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Reviewed Paper

Wine Yeast as Potential Biological Control Agent Against Downy Mildew of Grapevine (*Plasmopara viticola*)

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Abstract

Organic fruit growing still depends heavily on the use of copper-based fungicides. One of the most prominent examples of destructive diseases is downy mildew of grapevine. To avoid the environmental impact of copper accumulation, sustainable alternatives for preventing downy mildew epidemics are needed, such as biological control agents (BCAs). Yeasts are fierce competitors in natural systems and represent promising candidates as BCAs. In this study, greenhouse trials were carried out to test the efficacy of three strains of yeast against P. viticola*, namely,* Pichia kluyveri *A,* P. kluyveri *B, and* Saccharomyces cerevisiae *S101. A novel leaf-fertiliser Aqua-Hort® was also tested. This contains a copper microdose that could reduce pollution with copper. All yeasts and the copper treatment reduced disease severity of downy mildew significantly when applied once before inoculation with* P. viticola *sporangia. The highest efficacy was obtained with copper. Moreover, application of* P. kluyveri *B reduced the number of indigenous fungi in the phyllosphere of grapevines significantly, compared to* S. cerevisiae*. The results indicate fungal interactions and a putative killer activity by* P. kluyveri*. Conclusively, indigenous grapevine yeast showed potential as a BCA against* P. viticola*.*

Keywords: Yeast, biological control, downy mildew, copper alternatives, microdose.

Introduction

Grapevine downy mildew, caused by the pathogen *Plasmopara viticola*, poses a significant threat to viticulture, often resulting in substantial crop losses (Gessler et al., 2011). Traditional *Vitis vinifera* cultivars are highly susceptible, and common cultural practices such as shoot and leaf thinning offer limited disease control (Peressotti et al., 2010; La Torre et al., 2018). Consequently, chemical fungicides, though effective, are extensively employed in conventional viticulture (Gessler et al., 2011). While prohibited in organic farming, copper-based fungicides are permitted in many European countries for organic viticulture (La Torre et al., 2018). Organic farmers heavily rely on concentrated copper-based fungicides applied every 7-10 days (Agrios, 2004), raising environmental concerns due to copper accumulation (Komárek et al., 2010; Bardgett, 2010). Exploring innovative approaches, we aim to investigate if a novel microdose method can meet copper nutritional needs while providing disease control.

Biological control agents (BCAs) offer an alternative to reduce pesticide use in agriculture (Collinge et al., 2022). Several promising BCAs against grapevine downy mildew have been identified, including the mycoparasitic soil fungus *Trichoderma harzianum* (Dagostin et al., 2011; El-Sharkawy et al., 2018). It has been proposed that the mode of action exhibited by *T. harzianum* is induced resistance, as observed by upregulated plant defence genes and enhanced enzyme activity (El-Sharkawy et al., 2018). Wine yeast *S. cerevisiae*, explored by El-Sharkawy et al. (2018), demonstrated a ≈40% reduction in disease severity when applied four times during the growing season. Additionally, the entomopathogenic fungus *Beauveria bassiana* showed potential as a BCA, with significant disease severity reduction in

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greenhouse trials and induced resistance was in this case documented through RT-qPCR (Rondot and Reineke, 2019). Furthermore, attention to the symbiotic relationship between plants and mycorrhizal fungi has grown, with Cruz-Silva *et al*. (2021) highlighting the ability of *Rhizophagus irregularis* to significantly reduce *P. viticola* infection.

Our research focuses on exploring yeast as a BCA for control of downy mildew as well as assessing potential disease prevention through the application of leaf fertilizer microdoses with the AquaHort® system.

Material and Methods

Plant Materials: Potted plant were prepared of *V. vinifera* cv. Bolero the cultivar was chosen due to its resistance to powdery mildew (*Erysiphe necator*) but susceptibility to downy mildew (Ruehl et al., 2015). Hardwood cuttings were taken from Vrangbækgård Vin Aps (DMS 55°7'14.4"N 10°45'57.7"E) by making two-bud cuttings from the woody vines in the winter (10/01-21). The cuttings were submerged in a 1% acetic acid solution for 30 minutes to avoid Botrytis (Farkas and Kocsis, 2013). After disinfection, cuttings were kept at 2-4°C until planted. 3 batches of cuttings were produced (14/04-21, 10/05-21 and 08/06-21) by placing them in soil to allow rooting at 25°C/20°C with a 14 hour-photoperiod. All side-shoots and young leaves were pruned off so each plant had 10 fully developed leaves. This was done 2 days before the start of the experiments.

Production of Sporangial Suspension: Batches of frozen leaves infected with *P. viticola* were generously provided by Hochschule Geisenheim and Julius Kühn-Institut (JKI). The leaves were placed in a funnel with the abaxial side facing up, which exposed the sporangia. They were then sprayed with sterile MilliQ water by a handheld spray bottle (Bürkle GmbH, DE), so that the sporangia ran off the leaf with the water. The liquid was then inspected in a microscope (DM/LS, Leica Microsystems, Wetzlar GmbH, DE) and the sporangia counted with a Bürker Türk 0.100mm Haemocytometer (E. Hartnack, DE) The sporangial concentration was adjusted to a minimum of $1.0x10^4$ sporangia/ml^{-1} and used to infect new plants to maintain a flow of inoculum for the experiments.

Yeast Preparation: The yeast strains were streaked on plates with fungal selective yeast peptone glucose growth media (YPG) (10 g D(+)-Glucose, 10 g Bacto Peptone and 3 g Yeast Extract L^{-1}), enriched with 100 mg chloramphenicol and 50 mg tetracycline hydrochloride per L of YPG. The plates were incubated for 3 days at 25°C and visually inspected for contaminants before the yeast were scraped off into sterile MilliQ water. The yeast concentration was adjusted to $1.3x10^7$cells/ml^{-1} with a haemocytometer.

Greenhouse Experiments: The greenhouse experiments were conducted in a greenhouse at 20°C/18°C with a 16-hour photoperiod. The greenhouse was treated with sulphur before the last experiment because of a powdery mildew (*Erysiphe necator*) attack. A randomized block design with 15 potted plants per treatment arranged in 3 crates was used. Negative controls comprised only 3 plants.

Yeast treatments involved spraying plants with $1.3x10^7$ cells/ml^{-1} using GLORIA-pumps (No 89), while copper treatments used Aqua-Hort® water at 35-50 mg Cu/L^{-1}, applied 3 hours before infection. Both treatments were administered as a fine mist until run-off, maintaining separation between crates.

P. viticola infection was induced by applying sporangial suspension to the abaxial side of leaves. Plants were covered with moist bags for 48H in darkness and high humidity conditions. These steps were done to ensure that the zoospores had a minimum of 4 hours of darkness at 95-100% relative humidity (RH) and a temperature of 18-22°C (Smith, 1988, Cantoral and Collado, 2011). 6 days after infection sporulation was induced by reapplying a high humidity treatment in combination with 48H darkness (Dagostin et al., 2011).

Assessment of disease severity relied on sporangia percental coverage using the EPPO disease scheme (EPPO, 2001). The treatments were repeated in 3 experiments throughout the season. The viability of yeast strains and indigenous fungi was determined by dilution plating of washing water on agar both from detached leaves and from leaves on whole plants. The viability was expressed as CFU/cm^2 leaf area.

Statistical analysis: The efficacy of the treatments in the greenhouse trials were determined to be significant or not by running a one-way ANOVA Tukey's honestly significant difference (HSD) test with a 0.95 confidence level used. Analyses were done in R version 4.1.1 (GUI 1.77 High Sierra build (7985)).

Results

The 3 greenhouse trials all showed a significant reduction in the severity of downy mildew symptoms, when yeast or Aqua-Hort® copper microdoses, were used once prior to infection with *P. viticola* (Figure 1). In the first trial a 20% reduction was observed for the plants treated with *S. cerevisiae* and 10% for the plants treated with *P. kluyveri* A. In the second trial the reduction in disease severity was significant in all treatments with a 33% and 26% drop for *S. cerevisiae* and *P.kluyveri A*, respectively. However, the Aqua-Hort® copper treatment proved to have a significantly higher efficacy with a 61% reduction.

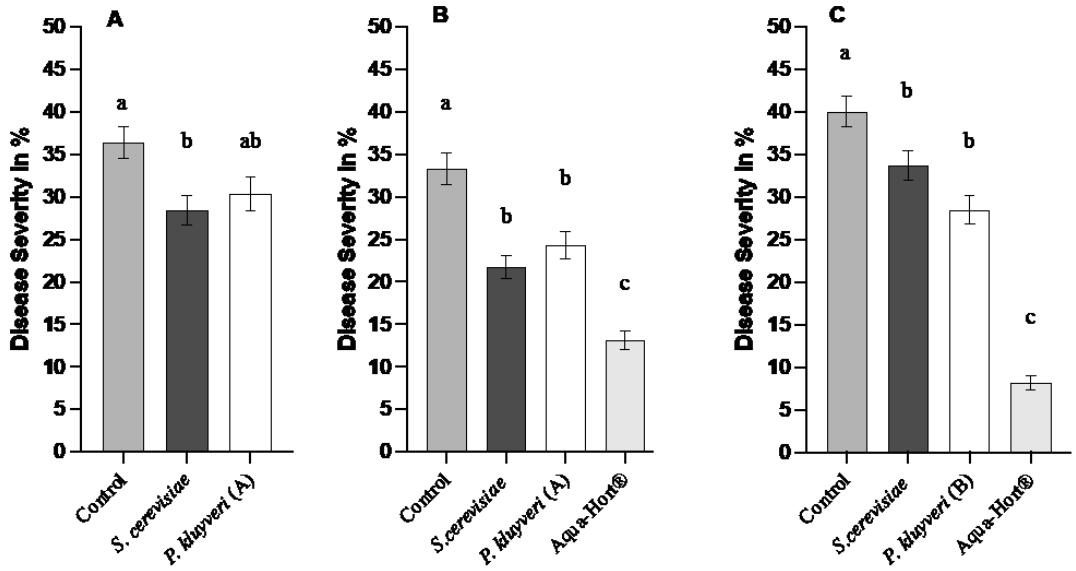

Figure 1: Disease severity in the 3 greenhouse trials. Illustrated are the positive controls, BCA treated plants (*S. cerevisiae*, *P. kluyveri* A and B) and plants treated with Aqua-Hort®. Same letters indicate values that are not significantly different according to Tukey's HSD test. A: 1st trial. P=0.009. B: 2nd trial. P<0.001 C: 3rd trial. P<0.001. Error bars = SEM. n=15.

The third and final trial also showed significant disease severity reduction of all treatments. The three yeast treatments reduced disease severity with 16% (*S. cerevisiae*), 29% (*P. kluyveri B*). Again, Aqua-Hort® reduced the disease severity even further by 80%. The viability of yeast strains on leaves was tested by dilution plating of washing water of treated leaves. On detached leaves, For all three yeast strains viable yeast cells (CFU/cm^2) were detected up to 10 days after their application which showed that the yeast can survive for a relatively long period on detached leaves (figure 2). However, the amount of *S. cerevisiae* CFU were reduced with more than 50% after 1 day on the leaves, while *P. kluyveri* A

colonies were halved after 3 days whereas it took 5 days before the *P. kluyveri* B population was reduced by 50% on the detached leaves.

For the yeast viability on leaves of whole plants (*in planta*), 11% of the *S. cerevisiae* had survived without application of *P. viticola* after 8 days (T_8) and 10% with application of *P. viticola,* which was not statistically significant (Figure 3). On the other hand, the viability of *P. kluyveri* B at T_8 was 4% without *P. viticola* and 1% with *P. viticola*. This difference was significant (P=0.03).

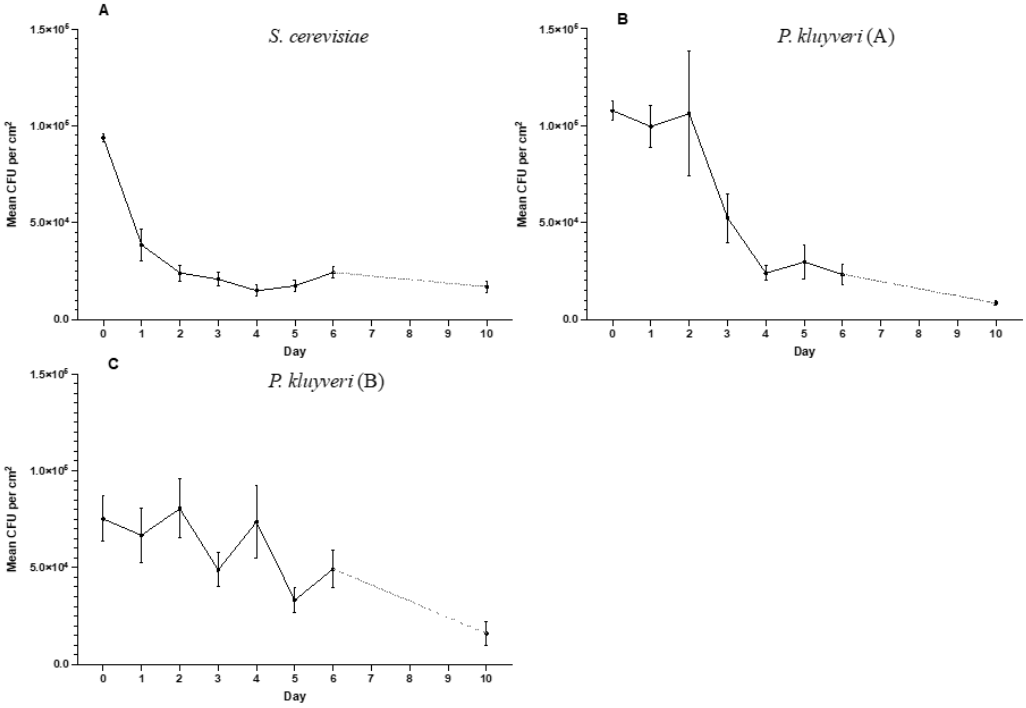

Figure 2: Survival of yeast up to 10 days on detached leaves (in vitro) Mean CFU per cm^2 of leaf. A: *S. cerevisiae,* B: *P. kluyveri* A, C: *P. kluyveri* B. Error bars = SEM. n=5.

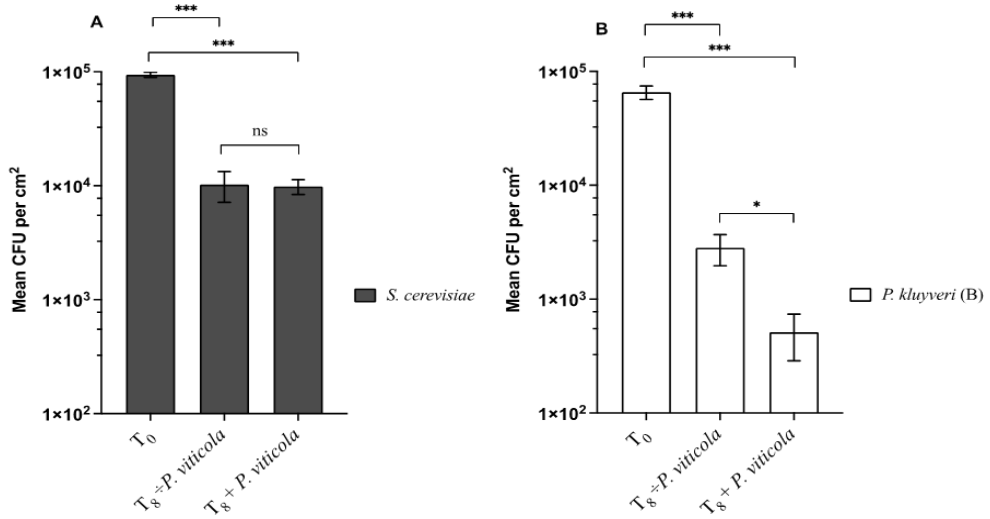

Figure 3: Yeast viability in planta of A: *S. cerevisiae* and B: *P. kluyveri* B. T_0 is at application time and T_8 is after 8 days. (Y-axis is logarithmically transformed). Asterisks indicate significant difference according a one-way ANOVA (*=P≤0.05. **=P≤0.01 ***=P≤0.001.) Error bars = SEM. n=5.

Besides the yeast, indigenous fungal colonies were also found on the plates from the *in planta* viability assays. These fungal colonies were quantified based on simple morphology traits (filamentous fungi and yeast like fungi). The populations for the plants infected with *P. viticola* and treated with *P. kluyveri* B, were significantly lower than the populations of the *S. cerevisiae* treated plants. Fewer filamentous fungi were also observed on *P. kluyveri* B treated plants compared to the *S. cerevisiae* treated (figure 4).

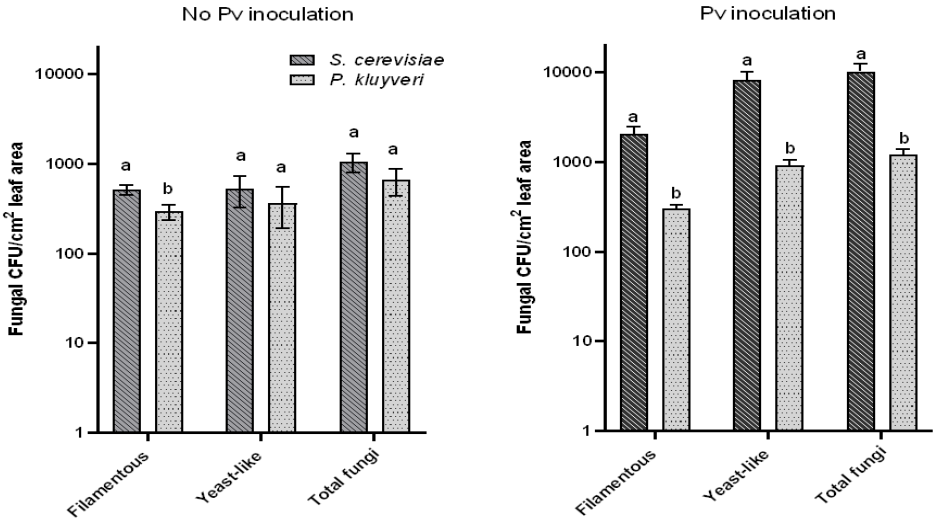

Figure 4: Indigenous fungi isolated from the phyllosphere. (CFU/cm^2 leaf area) on grapevine leaves sprayed with the yeast isolates *S. cerevisiae* and *P. kluyveri* B, respectively. A: no *P. viticola* applied after yeast treatment. B: *P. viticola* suspension applied on plants 3 hours after yeast treatment. Data were square root transformed (√((Y+1))) before statistical analysis. Error bars = SEM. n=5

Discussion

Efficacy of yeast strains and Aqua-Hort® in greenhouse: All treatments in the greenhouse trials significantly reduced disease severity across three repetitions. Notably, *S. cerevisiae* and *P. kluyveri* A and B demonstrated comparable efficacy (Figure 1), indicating their potential as biological control agents (BCAs). Optimization of dosages and timing of application(s) might further improve the efficacy of the yeast strains. However, in the current trials, the Aqua-Hort® treatment stood out, reducing downy mildew by 61%. Since it was applied with a Cu^{2+} concentration of only 35-50 mg/L^{-1} an implementation of this method would release much less copper into the vineyard soil, than traditional copper fungicides. The Bordeaux mixture, *e.g.*, contain 2.500-10.000 mg Cu^{2+}/L^{-1} (Gessler *et al.*, 2011).

Viability of *P. kluyveri* strains: Viability assays revealed all three yeast strains surviving over 10 days on detached leaves. *S. cerevisiae* showed a rapid decline after one day, while *P. kluyveri* A decreased by 59% after 3 days, and *P. kluyveri* B did not halve until the 5th day (Figure 2). These distinct survival patterns align with *S. cerevisiae*'s domesticated nature in fermentations, but seldom found in nature, while *P. kluyveri* show widespread distribution on plants (Vaughan-Martini and Martini, 2011; Kurtzman, 2011). Discrepancies between in vitro and in planta viability suggest the need for field trials to provide a realistic assessment of yeast survival as BCAs.

Impact of environmental factors on viability: In the greenhouse trial, *S. cerevisiae* exhibited higher viability than *P. kluyveri* B, contrary to detached leaf assays. The application of a sulphur spray may explain this difference, as the two species show very different sulphur

tolerance (Vicente *et al*., 2021). Thus, highlighting the importance of environmental factors in yeast viability. Repetition without sulphur treatment is essential to validate this observation.

Microbial interaction and phyllosphere fungi: The addition of *P. viticola* affected yeast viability differently, raising questions about potential microbial interactions (Figure 3 and 4). Increased fungi in *P. viticola*-treated plants suggest a microbial interaction, with lower CFU for *P. kluyveri*. Putative killer activity in *P. kluyveri*, observed in previous trials, could explain these results. However, further studies, including field trials, are necessary to confirm these observations.

Mechanism of action and plant defence: The mechanism of action (MoA) of the BCAs was not investigated, but induced resistance is suggested, given the comparable efficacy of distinct yeast strains. Induced resistance may simply occur due to a general pathogen-associated molecular pattern, and may therefore not be dependent on yeast strain or even species (Vidhyasekaran, 2014). However, if the MoA is based on antagonistic competition a combination of the two yeast could provoke them to boost the production of antimicrobial compounds (Pintar and Starmer, 2003). To explore this further, testing stilbene production, stomatal closure, and reactive oxygen species (ROS) accumulation can provide insights into the defence mechanisms induced by yeast application (Alonso-Villaverde *et al.*, 2011; Gindro *et al.*, 2003; Kortekamp and Zyprian, 2003).

Enhancing BCA efficacy: Recommendations for improving BCA efficacy include earlier application to allow for increased upregulation of plant defence genes (Wingerter *et al.*, 2021). Additionally, the use of surfactants like TWEEN® or Triton™ X could enhance dispersal on leaves, acting as a physical barrier for stomatal penetration (El-Sharkawy *et al*. 2018). The potential benefits of adding surfactants need further investigation, considering potential phytotoxic effects.

Combining biological control and copper microdose: Biological control alone may be insufficient (Gessler et al., 2011), and a combination with a copper microdose, such as the Aqua-Hort® system, is proposed for effective disease control. Field trials are crucial to validate the efficacy of this combined approach in real-life viticulture conditions. Legislative processes for BCA approval should be streamlined to encourage sustainable disease control practices.

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