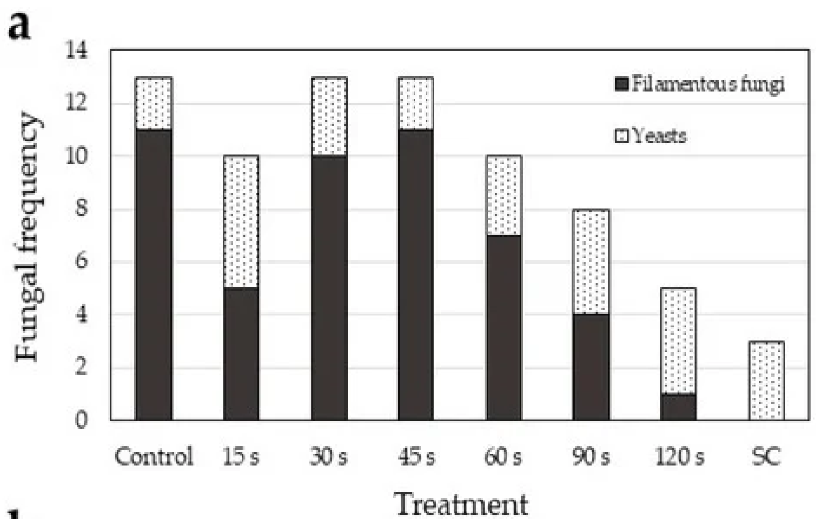
Josef Stefan Institute (prof. Mozetič)

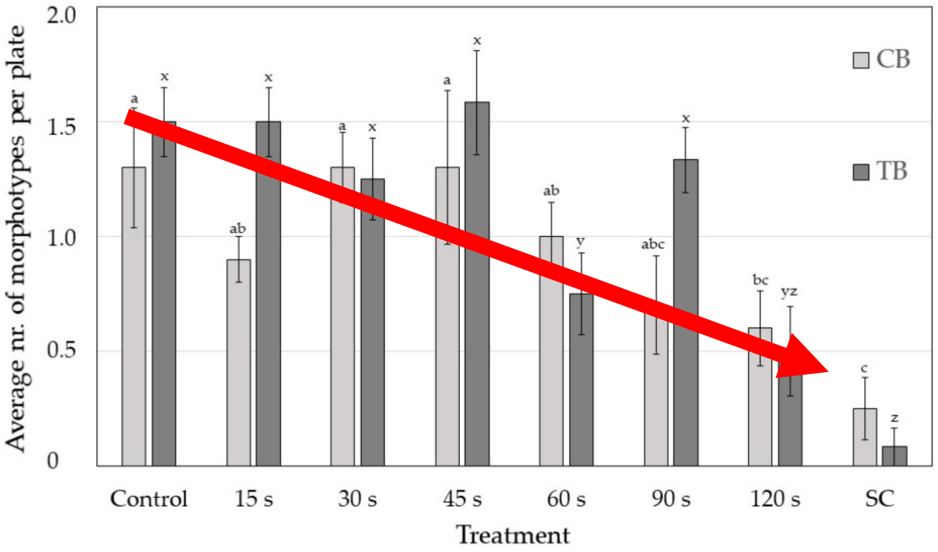


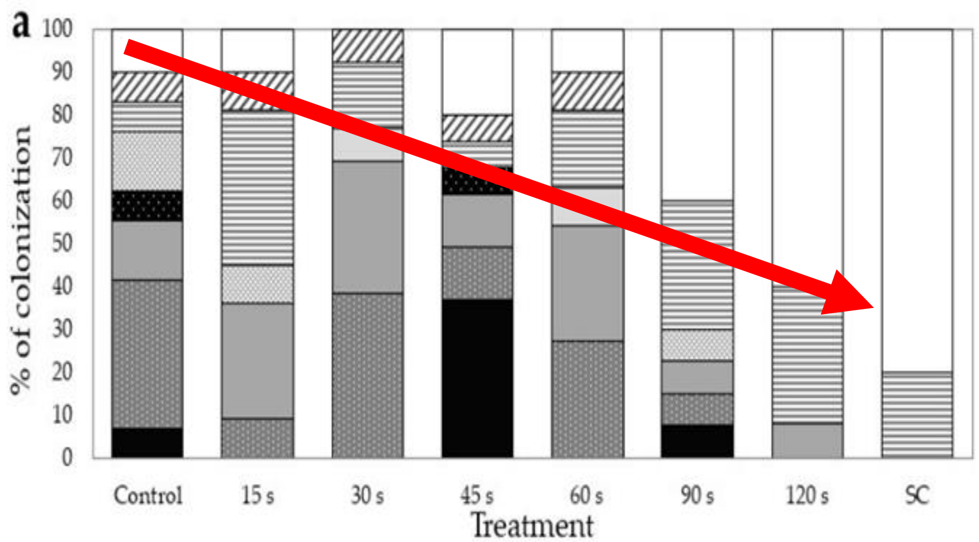


III. Results: Natural Microbiota

- **Natural fungal communities**
  - Reduction in fungal frequency and diversity [1,2]
  - More effective for FF than yeasts [1,2]
  - Longest exposures comparable to classical S





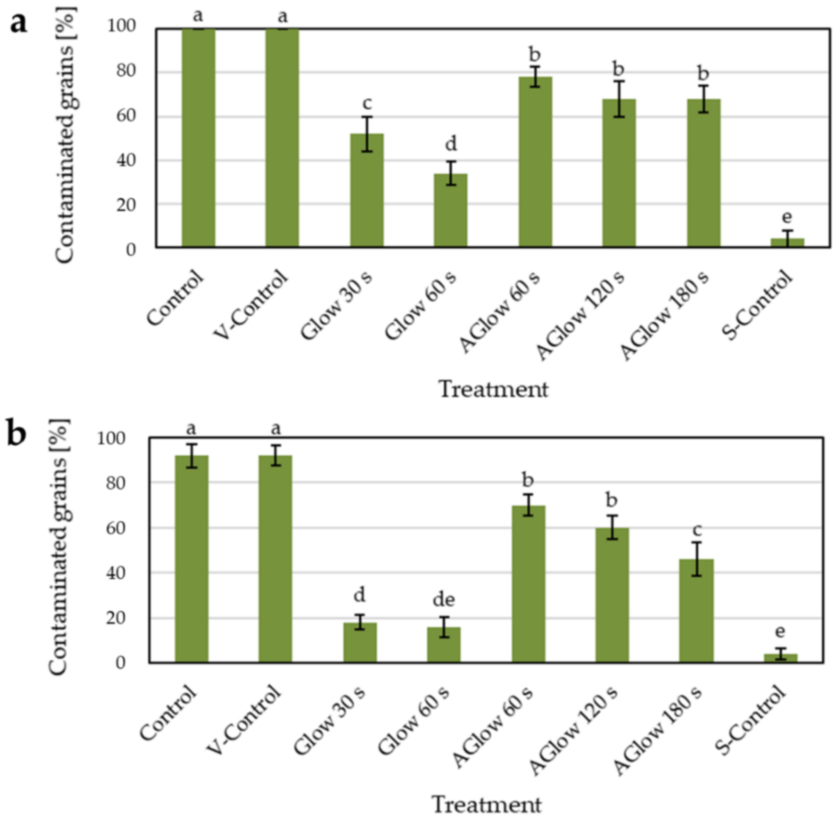


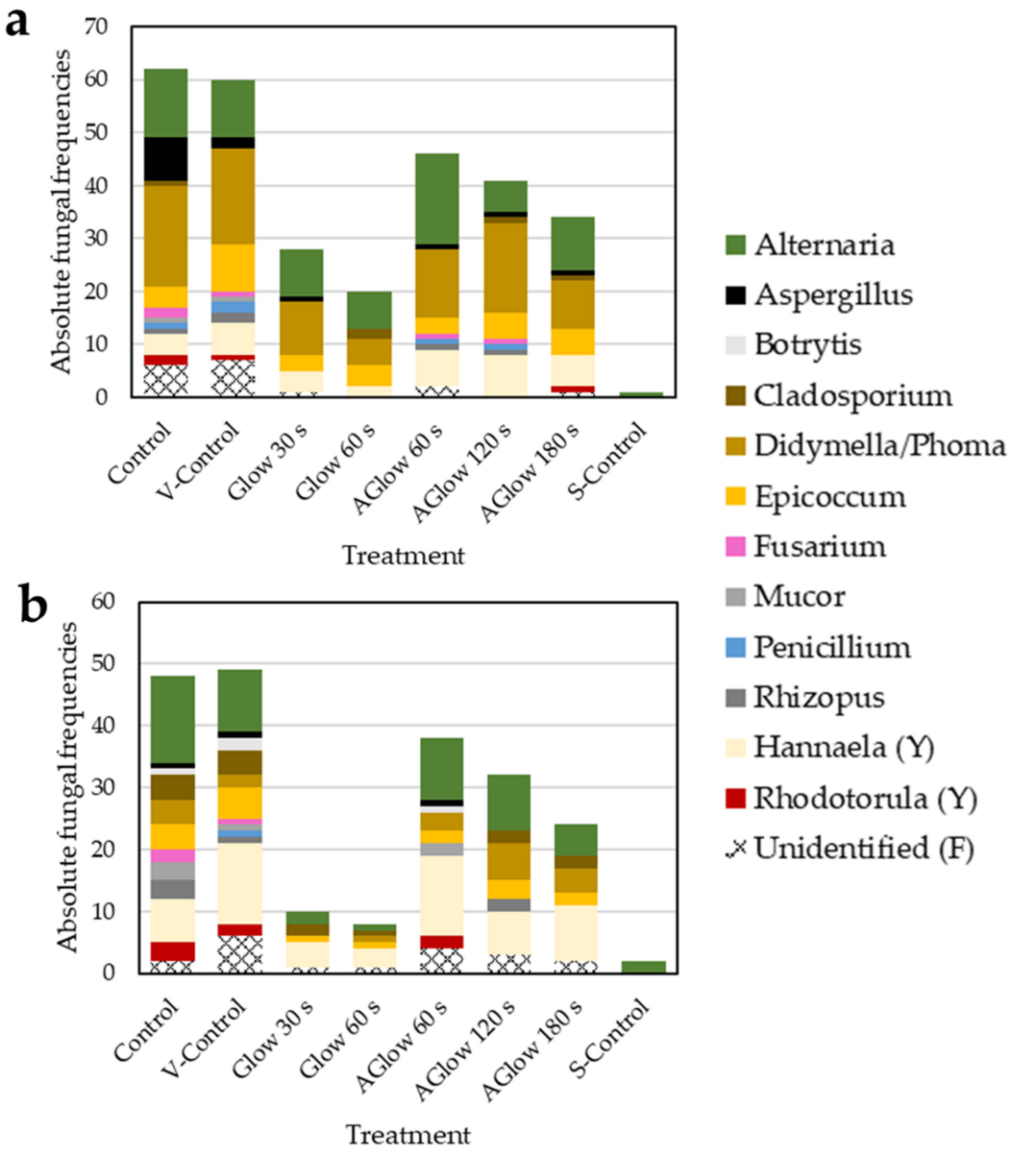
1 Mravlje et al. 2021 (DOI: 10.3390/plants10050851)
2 Mravlje et al. 2022 (DOI: 10.3390/plants11101366)

BF UNIVERSITY OF LJUBLJANA
Biotechnical Faculty

III. Results: Natural Microbiota

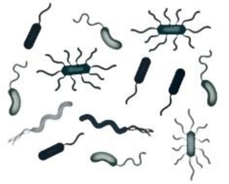
- **Natural fungal communities**
  - Glow more effective than afterglow [2]



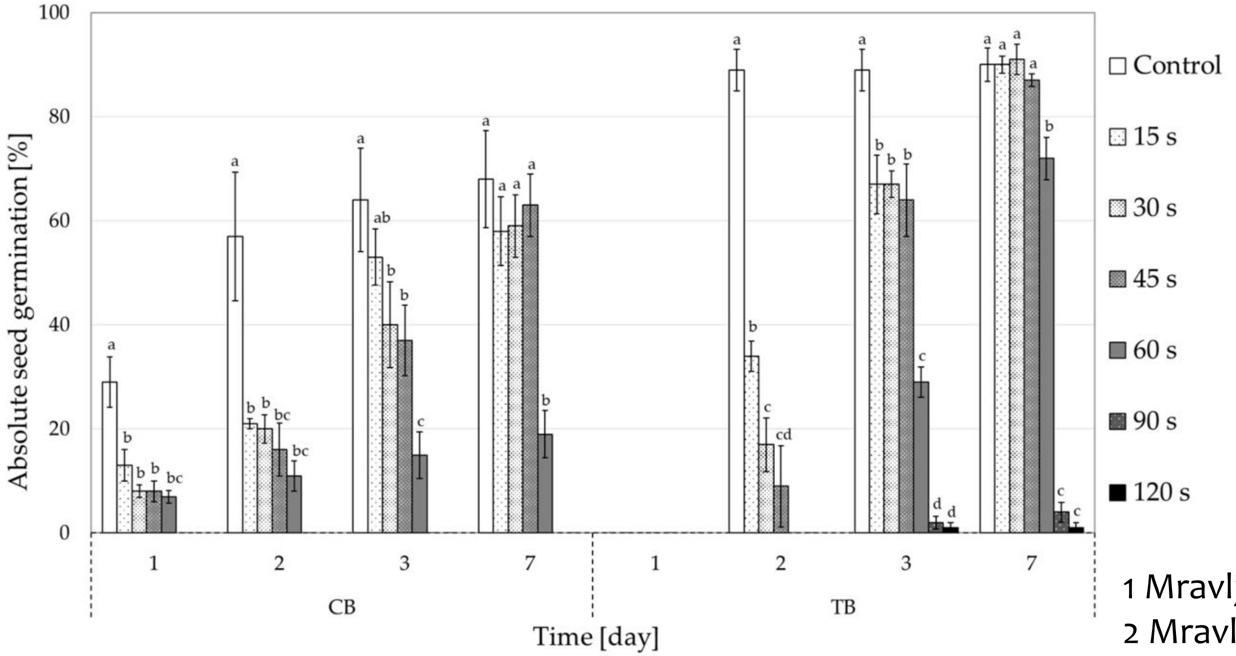


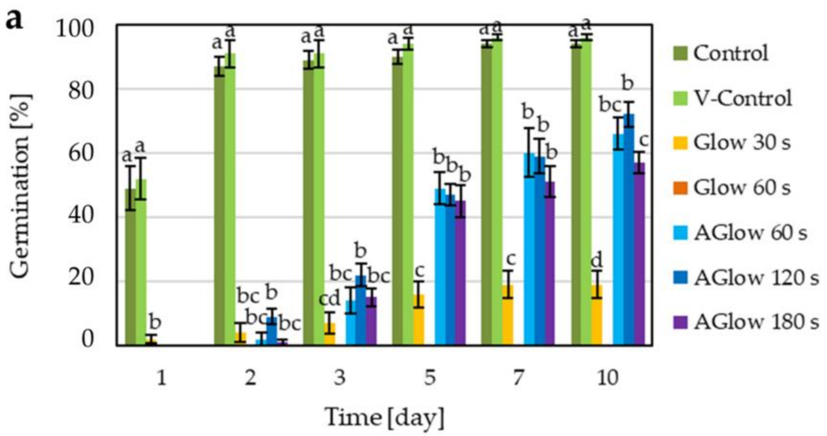
1 Mravlje et al. 2021
(DOI: 10.3390/plants10050851)
2 Mravlje et al. 2022
(DOI: 10.3390/plants11101366)

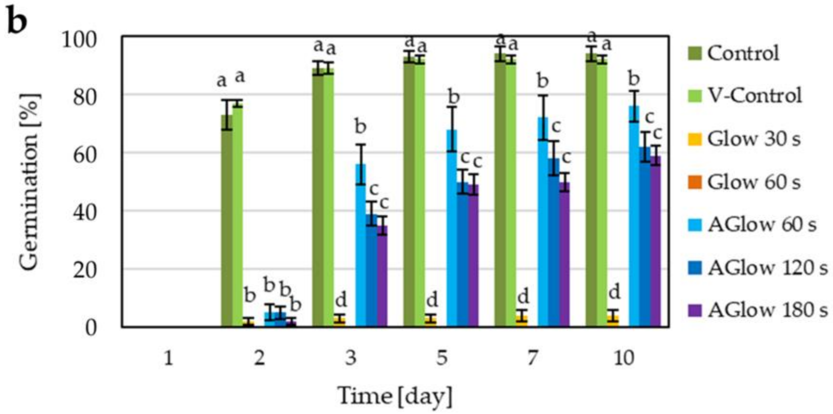
III. Results: Germination



- **Effect on germination ☹**
  - - effect on germination (↓ %, delay in MGT and T50) [1,2]
  - Afterglow less than glow [2]
  - Effect of species or reactor ???

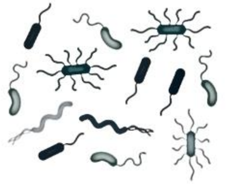






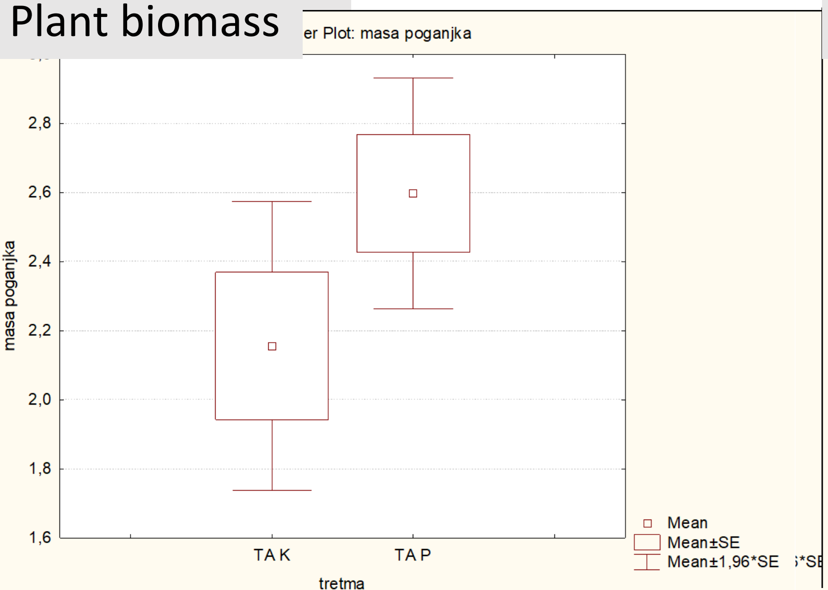
1 Mravlje et al. 2021 (DOI: 10.3390/plants10050851)
2 Mravlje et al. 2022 (DOI: 10.3390/plants11101366)

III. Results: Germination

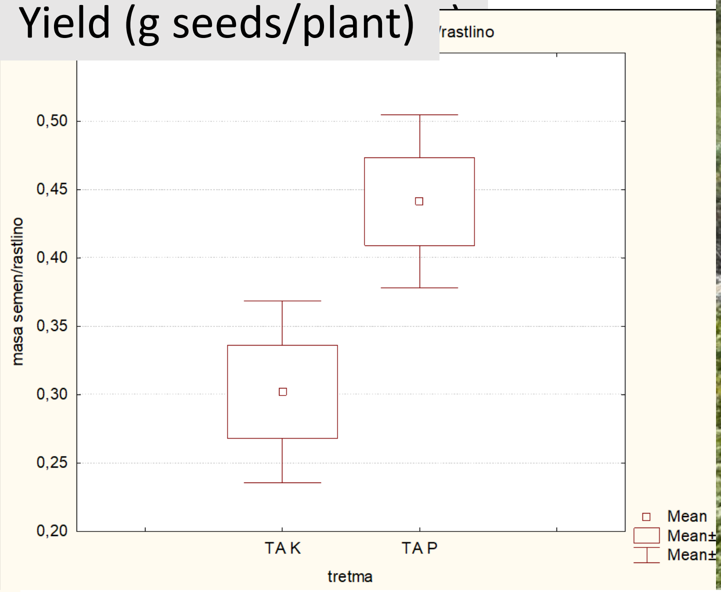


- **Effect on germination in the field studies**
  - Neutral or even positive!
    - Less % emerged plants / m2
    - ↑ yield (g of grains/plant)
    - Growth (+ trend)
  - Plasma does the "selection"???

Plant biomass



Yield (g seeds/plant)

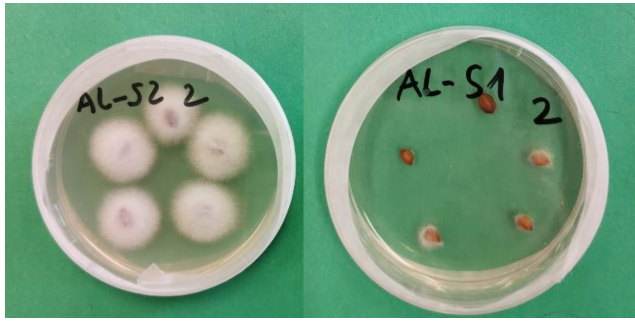


IV. Evaluating decontamination efficacy

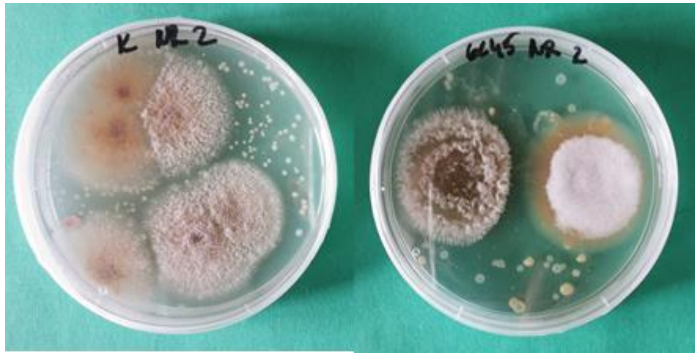
- **Methods/Techniques for evaluation**
  - **Direct plating method**
    - Infection rate,
    - infection degree
  - **Indirect method**
    - CFU per g

Direct – natural MO

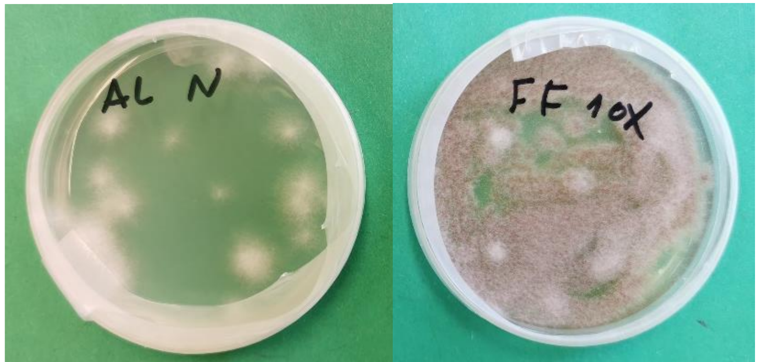
Direct – artificial MO

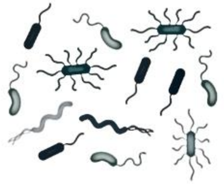


CFU – natural MO



CFU – artificial MO





IV. Evaluating decontamination efficacy

- **Methods/Techniques for evaluation**

Literature review

Table 1. An overview of experiments studying the effects of cold plasma on fungal decontamination in various plant species. AI: Artificially infected. NO: naturally occurring. CP: cold plasma. LP: low-pressure. AP: atmospheric-pressure. SMD: Surface micro-discharge. FB: fluidized bed. DCSBD: Diffuse Coplanar Surface Barrier Discharge. RF CC: radio-frequency capacitively coupled. (S)DBD: (Surface) Dielectric Barrier Discharge. CDPJ: Corona Discharge Plasma Jet. P: power. F: frequency. V: voltage. SPD: surface power density. PVD: power volume density. TT: treatment time. NT: not tested.

Fungal Species	Seed Type	Plasma Source and Properties	Gas Type and Exposure Time	Key Findings	% of Germination	Ref.
AI *Aspergillus* spp. and *Penicillium* spp.	Tomato (*Lycopersicon esculentum*), wheat (*Triticum durum*), bean (*Phaseolus vulgaris*), chick pea (*Cicer arietinum*), soybean (*Glycine max*) barley (*Hordeum vulgare*), oat (*Avena sativa*), rye (*Secale cereal*), lentil (*Lens culinaris*), and corn (*Zea mays*)	LP CP (500 mTorr); P: 300 W; F: 1 kHz; V: 20 kV	Air gas and SF_6; 5–20 min	Significant reduction to below 1% of initial fungal load	No significant effect on wheat and bean seeds	[6]
AI *Aspergillus parasiticus*	Hazelnuts (*Corylus avellane*), peanuts (*Arachis hypogaea*) and pistachio nuts (*Pistacia vera*)	LP CP (500 mTorr); P: 300 W; F: 1 kHz; V: 20 kV	Air gas and SF_6; 1–20 min	Air plasma 1-log reduction of initial load, SF_6 plasma more effective with app. 5-log decrease (after 5 min TT)	NT	[70]
NO microbiota	Chickpea (*Cicer arietinum*)	AP SMD CP; SPD: 10 mW/cm^2; V: 5–17 kV	Ambient air; 0.5–5 min	Significant reduction of 1–2 log of microbial contamination	Increased up to 3 min TT	[4]
AI *Aspergillus flavus* and *A. parasiticus*	Hazelnuts (*Corylus avellane*)	AP FB CP; P: 460–655 W; F: 25 kHz; V: 5–10 kV	Air gas and N_2; 1–5 min	Significant reduction of app. 4-log (CFU/g) after 5 min of air gas plasma TT	NT	[83,84]
AI *Fusarium circinatum* (pine pest)	Pine (*Pinus radiata*)	AP DCSBD CP; F: 14 kHz; V: 10 kV	Air gas; 5–300 s	Reduction of seedborne pathogens (14–100%)	No significant effect	[85]
NO microbiota and AI *Fusarium nivale*, *F. culmorum*, *Trichothecium roseum*, *A. flavus*, *A. clavatus*	Wheat (*Triticum aestivum*)	AP DCSBD CP; PVD: 100 W/cm^3; P: 400 W	Ambient air; 10–600 s for NO microflora; 1–300 s for AI fungi-infected seeds	Increased inhibition of microflora with increased treatment time; Total devitalization of NO filamentous fungi after 120 s TT	Increased up to 40 s TT, then decreased	[18]
NO microbiota and AI *A. flavus*, *Alternaria alternata*, *F. culmorum*	Maize (*Zea mays*)	AP DCSBD CP; PVD: 80 W/cm^3; P: 400 W	Ambient air; 60–300 s	Total devitalization of NO bacteria after 60 s and NO fungi 180 s TT	No significant effect up to 120 s TT, then decreased	[51]
NO fungi (*Fusarium*, *Alternaria*, *Stemphylium*)	Wheat (*Triticum aestivum*), spring barley (*Hordeum vulgare*), blue lupine (*Lupinus angustifolius*), soy (*Glycine soja*), and field pea (*Pisum arvense*)	AP RF CC CP; PVD: 0.6 W/cm^3; F: 5.28 MHz	Air gas; 2–20 min	Reduction of fungal infection; the most effective TT at 10 and 15 min	Little enhancement in blue lupine and field pea	[86]
NO fungal microbiota	Wheat (*Triticum aestivum*)	AP CP; F: 100 Hz–83 kHz; V: 8 kV	Air gas; 3–30 s	Reduction of fungal colonies on wheat grains at the optimum 10 s TT	No significant effect	[87]
NO microbiota	Sweet basil (*Ocimum basilicum*)	AP SDBD CP; SPD: 80 mW/cm^2; F: 5 kHz	Humid air; 10–600 s	Significant decrease in microbial load (up to 50% in 300 s TT)	No significant effect	[88]
NO microbiota (molds and yeasts)	Rapeseed (*Brasica napus*)	AP CDPJ CP; F: 58 kHz; V: 20 kV, 58 kHz	Air gas; 0.5–3 min	Reduction by 2-log units compared to initial count	Positive effect up to 1 min TT	[61]
NO bacteria and fungi	Ginseng (*Panax ginseng*)	AP DBD; F: 60 Hz; V: 120 V	Ar and Ar/O_2 mixture (80:20); 10 min each day, 3 days in a row	Ar/O_2 plasma mixture had better bactericidal and fungicidal effect	Positive effect in both mixtures	[89]
NO fungi (molds and yeasts)	Common buckwheat (*Fagopyrum esculentum*) and Tartary buckwheat (*F. tataricum*)	LP RF CP (50 Pa); P: 1400 W; F: 27.12 MHz	Pure O_2 plasma; 30–120 s	Reduction of seedborne fungi to below 50% of control after 120 s	No significant effect up to 45 s TT, then decreased	[33]

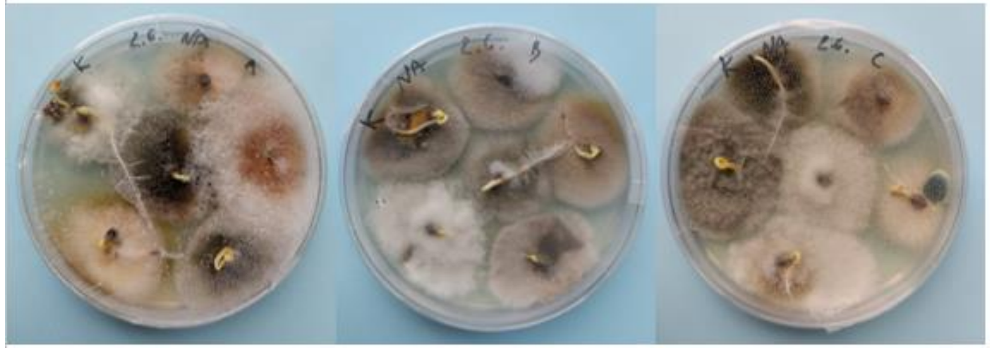
- Orange – native seed microbiome
- Red – artificially contaminated seeds
- Green – direct plating
- Blue – CFU (indirect) method

© Mravlje et al., 2021, Journal of Fungi, 7(8), 650
https://doi.org/10.3390/jof7080650

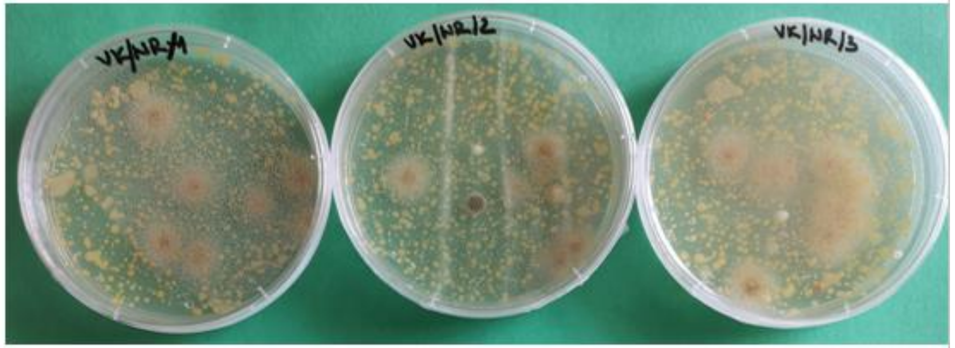
IV. Evaluating decontamination efficacy

- **Comparison of direct / CFU**
  - **Biological point of view**: same seeds (control) → different evaluation method → different results?
  - **Ecological relevance!** (diversity, lifestyle: S/E, P)

	Direct plating	CFU method
+	Eco-relevance, diversity	Quick, easy eval. and compare (log-reduction)
-	Time, N, experience with fungi (taxonomy)	Developed for bacteria, Misleading?(eco-relev.?)



A: direct plating of buckwheat seeds on PDA medium; after 1 week of growth



B: CFU count of buckwheat seeds (1g of seeds suspended in saline → diluted); 1 week

V. Comparison of Methods

- **Is sensitivity to CP species specific?**
  - 1st problem = different attachment rate of spores!

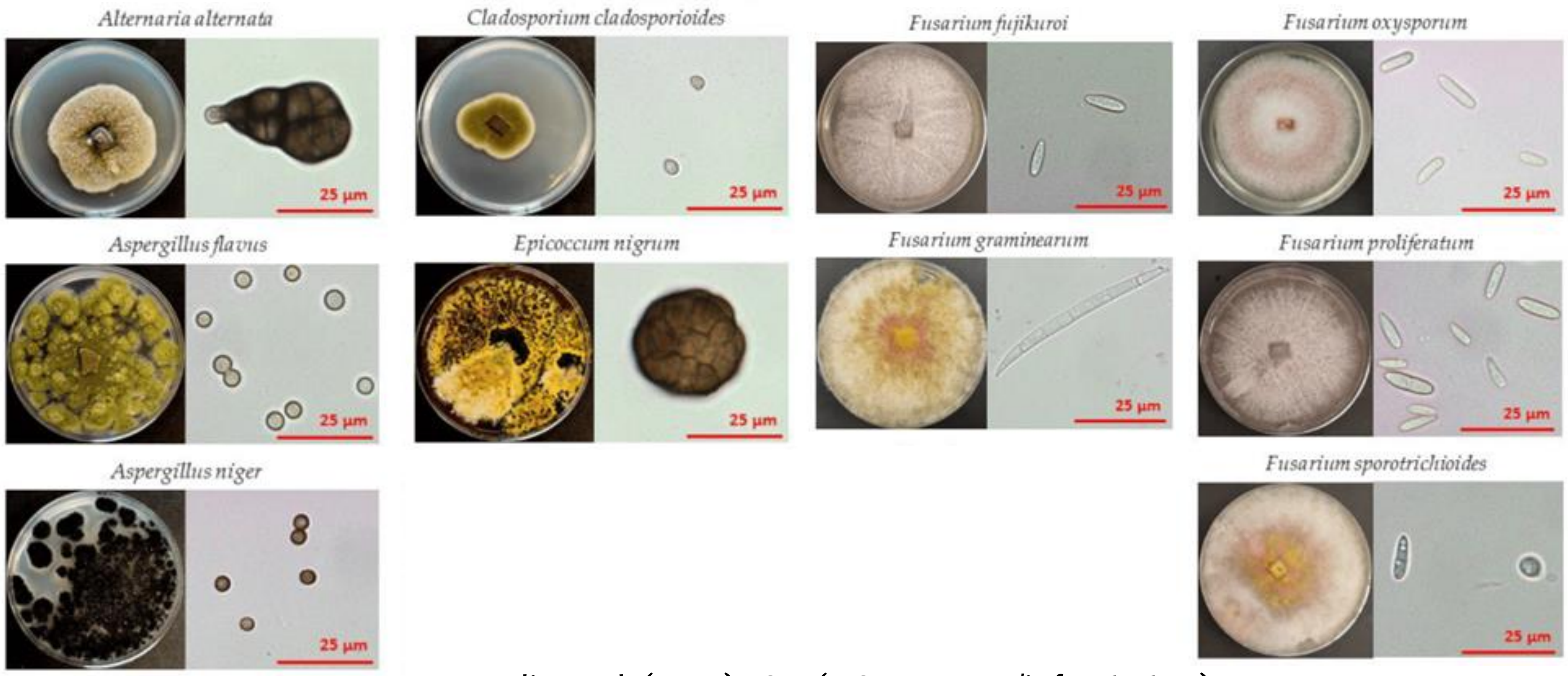


Table 1. Colony forming units (CFU) in control and after different cold plasma treatment (CPT) exposure times for each fungus expressed in log CFU g^{-1} grains. Different letters (superscripts) indicate statistically significant differences between each fungus's control and respective CPT. AA—*Alternaria alternata*; AF—*Aspergillus flavus*; AN—*Aspergillus niger*; CL—*Cladosporium cladosporioides*; EN—*Epicoccum nigrum*; FF—*Fusarium fujikuroi*; FG—*Fusarium graminearum*; FO—*Fusarium oxysporum*; FP—*Fusarium proliferatum*; FS—*Fusarium sporotrichioides*.

Fungi	Control	30 s CPT	60 s CPT	120 s CPT	180 s CPT
AA	3.57 ± 0.1 ^{a}	3.21 ± 0.1 ^{a}	0.77 ± 0.8 ^{b}	0.0 ± 0.0 ^{c}	0.0 ± 0.0 ^{c}
AF	4.18 ± 0.1 ^{a}	3.01 ± 0.1 ^{b}	0.67 ± 0.7 ^{c}	0.0 ± 0.0 ^{c}	0.0 ± 0.0 ^{c}
AN	4.41 ± 0.0 ^{a}	1.33 ± 0.7 ^{b}	1.33 ± 0.7 ^{b}	0.0 ± 0.0 ^{b}	0.0 ± 0.0 ^{b}
CL	3.98 ± 0.0 ^{a}	2.43 ± 0.1 ^{b}	0.67 ± 0.7 ^{c}	0.0 ± 0.0 ^{c}	0.0 ± 0.0 ^{c}
EN	3.91 ± 0.1 ^{a}	3.62 ± 0.0 ^{a}	1.33 ± 0.7 ^{b}	0.0 ± 0.0 ^{b}	0.0 ± 0.0 ^{b}
FF	6.34 ± 0.1 ^{a}	5.85 ± 0.1 ^{b}	4.99 ± 0.0 ^{c}	3.70 ± 0.0 ^{e}	4.0 ± 0.1 ^{d}
FG	3.58 ± 0.0 ^{a}	0.0 ± 0.0 ^{b}	0.0 ± 0.0 ^{b}	0.0 ± 0.0 ^{b}	0.0 ± 0.0 ^{b}
FO	5.68 ± 0.0 ^{a}	5.44 ± 0.0 ^{b}	4.50 ± 0.0 ^{c}	3.96 ± 0.0 ^{d}	2.46 ± 0.1 ^{e}
FP	5.94 ± 0.1 ^{a}	5.70 ± 0.1 ^{a}	4.78 ± 0.0 ^{b}	2.80 ± 0.1 ^{c}	2.93 ± 0.1 ^{c}
FS	5.27 ± 0.0 ^{a}	4.40 ± 0.0 ^{b}	3.99 ± 0.0 ^{c}	2.26 ± 0.1 ^{e}	2.71 ± 0.1 ^{d}

Mravlje et al. (2023) JOF. (DOI: 10.3390/jof9060609)

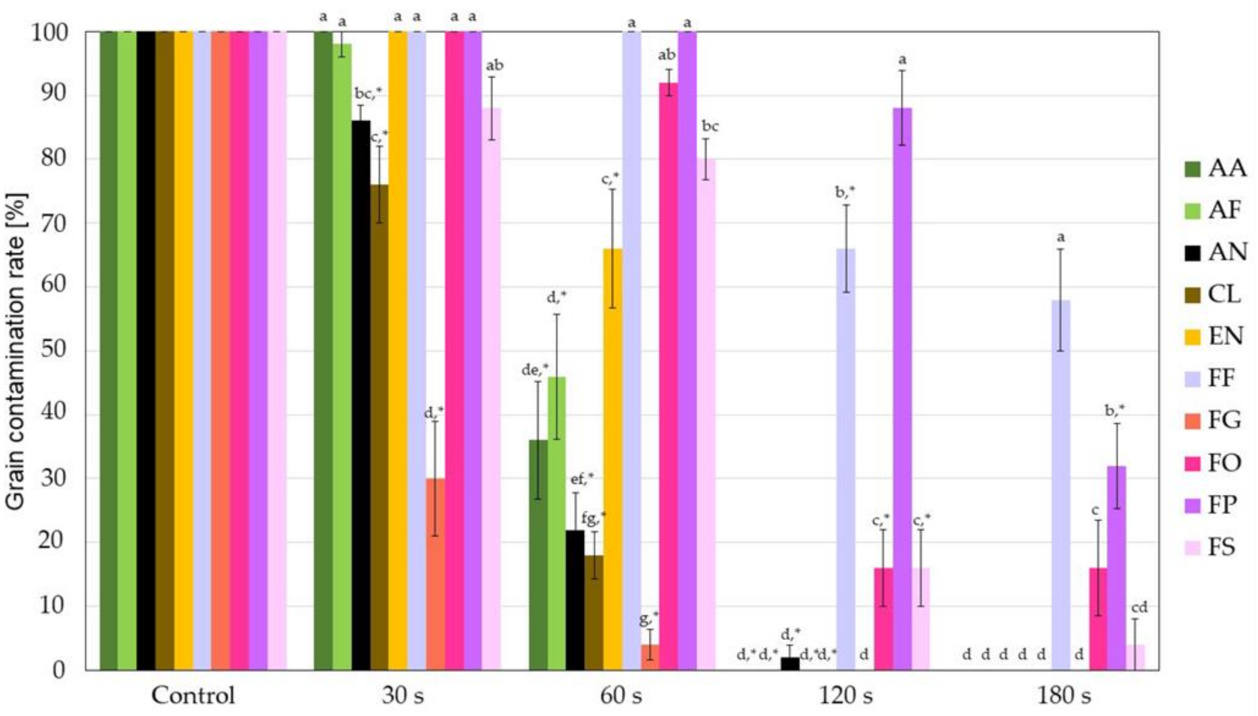
V. Comparison of Methods

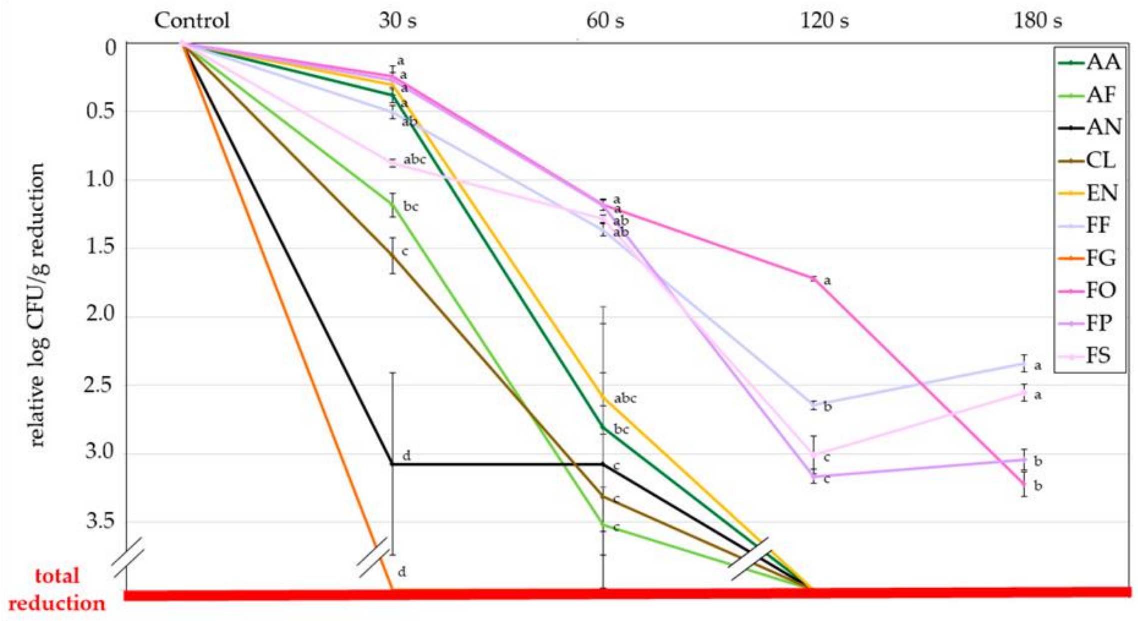
- **<u>Is sensitivity to CP species specific?</u>**
  - 2nd problem = a bit different results,
  - Similair trends!

F. graminearum – the most sensitive ✓
- Larger/multicellular spores → ↑ surface → CP ↑ efficient?

other *Fusarium* species – microconidia
- Advantage in CP treatment?

Melanised spores (*Alternaria, Epicoccum*) – advantage to some point? Not in LP CP?



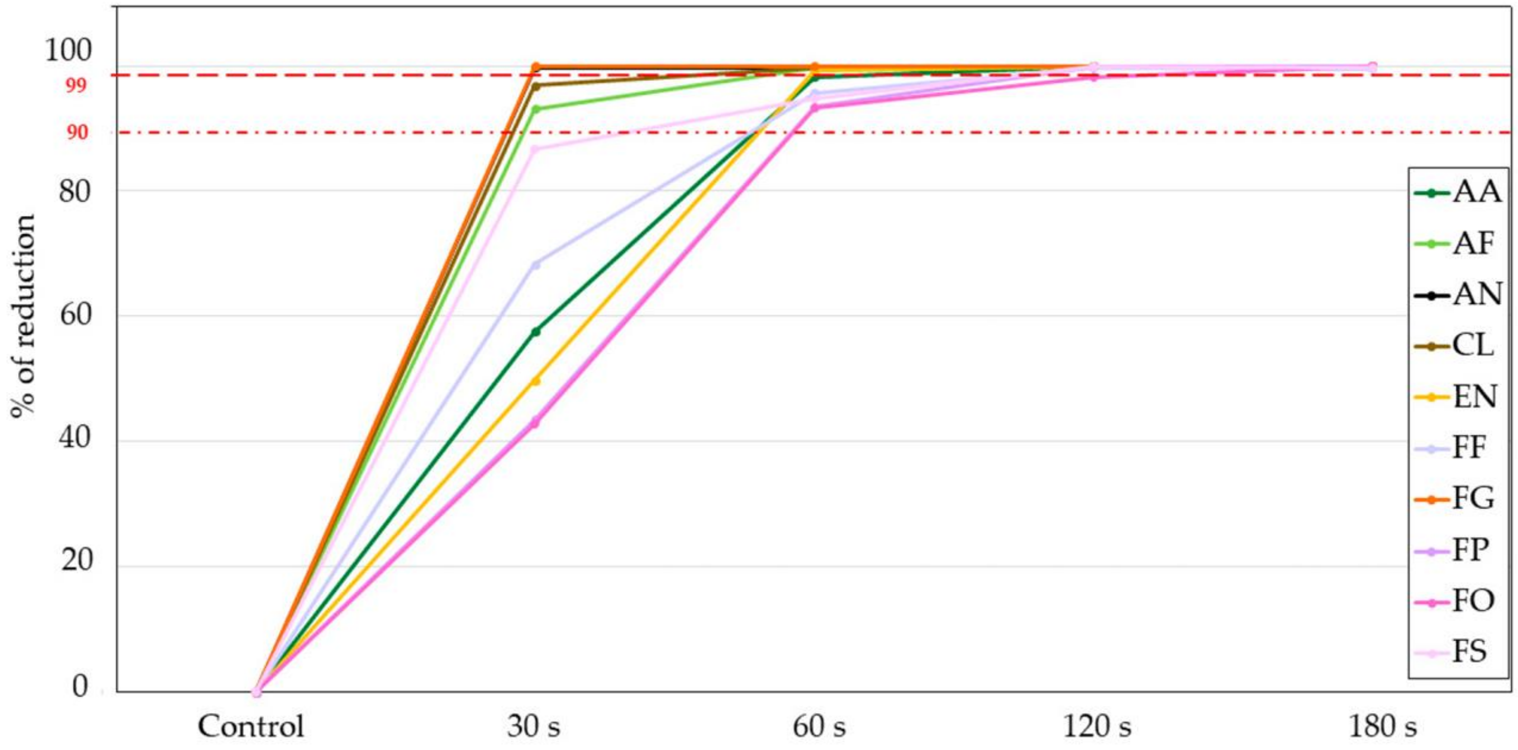


Mravlje et al. (2023) JOF. (DOI: 10.3390/jof9060609)

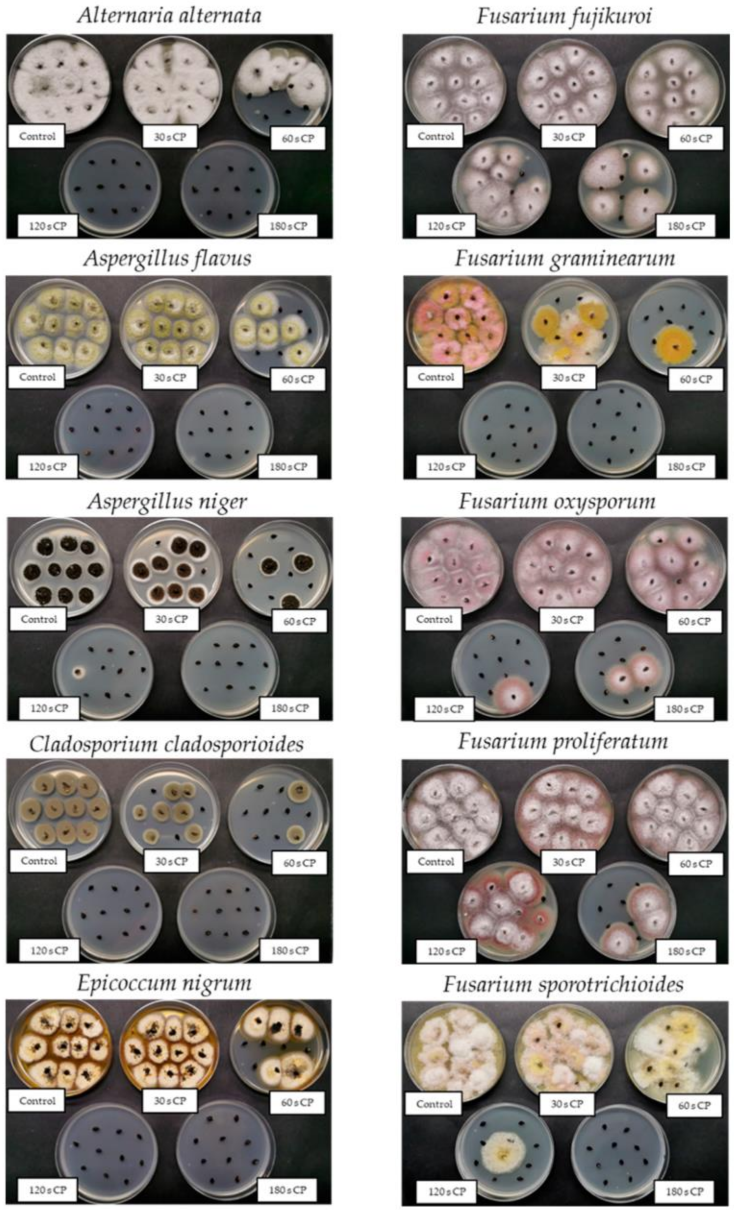
UNIVERSITY OF LJUBLJANA
Biotechnical Faculty

V. Comparison of Methods

- **Is sensitivity to CP species specific?**
  - 3rd problem = how to report your results…

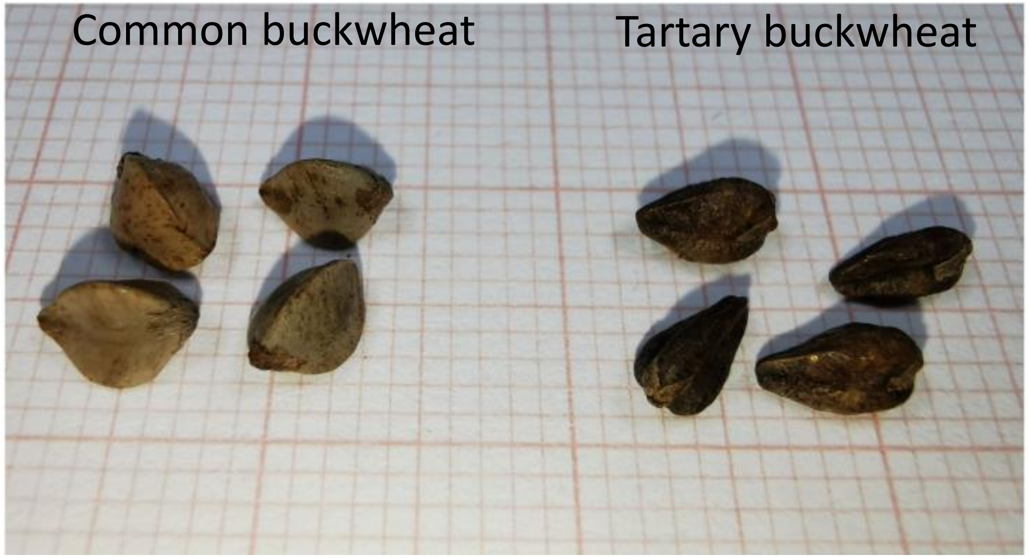


Mravlje et al. (2023) JOF. (DOI: 10.3390/jof9060609)



VI. Problems in CP seed decontamination

- **SEEDS**
  - **“Internal”:**
    - Seed type (species): size, shape, surface, color…
    - Dormancy, vernalization
    - Maturation (seed age) →
    - Viability
  - **“External”:**
    - Seasonal effect on germination
    - Experimental conditions (besides CP treatment)
    - Light, temperature
    - Storage conditions
    - …

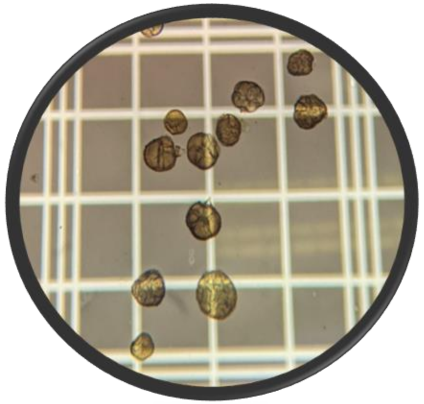


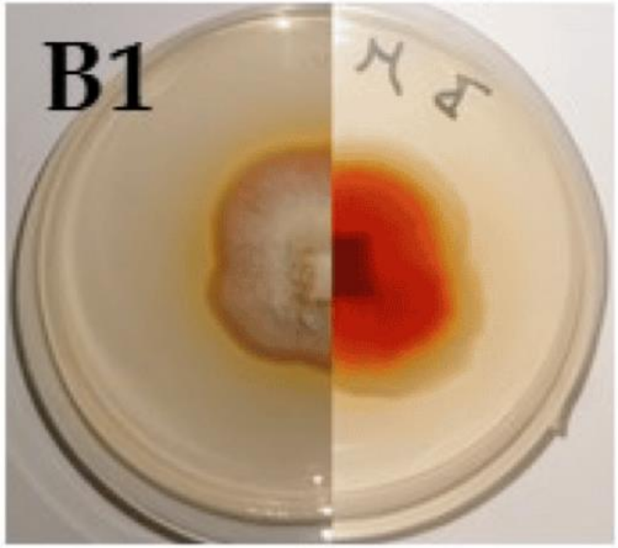
VI. Problems in CP seed decontamination

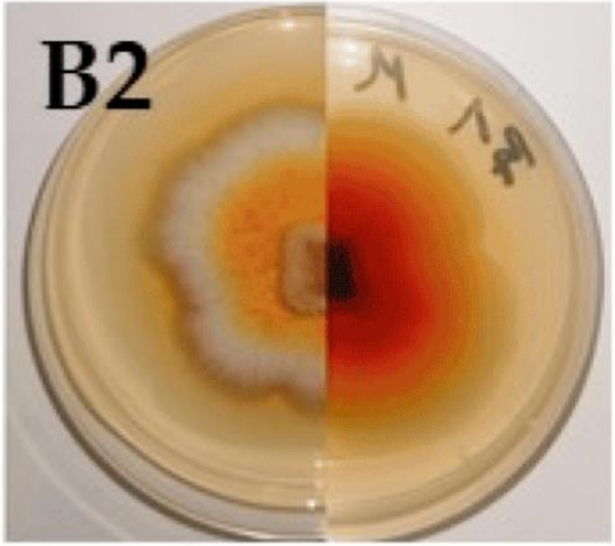
- **FUNGI**
  - COUNTLESS
    - Species → subspecies → strain → … changing with time! (lab?)
    - Growing conditions
      - Light, temperature, humidity … growing medium!
    - Growth type:
      - Teleomorph (sexual) / anamorph (asexual stage)
    - Growth stage:
      - Mycelia (hyphae)
      - Spores:
        - Asexual (mitospores): conidia, chlamydospores…
        - Sexual (meiospores): asco-, basidio-, zygospores…

VI. Problems in CP seed decontamination

- Example: *Epicoccum nigrum*







Epicoccum nigrum – cosmopolitan fungus, endophyte: medical, industrial and agricultural importance – source of many secondary metabolites.

VI. Problems in CP seed decontamination

- And then comes the PLASMA… :O :O :O

VI. Problems in CP seed decontamination

- **Review Paper**
  JoF, 2021

https://doi.org/10.3390/jof7080650

Journal of *Fungi*

MDPI

Review

Development of Cold Plasma Technologies for Surface Decontamination of Seed Fungal Pathogens: Present Status and Perspectives

Jure Mravlje [1,*], Marjana Regvar [1] and Katarina Vogel-Mikuš [1,2]

[1] Biotechnical Faculty, University of Ljubljana, 1000 Ljubljana, Slovenia; marjana.regvar@bf.uni-lj.si (M.R.); katarina.vogelmikus@bf.uni-lj.si (K.V.-M.)
[2] Jožef Stefan Institute, 1000 Ljubljana, Slovenia
* Correspondence: jure.mravlje@bf.uni-lj.si

Abstract: In view of the ever-growing human population and global environmental crisis, new technologies are emerging in all fields of our life. In the last two decades, the development of cold plasma (CP) technology has offered a promising and environmentally friendly solution for addressing global food security problems. Besides many positive effects, such as promoting seed germination, plant growth, and development, CP can also serve as a surface sterilizing agent. It can be considered a method for decontamination of microorganisms on the seed surface alternative to the traditional use of fungicides. This review covers basics of CP technology and its application in seed decontamination. As this is a relatively young field of research, the data are scarce and hard to compare due to various plasma setups and parameters. On the other hand, the rapidly growing research field offers opportunities for novel findings and applications.

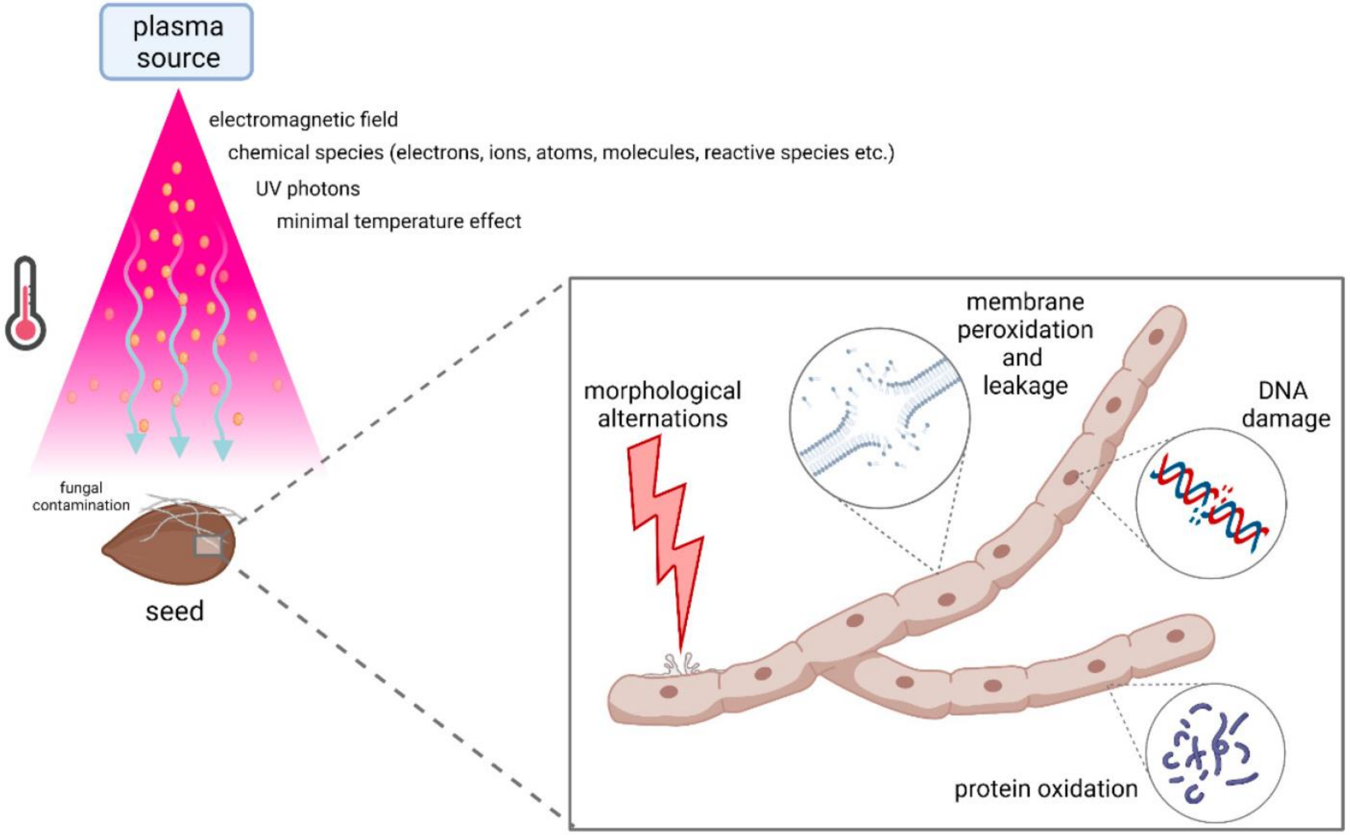
Keywords: cold plasma; seeds; grains; microorganisms; fungi; agriculture; plant production; food security

check for updates

Citation: Mravlje, J.; Regvar, M.; Vogel-Mikuš, K. Development of Cold Plasma Technologies for Surface Decontamination of Seed Fungal Pathogens: Present Status and Perspectives. *J. Fungi* **2021**, *7*, 650.

VI. Problems in CP seed decontamination

- **How does CP work on Fungi?**



- **Plasma apparatus!** (properties)
  - Atmospheric / reduced pressure
  - Glow (shorter) / afterglow (safer)
  - Source, feeding gas, operating pressure…
  - PD of plasma
  - MO!
- ***Non-linear survival curves*** (complex process)
- Efficient: many cellular targets, complex biological interactions → unlikely resistance mechanisms development
- Thermal effect?
- Different studies – different conclusions!

© Mravlje, Regvar & Vogel-Mikuš (2021) DOI: 10.3390/jof7080650

BF UNIVERSITY OF LJUBLJANA Biotechnical Faculty

The End ☺

*"(Ladies and) Gentlemen, **it is the microbes who will have the last word!**" (Louis Pasteur)*

Acknowledgements

- *Prof. dr. **Katarina Vogel-Mikuš** & prof. dr. **Marjana Regvar***
- Other **colleagues from Laboratory for Plant Physiology**, Dept. of Biology, Biotechnical faculty, University of Ljubljana
- Colleagues from "Jožef Stefan" Institute (group of prof. dr. Miran Mozetič)
- Slovenian Research Agency (ARRS) for funding: P1-0212 (Plant Biology), project J1-3014

Literature & Sources

- Brock Biology of Microorganisms, 16th ed. (2021) Pearson Education.
- Campbell Biology, 12th ed. (2021) Pearson Education.
- Plant Pathology, 5th ed. (2005) Elsevier Academic Press.
- All the Articles cited (DOI in text).
- Personal archive (J. Mravlje & colleagues)