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SAGWRI South African Grape and Wine Research Institute

Evaluation of biocontrol agents through isolation, characterization, and fermentation trials for sustainable wine production

Dr. Evelyn Maluleke

SASEV 2025

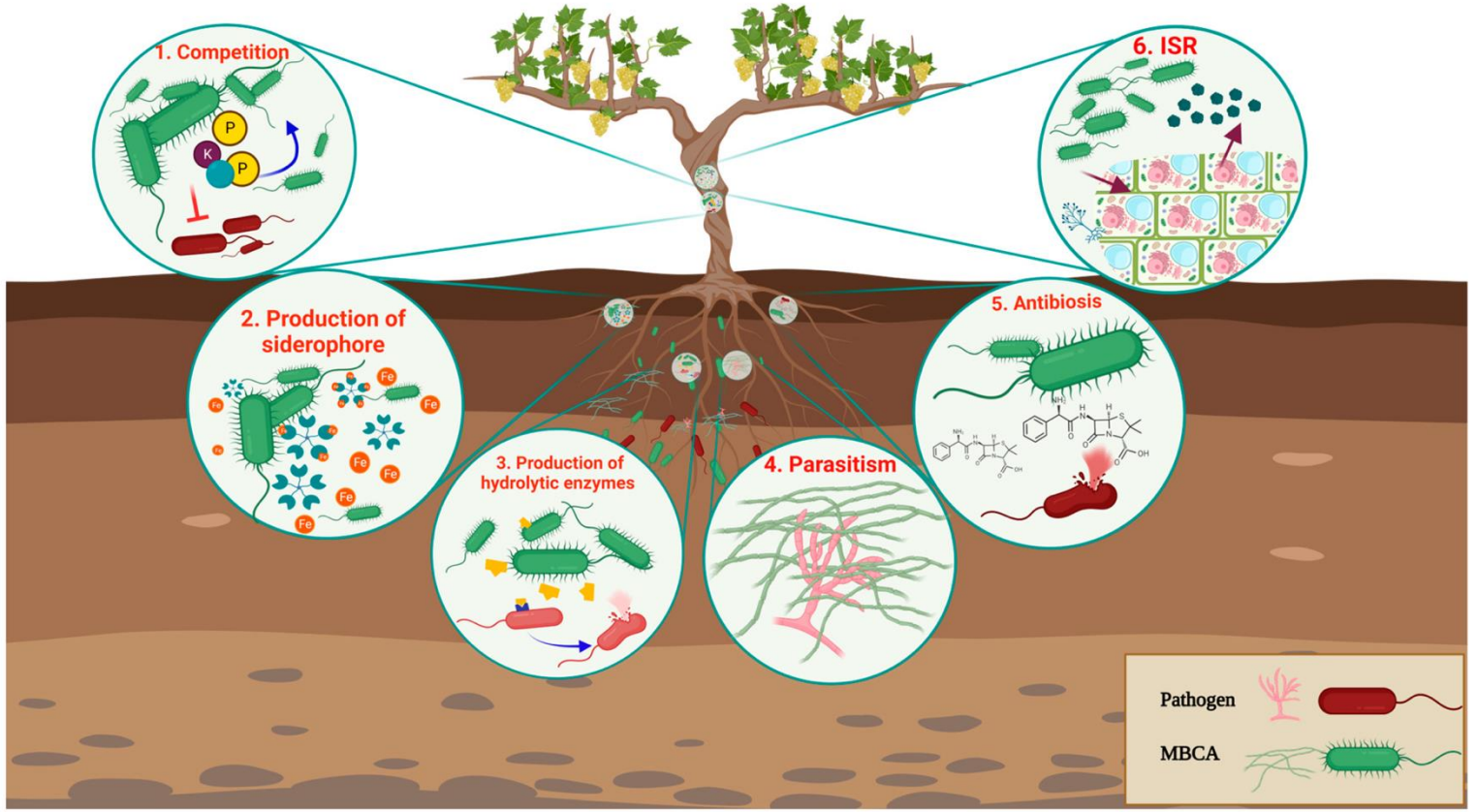
Biocontrol agents

- **Biological control agents** are living organisms (yeasts, bacteria, fungi, or even insects) derived from nature and used to suppress pests, weeds, or plant diseases.
- **In vineyards:** used against *Botrytis cinerea*, powdery mildew (*Erysiphe necator*), downy mildew (*Plasmopara viticola*), and sour rot organisms.
- **Application:** Soil inoculation, flowering sprays, pre harvest, postharvest
- **Integration:** used alone or in conjunction with fungicides in integrated pest management (IPM).
- BCAs don't fully replace chemicals, reduce the doses of fungicides, and lower the chemical residues on grapes.

Biocontrol agents

BCAs Modes of action:

- **Competition:** Outcompetes pathogens for nutrients and space.
- **Antibiosis:** Produce antifungal metabolites that inhibit pathogen growth.
- **Enzymes:** Secrete hydrolytic enzymes (chitinase, glucanase) that degrade fungal cell walls.
- **Induction:** Triggers plant defence responses
- **Biofilms:** Form protective biofilms on plant surfaces, enhancing persistence



(Quiza et al., 2023; Diez-Mendez et al., 2024; Tsioka et al., 2024; Fedele et al., 2025)

Limited success in vineyards

- **Limited success** in vineyards despite promising BCA mechanisms
- **Few candidates** reach commercialization due to performance limits
- Vineyard variability (environment, colonization, practices) lowers reliability
- Field results often inconsistent and sensitive to climate factors
- **Research continues** to find strains with stronger, stable efficacy

Research Aim

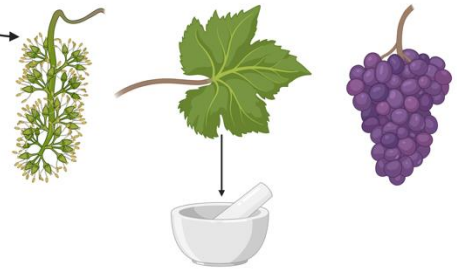
- This study forms part of the SA-Italy BiSus4Wine project, which focuses on sustainable viticulture through microbial innovation.
- This study aims to expand the portfolio of biological control agents by isolating and characterizing microorganisms from vineyards managed under different farming practices, to identify strains capable of performing effectively across diverse vineyard conditions.

Research Objectives

- Isolating microorganisms from grapevine tissues across various vineyards with different farming practices
- Screening isolates for antifungal activity against *Botrytis cinerea*
- Identifying promising isolates via Sanger sequencing
- To determine the modes of action of selected yeast and bacterial strains against *B. cinerea*
- Evaluating fungicide tolerance of potential biocontrol agents

Sample collection and microbial isolation (bacteria and yeasts)

Reyneke
De Toren
Welgevallen



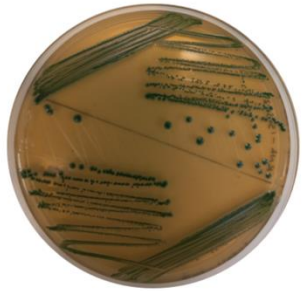
Washing-Saline

Agitate for 1 hour

Endophyte and epiphytes isolation

Bacteria:
NA and KB agar

Yeasts:
MEA
WLN
Grape juice agar



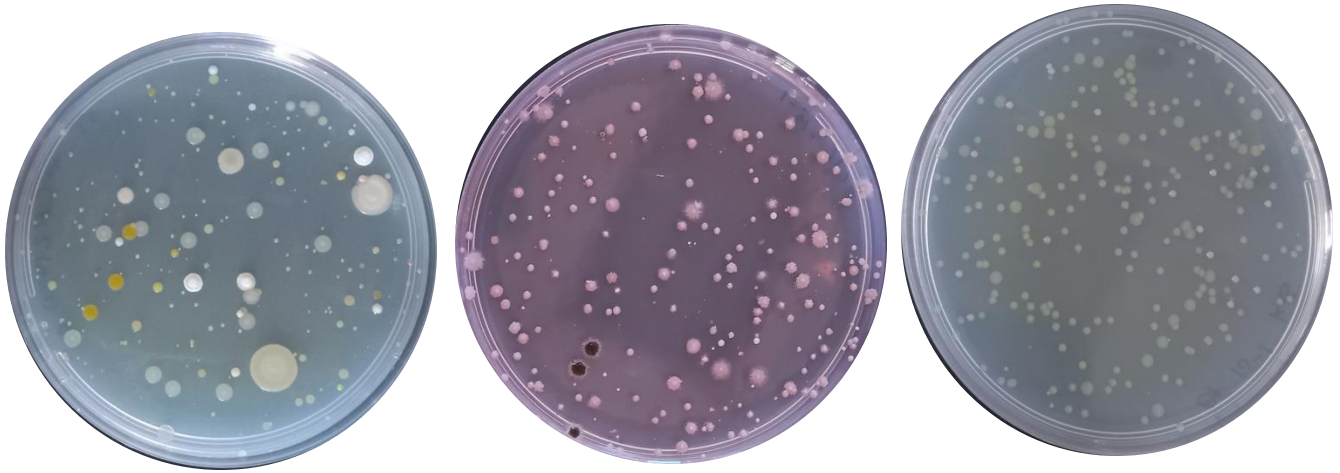
Sub-culturing

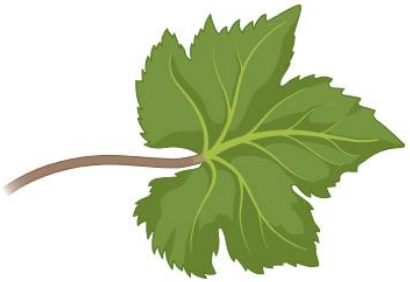
cryopreserved cultures at -80°C

Long-term storage

Isolates diversity

Bacteria = 134 isolates
Yeasts = 100 isolates





Bacteria = 155
Yeasts = 50 isolates

Bacteria = 94
Yeasts = 114

- Retrieved a **large number** of bacterial and yeast isolates from **grapevine tissues**
- **Yeasts** isolated mainly from **grapes** rather than leaves or flowers
- Grapes provide a nutrient rich, sugary environment → highly conducive to yeast growth and colonization
- Outcome: diverse pool of isolates available for screening

Botrytis as a model fungus

- ***B. cinerea*** is widely used as a model pathogen for screening microbial antagonism or BCAs.
- Easy to culture, reproducible infection assays, clear inhibition readouts.
- While *Botrytis* is a useful lab model, the wine industry's bigger concerns are powdery mildew and downy mildew.

(Hrycan et al., 2023; Díaz et al., 2023)

Primary screening- Preventive treatment against *B. cinerea*

Experiment Setup

- Seedless white grapes **disinfected and gently wounded**
- **Applied preventive yeast/bacteria treatment** applied to wounds (~10^6 cells/ml)
- **Pathogen inoculation:** *Botrytis cinerea* spores (~10^6 spores/ml) added after 24 h
- Incubation: 25 °C, airtight container, high humidity
- **Negative control:** grapes inoculated with *B. cinerea* only
- **Positive isolates:** those that completely inhibited *Botrytis* growth

B. cinerea

Number screened: 159 bacteria and 159 yeasts

- +ve isolates of yeasts = 28 yeast and 12 (yeast-like fungus), ***Aureobasidium*** (Not selected for further screening)- **commercial products**
- +ve isolates of bacteria = 39

Identification of positive isolates - DNA-based identification

Farming Method	Cultivar	Origin	Bacterial species Identified
Biodynamic	Chenin blanc	Flowers	*Pantoea ananatis, Pantoea agglomerans, Pantoea allii, Pantoea sp., Pseudomonas syringae, Curtobacterium plantarum, Erwinia aphidicola, Serratia marcescens*
Conventional	Cabernet franc	Leaves	*Pantoea agglomerans, Klebsiella pasteurii*
Conventional	Cinsaut Noir	Leaves	*Pantoea agglomerans, Klebsiella pasteurii, Klebsiella grimontii, Erwinia aphidicola (strain), Pseudomonas pudita*
Conventional	Mouverdre	Leaves	*Pantoea agglomerans, Klebsiella oxytoca, Erwinia billingiae (strain)*
Conventional	Muscat d'Alexandrie	Leaves	*Klebsiella grimontii*

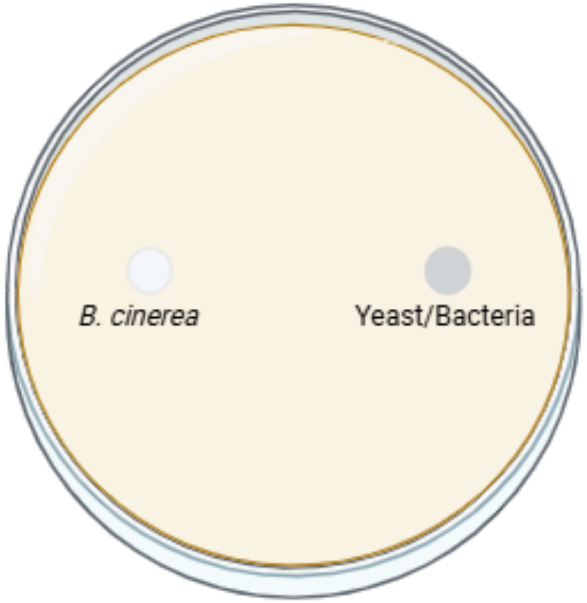
Farming Method	Cultivar/Varietal	Organ	Yeast species Identified
Biodynamic	Chenin blanc	Grapes	*Hanseniaspora opuntiae, Hanseniaspora uvarum, Metschnikowia hawaiiana, Aureobasidium pullulans, Candida africana, Hanseniaspora guilliermondii, Hanseniaspora pseudoguilliermondii*
	Cabernet Franc	Grapes	*Metschnikowia bicuspidata, Metschnikowia pulcherrima, Aureobasidium pullulans*
Organic	Cabernet Sauvignon	Grapes	*Aureobasidium pullulans*
	Cabernet Franc	Grapes	*Aureobasidium pullulans, Filobasidium magnum*
Conventional	Cabernet Franc	Flowers	*Aureobasidium pullulans, Metschnikowia reukaufii*
	Mouverdre	Flowers	*Rhodotorula glutinis, Metschnikowia sp., Aureobasidium pullulans*
	Cinsaut Noir	Flowers	*Filobasidium magnum*

- **Bacterial isolates:** *Pantoea agglomerans*, *Pseudomonas* spp among others.
- **Yeast isolates:** **Various** *Metschnikowia species: M. reukaufii, M. pulcherrima, M. hawaiiana* *Aureobasidium pullulans* and various *Hanseniaspora* spp.

(Zhao et al., 2022; Ayogu et al., 2024)

Secondary screening - *In vitro* inhibition of different *B. cinerea* strains

- All confirmed yeast and bacterial isolates were included in the secondary screening
- **Standardized concentrations** (~10^6 cells/ml) of yeast/bacteria co-inoculated opposite *B. cinerea* on Malt Extract Agar
- Assay tested against three ***B. cinerea* strains,** with **FF1** confirmed as **hypervirulent** (used for further assays)
- **Mycelial inhibition** measured relative to *Botrytis*-only controls

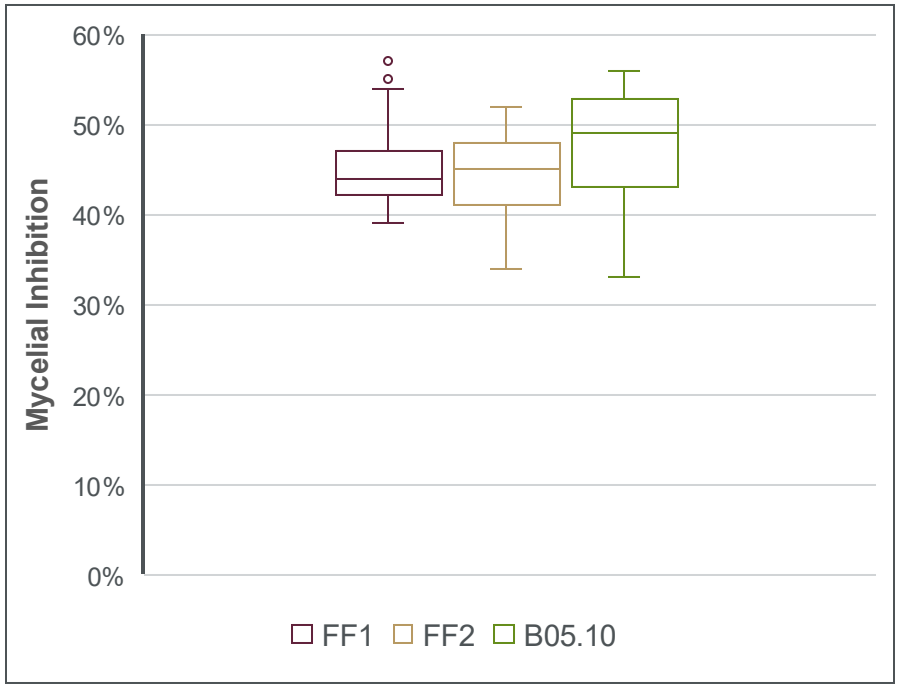


Dual culture plate assay

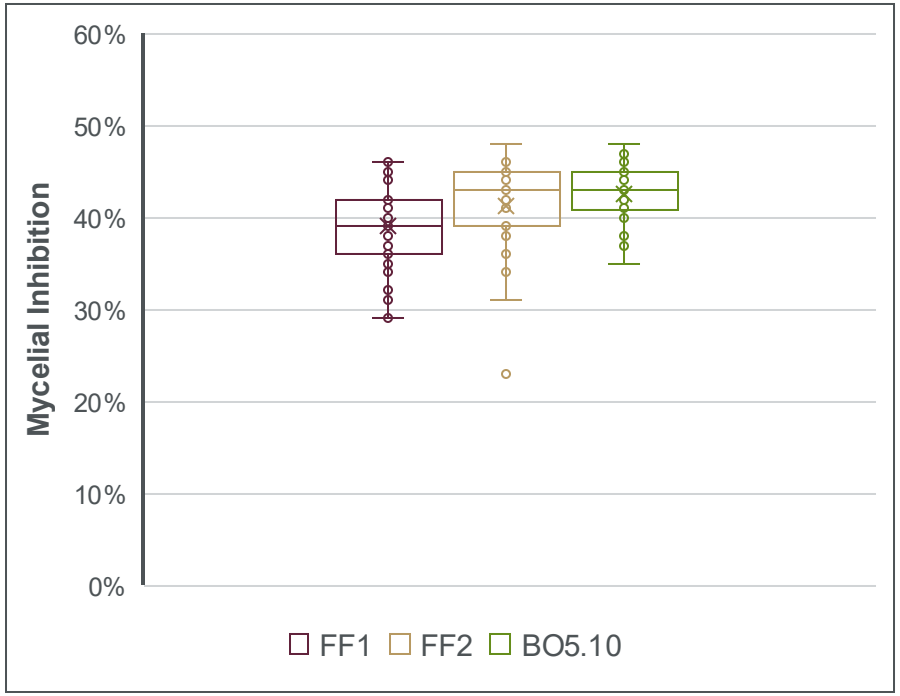
(Rowe and Kliebenstein, 2008; Chen et al 2018; Maluleke et al., 2022)

In vitro inhibition of different *B. cinerea* strains

Bacterial isolates



Yeast isolates

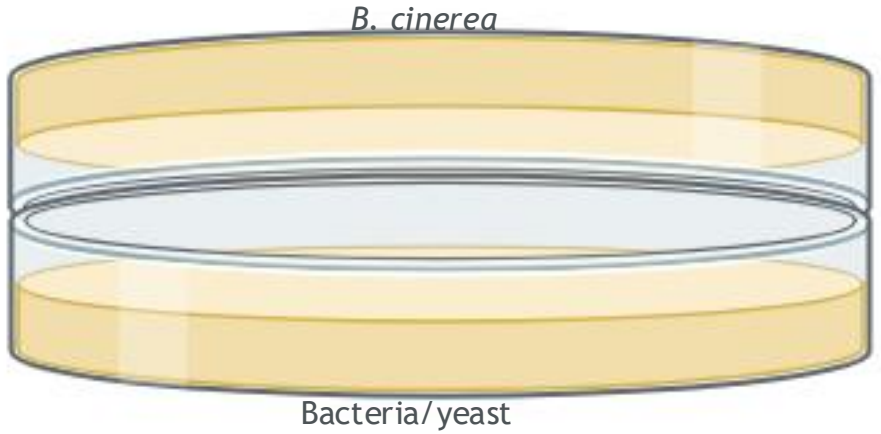


- **Bacterial isolates:** median inhibition **40-50%**; BO5.10 generally most sensitive
- **Yeast isolates:** median inhibition against BO5.10 **44-55%**, higher than FF1 (~46%) and FF2 (~45%)
- Several isolates consistently inhibited **multiple *Botrytis* strains** → potential **broad-spectrum biocontrol agents**

Modes of actions - Double petri dish assay

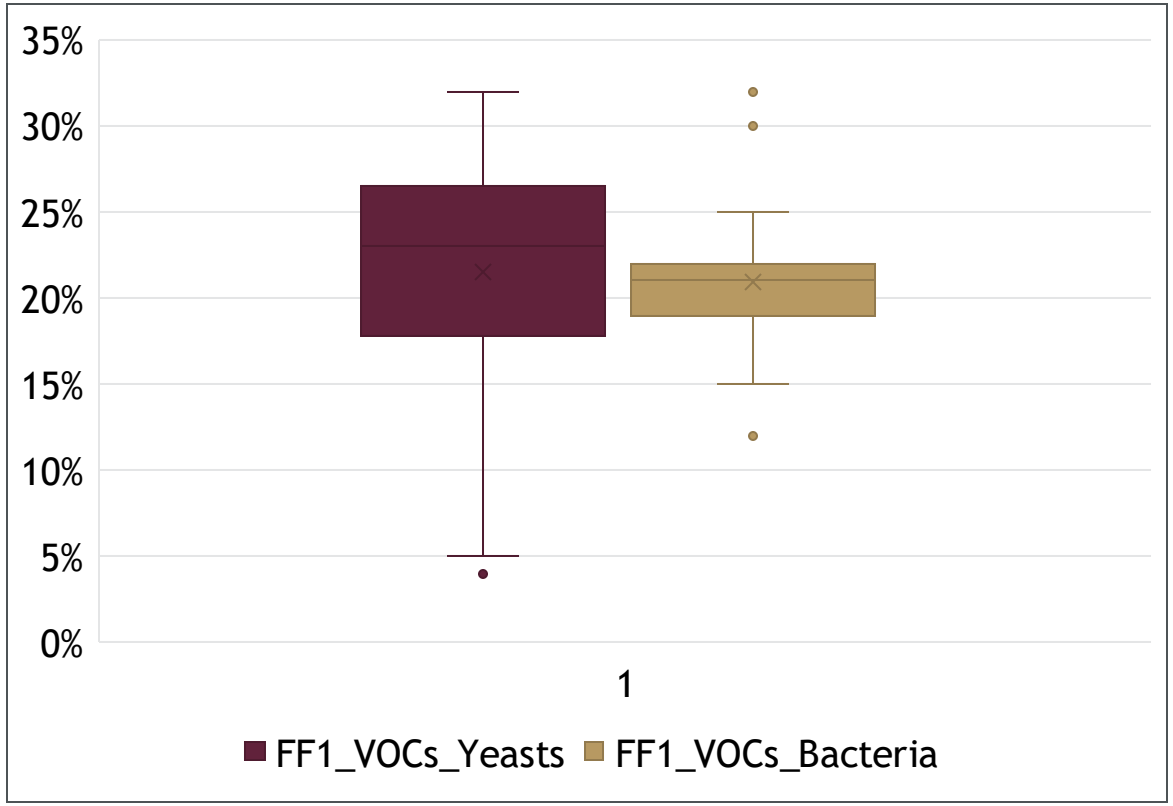
- **A double Petri dish setup is used in Volatile organic compounds (VOCs) assays to test how volatiles from yeast/bacteria affect *B. cinerea***

- Yeast or bacteria inoculated opposite *B. cinerea* on agar plates
- Plates sealed with **parafilm → prevents VOC escape and contamination**
- Incubation: **5 days at 25 °C**
- *Botrytis*-only plates used as **controls**
- Mycelial diameter measured



Effect of VOCs on *B. cinerea* growth

Mycelial growth inhibition of selected bacterial isolates against *B. cinerea* FF1



- VOCs showed **modest inhibition** of *B. cinerea* (~24%), weaker than direct-contact assays
- **Low activity is normal** and consistent with published ranges
- Effects mainly on **spore germination** and **hyphal extension**

Proven VOCs:

- Acetoin, 2,3-butanediol, 2-phenylethanol, isobutanol, Ethyl acetate, Butan-1-ol, 3-methyl-1-butanol (isoamyl alcohol).

(Cordero-Bueso et al., 2017; Ayogu et al., 2024)

Modes of actions - Hydrolytic enzyme assay

B. cinerea cell walls contain **chitin** and **β-1,3-glucan** as major components

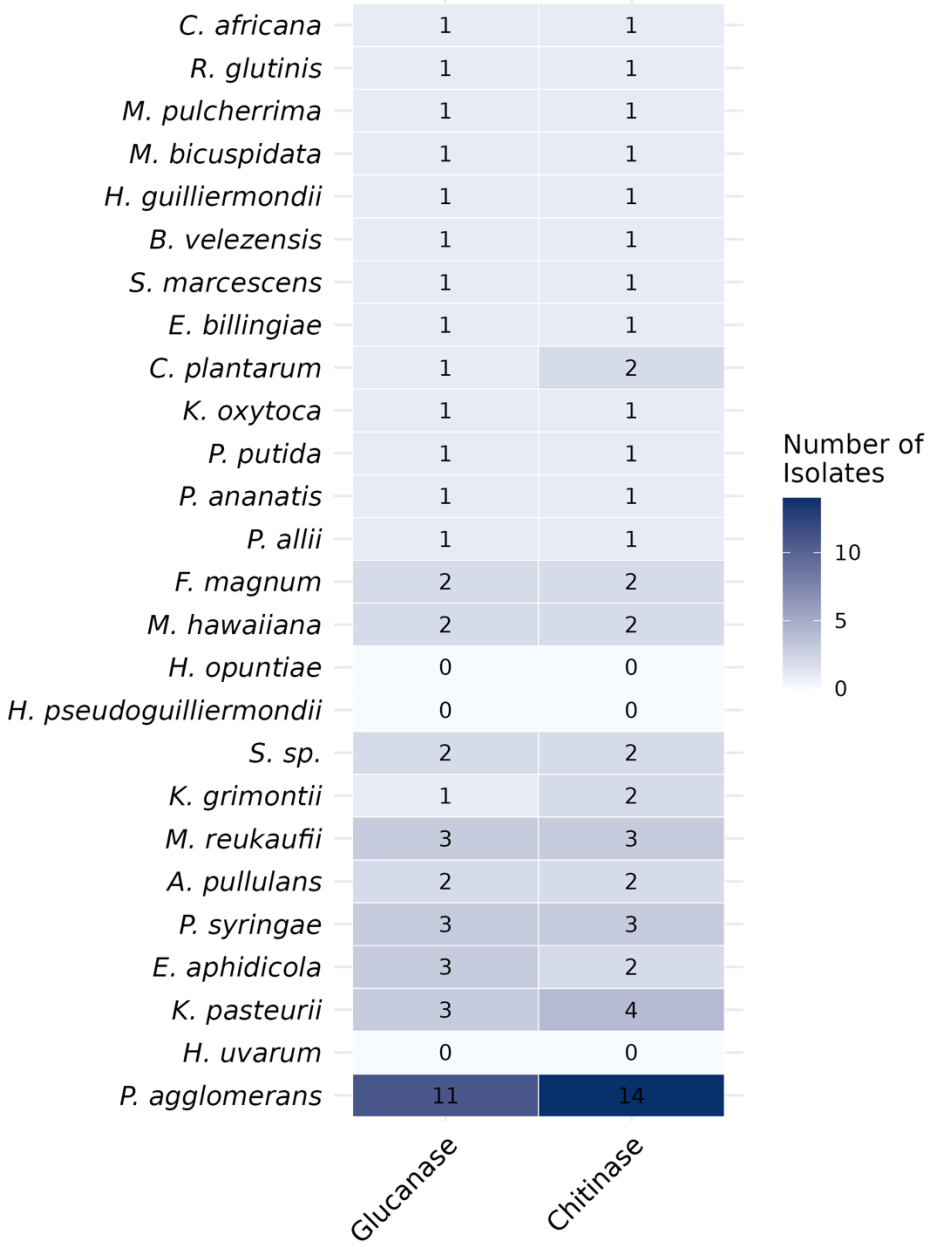
- Chitinase and β-1,3-glucanase degrade these polymers can **weaken pathogens**
- Isolates tested for **chitinase and glucanase** using **substrate-enriched dual plate assays**

Chitinase: purple zone on colloidal chitin plates

Glucanase: clear zone after congo red staining

(Maluleke et al., 2022)

Production of chitinase and glucanase by yeasts and bacteria

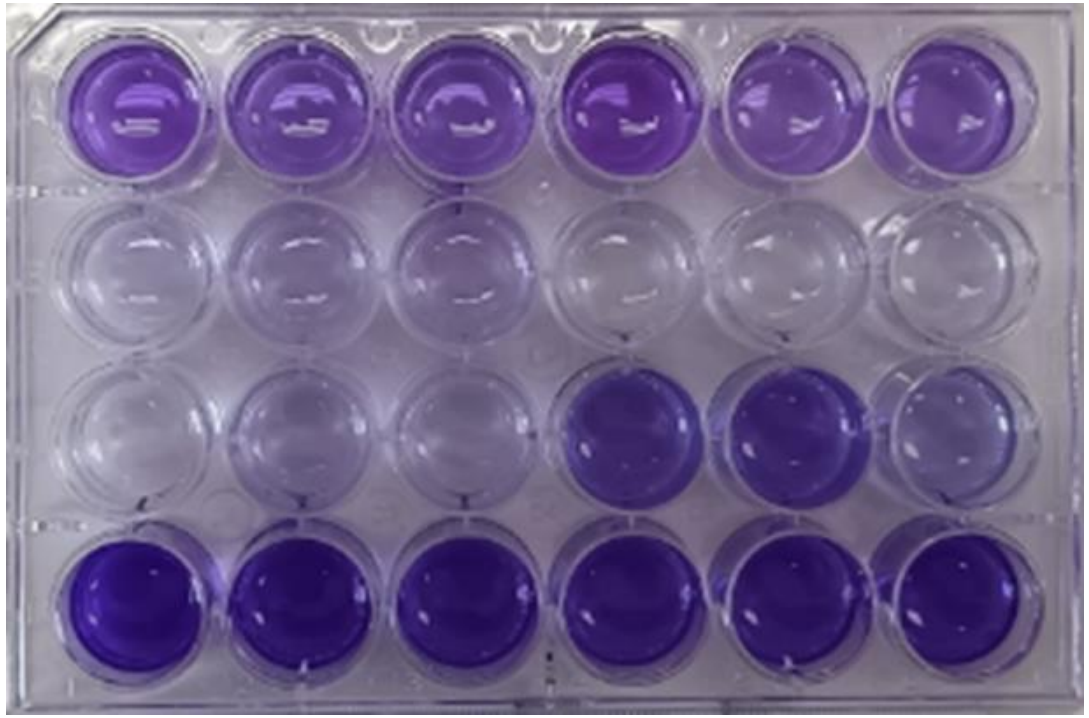


- Enzymes were **widely distributed** across bacteria and yeasts
- **Activity was variable across isolates** even within the same species
- Example: **all *P. agglomerans* isolates produced chitinase**, but **not all produced glucanase**

Modes of actions - Biofilm formation

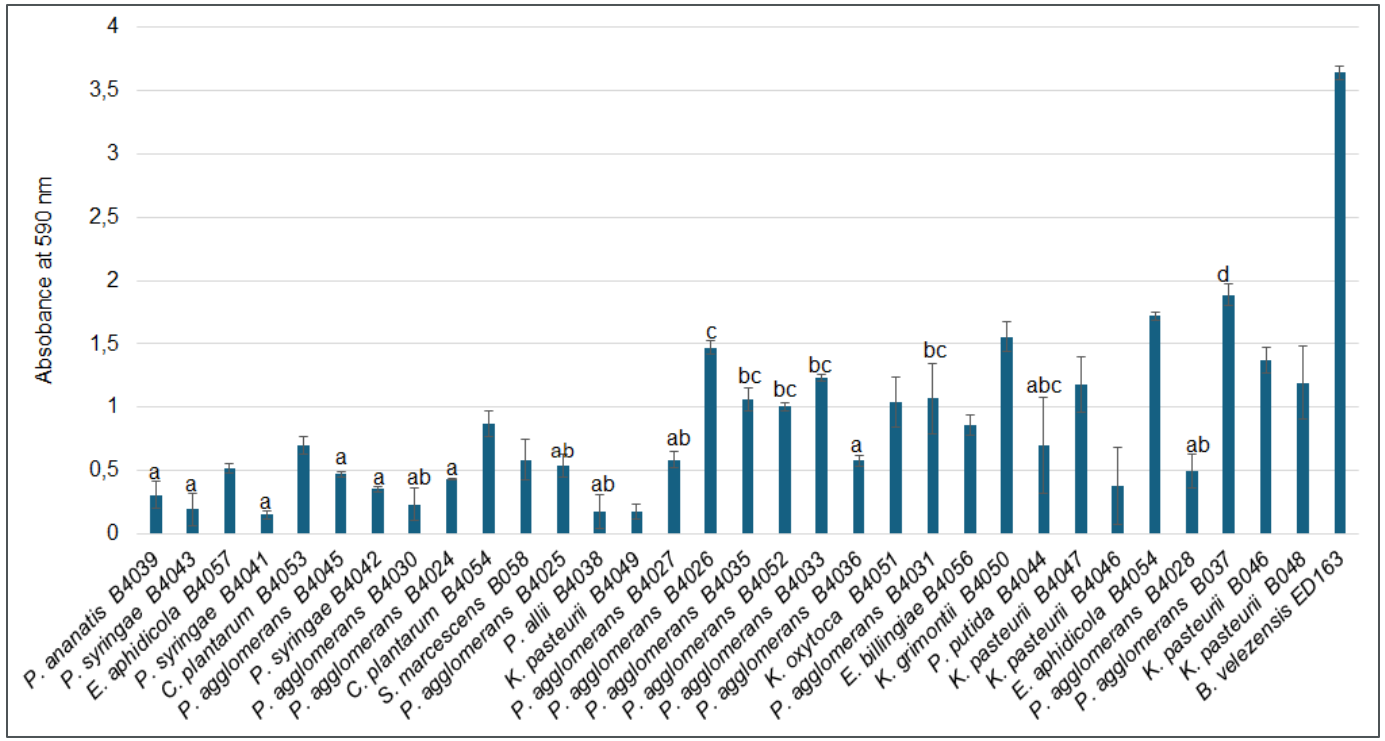
Crystal violet microtiter plate biofilm assay

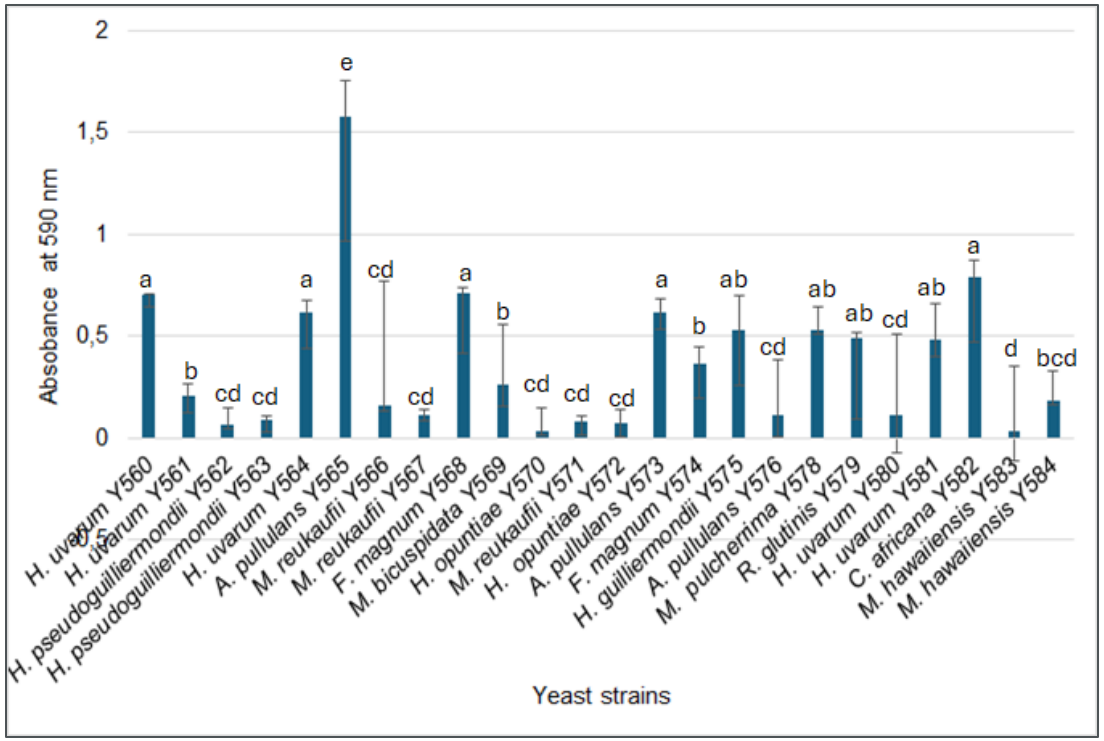
- Isolates cultured in Yeast Peptone Dextrose broth → biofilms formed via **exopolysaccharide attachment to the plate surface**
- **Free** (planktonic) cells washed away
- Attached biofilms stained with **crystal violet**
- Crystal violet binds to **polysaccharides and biomass** in the biofilm matrix
- **Standard method** to **quantify biofilms** by staining extracellular matrix and surface-attached cells



(O'Toole et al., 2011)

Biofilm formation by bacteria and yeast





- Observed clear differences across species
- Biofilm formation varied with microbial species
- Bacterial isolates showed significantly higher biofilm formation than yeast isolates
- Consistent with literature: bacterial pathogens often dominate biofilm biomass

Yeast tolerance to the commercial fungicide Switch (37.5% Cyprodinil + 25% Fludioxonil) and Copper

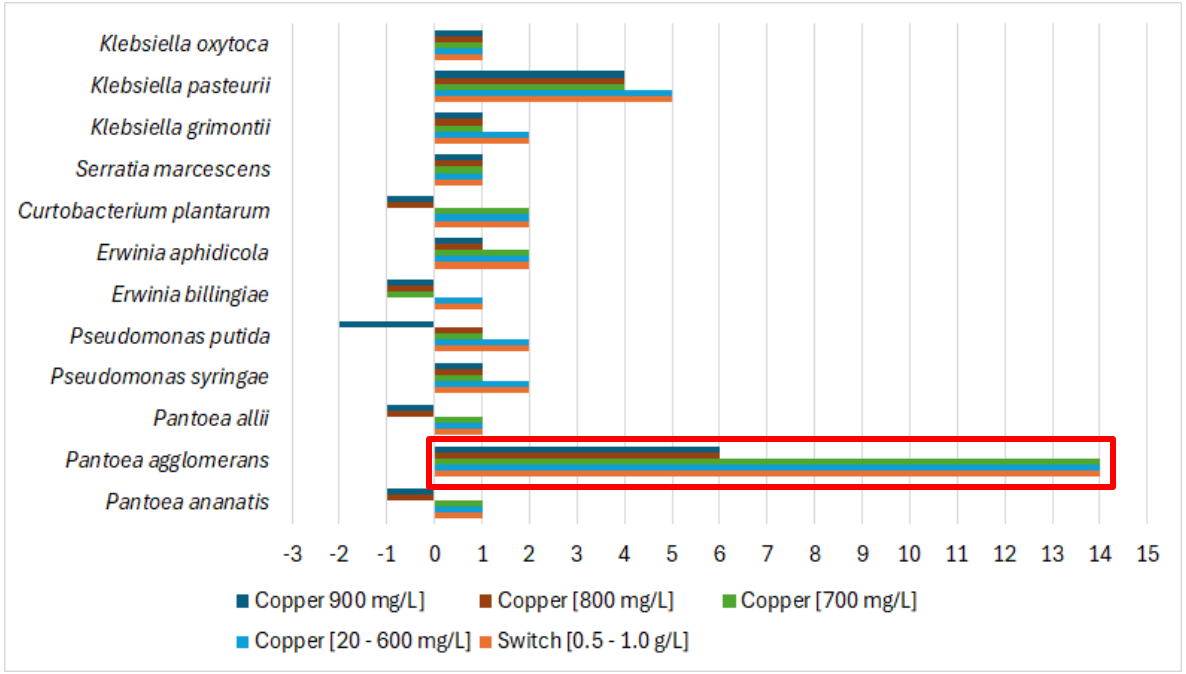
BCAs can be co-applied with chemical fungicides

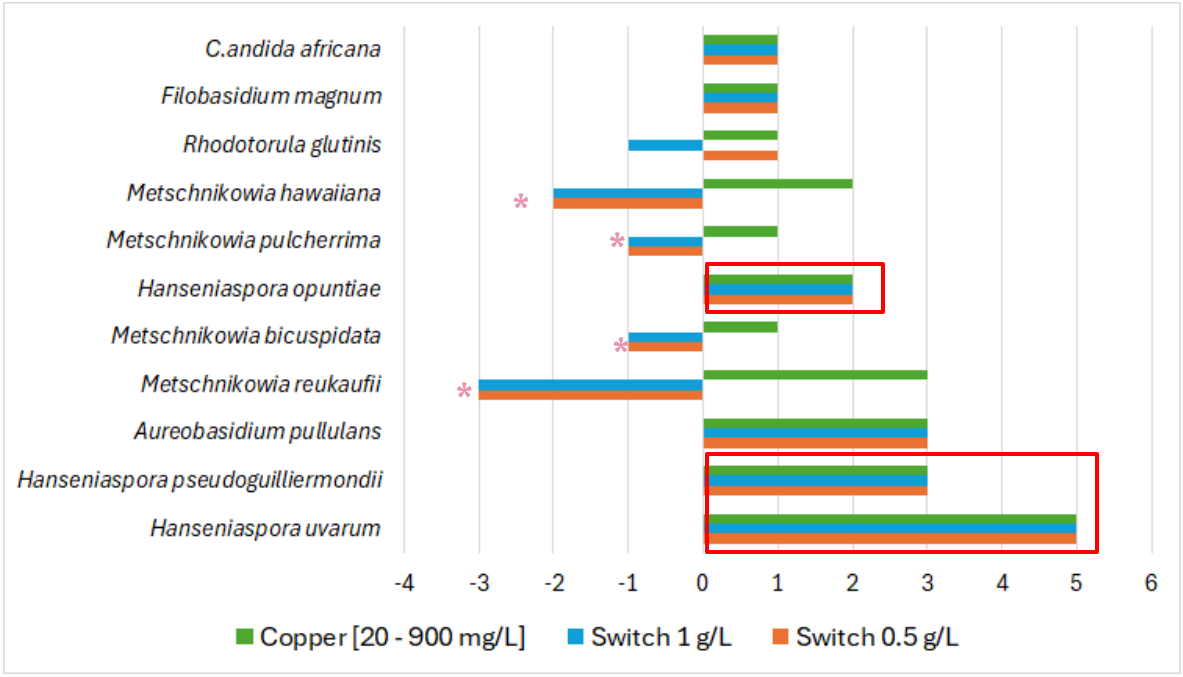
- **To identify BCAs compatible with commercial fungicides**

Experimental setup and growth conditions

- CuSO4 in 6-well YPD: 20, 64, 100–900 mg/L (100 mg/L steps).
- SWITCH in 24-well YPD: 0.5 and 1.0 g/L.
- Spotted 5 µL of 10^6 CFU/mL; incubated; scored growth after 5 days (triplicate).

Bacteria and yeast tolerance to copper and Switch® Fungicide





- *P. agglomerans* was tolerated both treatments, while other species showed variability

- All *Hanseniaspora* species tolerated all copper and Switch® treatments, while *Metschnikowia* species were sensitive to Switch® but tolerated copper.

From screening to selection

Following **isolation, screening, and characterization** of potential BCAs

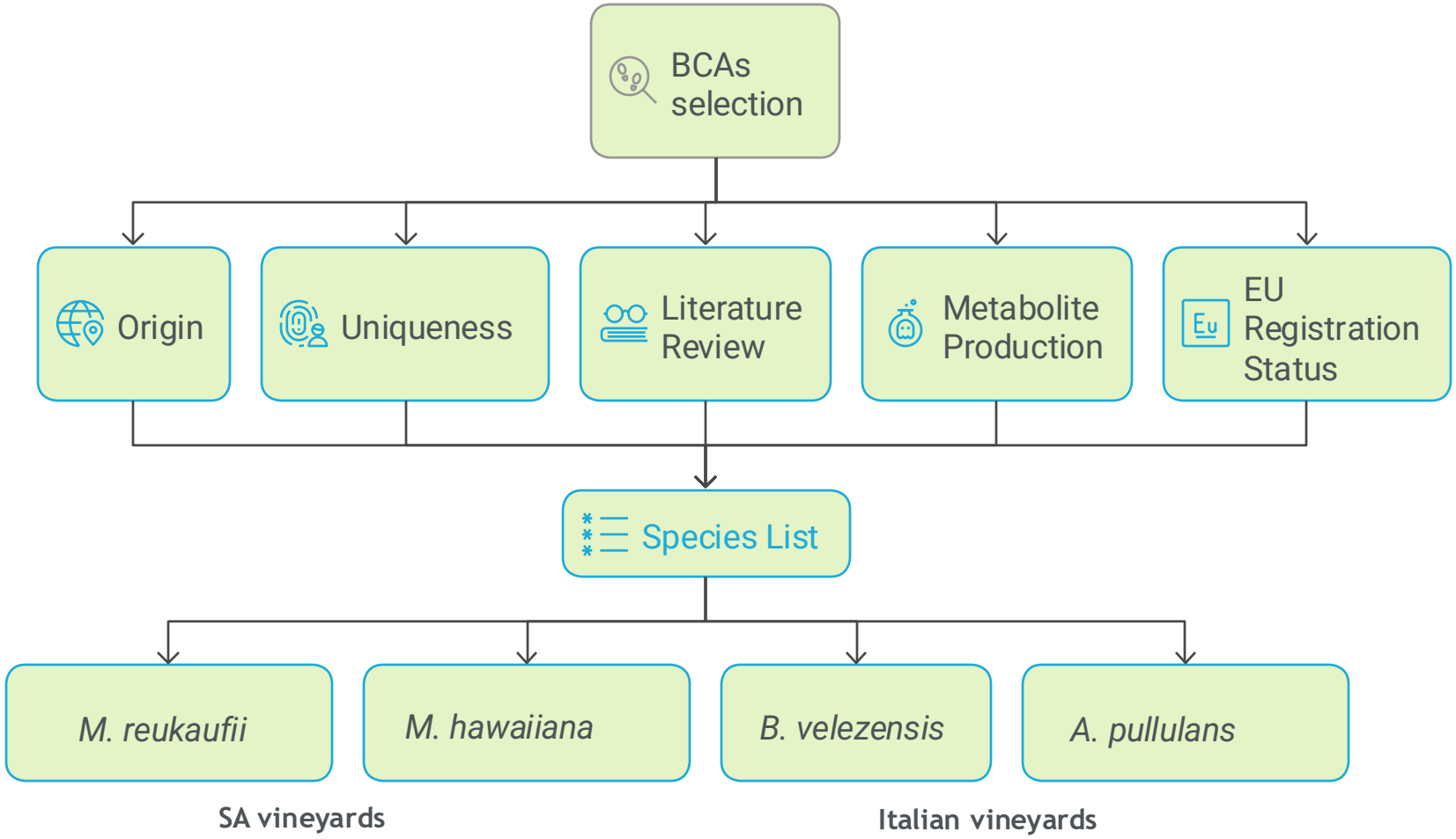
We **chose strains** that demonstrated:

- **Diverse antifungal modes of activity** (enzymes, VOCs, biofilms)
- **Multiple mechanisms of inhibition** against *Botrytis*
- **Tolerance to copper and SWITCH® fungicide**

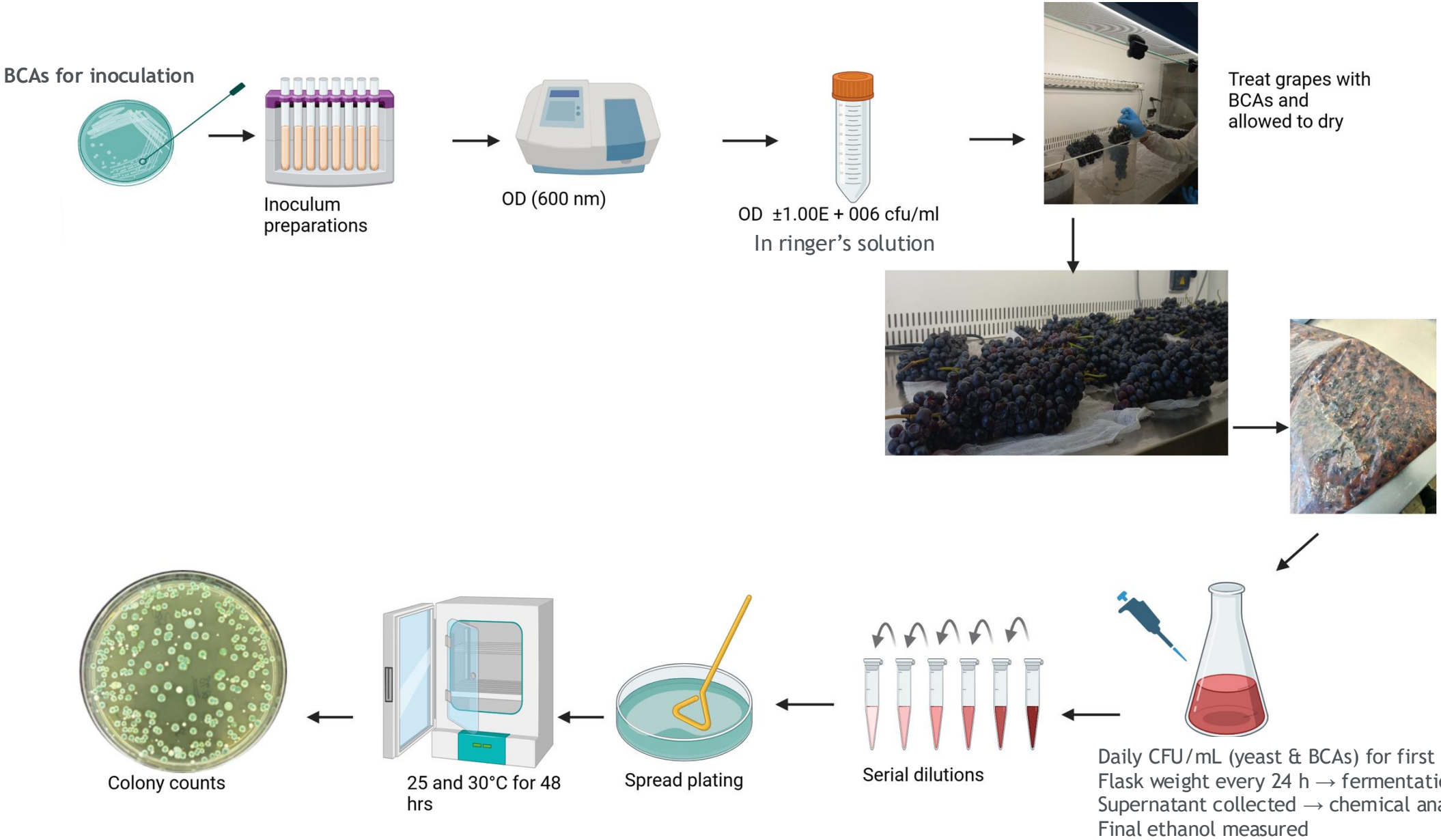
Selection combined **South African isolates** with strains from **Italian collections**

- Final candidates advanced to **vinification trials**

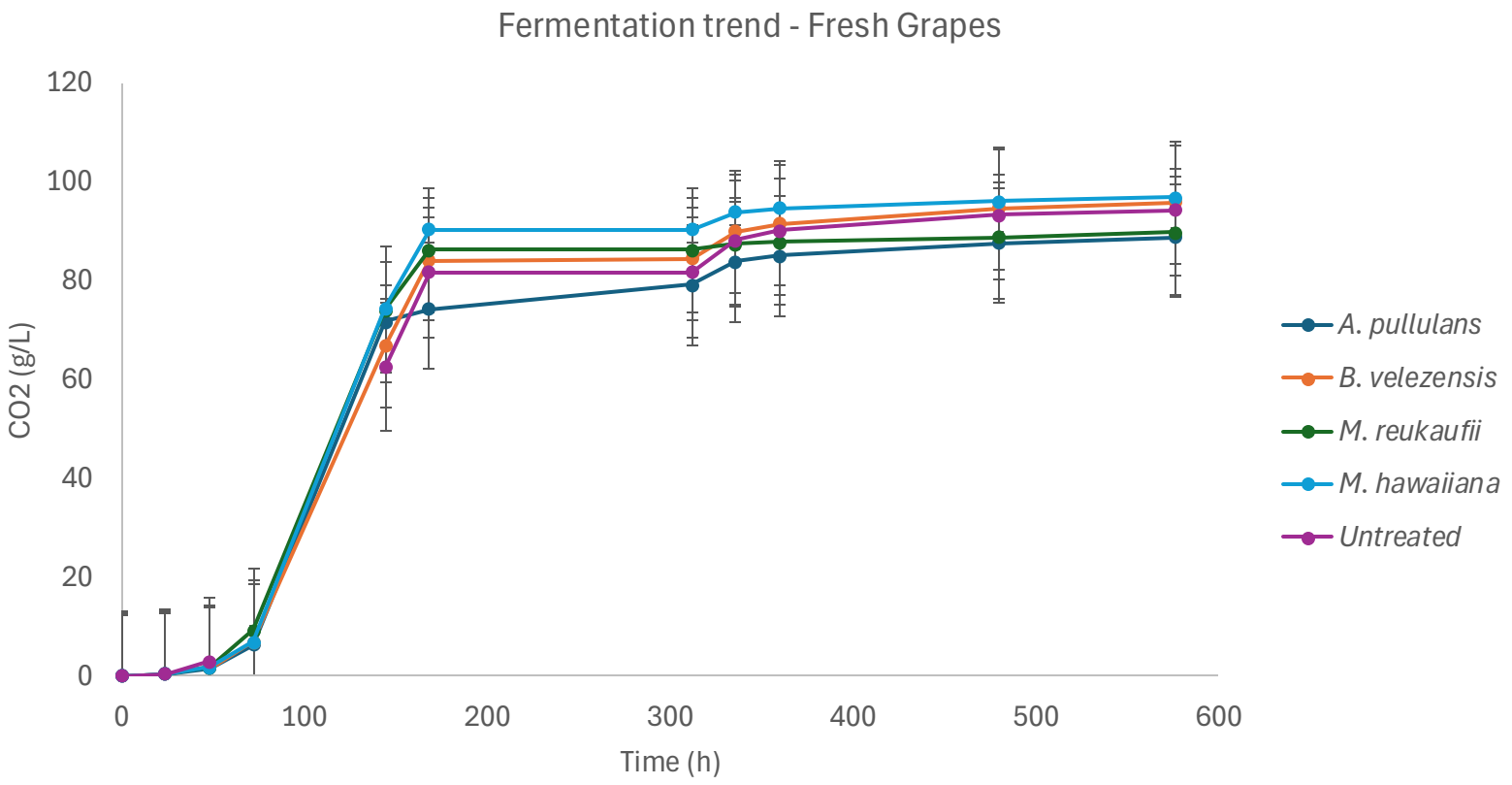
BCAs selection for vinification



Fermentation trails



Fermentation kinetics



Treatment	%EtOH	STD
Untreated	9,995751628	0,018575
M. hawaiiana	10,2690626	0,3025
M. reukaufii	9,523427819	0,172478
B. velezensis	10,16557592	0,002654
A. pullulans	9,42524815	0,573157

- All treatments followed a similar fermentation pattern.
- CO_2 production increased rapidly between 70–100 h and then plateaued.
- BCAs did not inhibit or delay fermentation.
- Minor strain-to-strain differences were observed but remained within normal fermentation ranges.
- Final ethanol levels were 9.4–10.3 g/L, with *M. hawaiiana* and *B. velezensis* slightly higher.

Conclusions

- This study sought to **identify biological control agents (BCAs) effective against *B. cinerea* and compatible with commercial fungicides.**
- We identified **promising microbial species** with antifungal activity
- **Key characteristics:** enzyme activity, volatile compounds, biofilm formation, and fungicide tolerance
- These candidates may also **enhance fermentation processes**
- Together, they provide the **foundation for sustainable biocontrol** in grape and wine production

Acknowledgement

- Reyneke Wines
- De Toren
- Welgevallen experimental farm
- CAF sequencing

BiSus4Wine
THE POWER OF BIOCONTROL

National Research Foundation

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SAGWRI South African Grape and Wine Research Institute

Thank you
Enkosi
Dankie

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Photo by Stefan Els