Description of an *in vitro* and *in vivo* combined methodology to determine the fungicidal activity of three essential oils against *Venturia inaequalis*

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1 Introduction

Venturia inaequalis, the causal agent of apple scab disease, is known to be one of the most economic threats to apple production without proper management (Caffier et al. , 2022 ; Patocchi et al. , 2020). Ten to fifteen chemical treatments are usually required to prevent severe infections. This number can even double during years favorable for apple scab development (Patocchi et al. , 2020). Developing innovative phytopharmaceutical products (PPP) based on natural active substances is stimulated by the issue of pesticide resistance to pathogens but also their potential negative impacts on ecosystems and on human health. Essential oils (EOs) count many interesting molecules that can take up this challenge. After screening among 91 EOs, three candidates are selected for their fungicide effect on apple scab disease. These three EOs are compared with conventional active matters throughout two *in vitro* experiments and one *in vivo* trial. *In vitro* experiments consist of calculating the concentration preventing 50% of the pathogen's growth (IC50) and the concentration killing 50% of the conidia (LD50). The *in vivo* trial measures the scab infection on apple tree leaves after a preventive application of EOs followed by an artificial inoculation of *V. inaequalis*.

2 Methodology

In vitro **:** Three EOs (A, B, and D) are mixed in the liquid growth medium of *V. inaequalis* conidia. Four different strains were tested for IC50 determination and only one for LD50 determination. In both experiments, conidia are incubated with several EOs concentrations to calculate IC50 and LD50 thanks to a regression. For IC50, the effect of EOs on the pathogen's growth is measured with a microplate reader at 630 nm after seven days of incubation. Just one day of incubation is required for LD50 determination. The living rate is measured by numerical analysis of conidia colored with fluorescein diacetate (FDA).

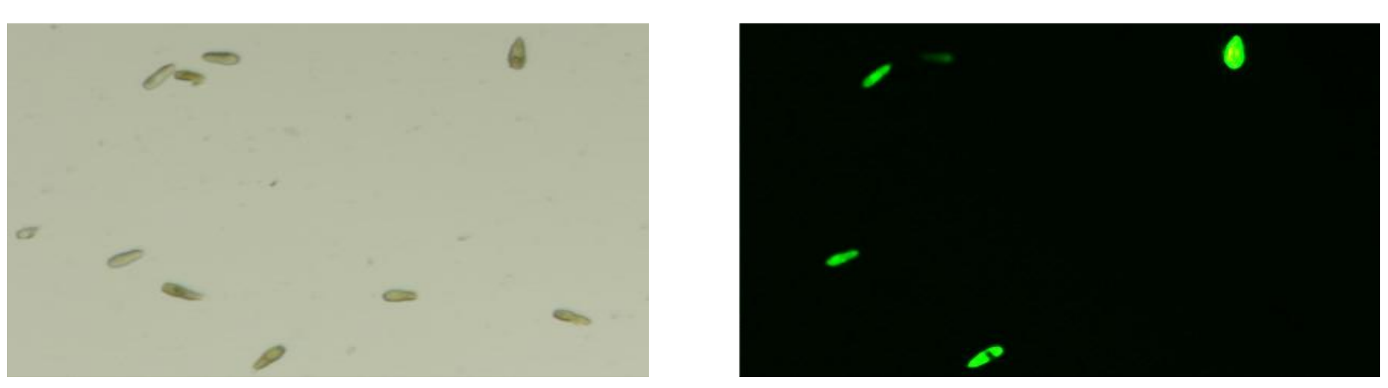


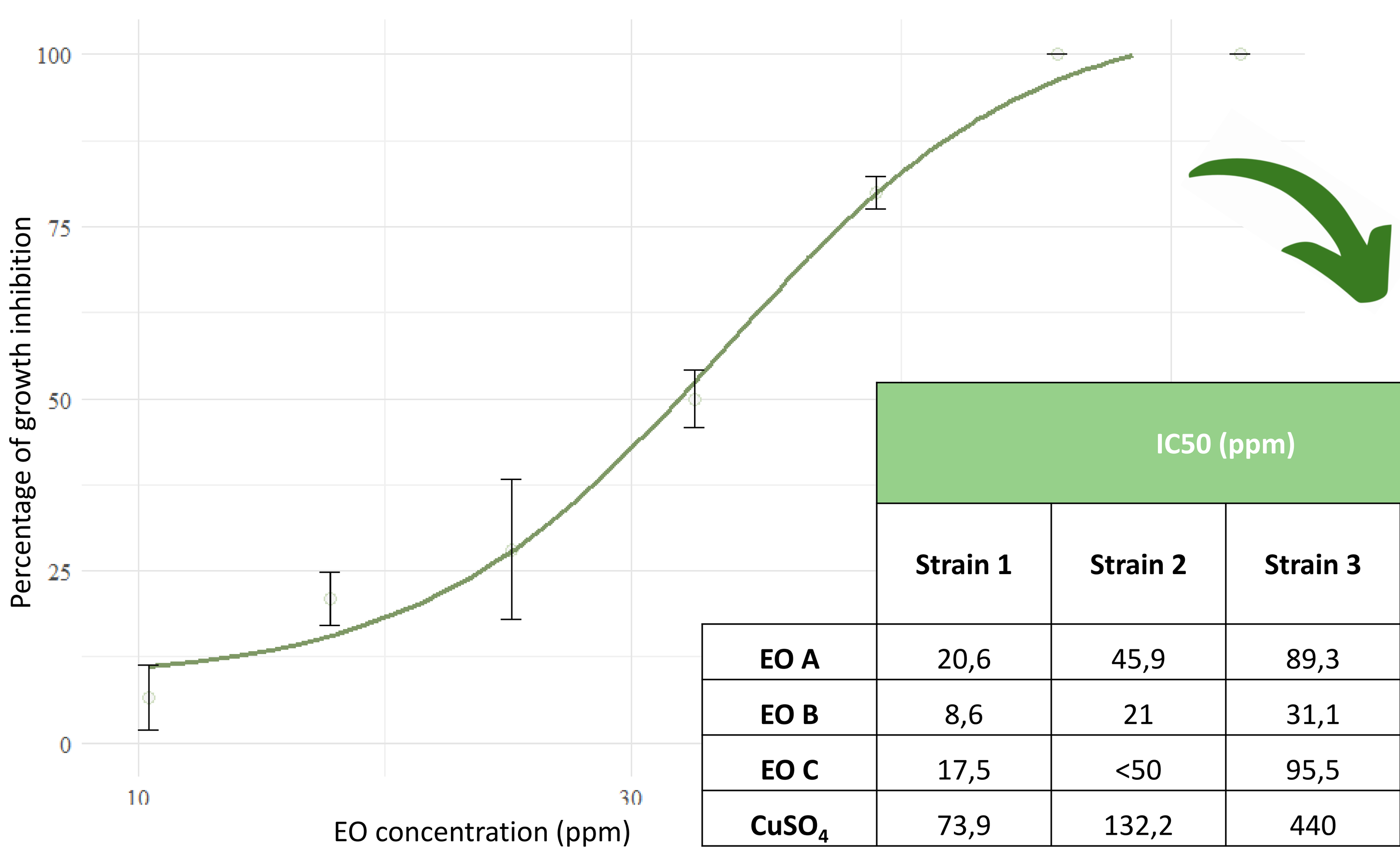
Fig 1 : Picture of V. inaequalis conidia captured with Nikon SMZ18 colorated with FDA in white light (left) and under UV light (right)

In vivo **:** Three EOs are applied as preventive treatments on Jonagold apple plants in controlled conditions. They are sprayed directly on leaves 24 and 48 hours after scab inoculation. For a successful germination and infection of sprayed conidia, leaves need to stay moistened for 48 hours after inoculation. To achieve this, inoculated plants are individually wrapped in a plastic bag and the growth chamber is plunged into the dark. The temperature is fixed at 20°C to maximize the development of the phytopathogenic mushroom. After an incubation period of 15 days, infected leaves are pictured, and symptoms are visually assessed based on a scale from 0 to 10. A quotation of 0 means no infection and a 10 stand for symptoms covering 100% of a leaf. A reproductible photography of symptoms enables rigorous comparisons of symptoms by numerical analysis of scab infections.

Fig 2 : macro photography of apple scab symptoms quoted at 0 (left), 5 (middle) and 10 (right)

3 Result

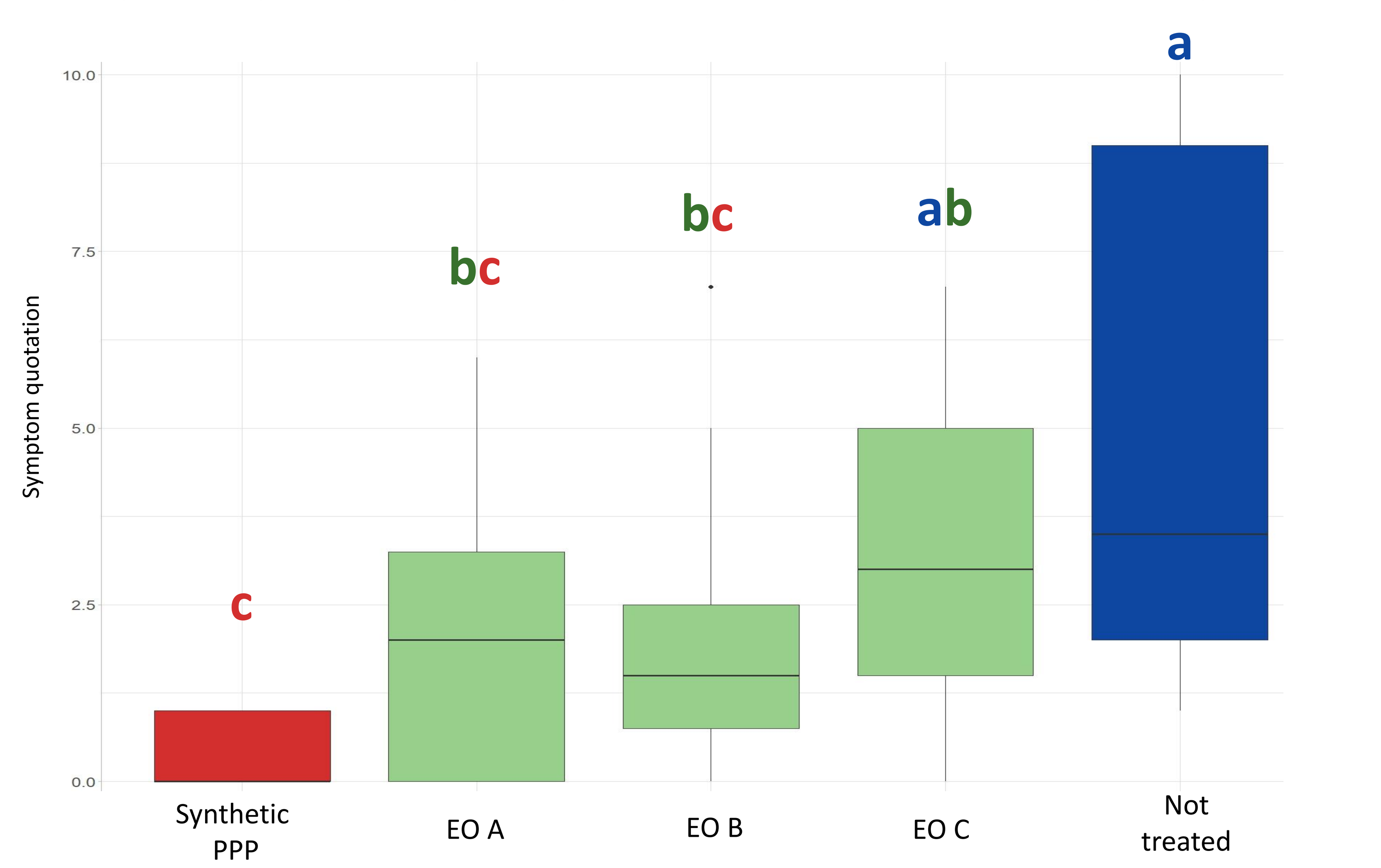
Fig 3 : Example of regression calculated for the IC50 estimation



	IC50 (ppm)				LD50 (ppm)
	Strain 1	Strain 2	Strain 3	Strain 4	Strain 2
EO A	20,6	45,9	89,3	37,8	193
EO B	8,6	21	31,1	33,5	306
EO C	17,5	<50	95,5	82	< à 100
$CuSO_4$	73,9	132,2	440	432	< à 200

Table 1 : Calculated IC50 and LD50 of the three EO studied

Fig 4 : Boxplot presenting the fungicidal effect of three EOs applied on apple tree leaves 24 hours before an artificial infection of V. inaequalis, the letters correspond to an SNK test (α = 0,05)



The three EOs shows an effective control of *V. inaequalis* both *in vitro* and *in vivo*. For each EO, and for each tested apple scab strain, the IC50 calculated is systematically lower than $CuSO_4$, an active matter commonly used as a fungicide against scab disease in organic farming. IC50 calculation also shows that EO B has a better capacity to prevent conidia's growth than EO A and EO C. For every treatment, important differences in efficiency are observed between scab strains. This considerable variability in strain sensitivity is also observed in the bibliography (Muchembled et al., 2018 ; Montag et al. , 2006). The study of the lethal dose shows that only EO A and EO C are more lethal on *V. inaequalis* conidia than $CuSO_4$. To obtain 50% of lethality with EOs, the IC50 concentration needs to be multiplied by a factor four or even more. Better precision in LD50 determination for $CuSO_4$ is required to make the same observation.

In vivo results of EOs treatment applied 48 hours before inoculation are not presented because there are not significantly different from the not treated modality. There is no significant fungicidal effect of EOs for this modality of application. However, EOs applied 24 hours before inoculation, help to protect apple trees in a significant way. EO A and B seem more efficient than EO C even if they belong to the same statistical group. A visual quotation of scab symptoms is presented in the graph above; numeral analysis of photography is still in progress.

4 Conclusion

The *in vivo* efficiency of EOs is compared with $CuSO_4$, a commercial reference commonly applied in organic orchards. Results confirm the EOs potential to be used as an innovative active matter in scab management. Preventive application of the same three EOs on leaves before *V. inaequalis* inoculation consolidates the interest in EOs for the substitution of synthetic PPP. The fact that EOs selected for their *in vitro* properties also reduce *V. inaequalis* development once spread on apple tree leaves, approved the *in vitro* methodologies used for EOs screening. However, EOs scab efficiency after being sprayed on plants needs to be improved to be competitive with synthetic PPP. Indeed, tested EOs formulations have no longer fungicide effects once spread two days before the inoculation. In practice, a contact fungicide should protect plants for 4 to 5 days. An important work on the formulation still to be done. Non presented results show that EOs concentrations in the sprayed product could be slightly increased without apparition of phytotoxicity. Various co-formulants could also be added to the mix to optimize several rheological parameters of the prototype product. Ongoing research also concerns the persistency of the EOs protection. Finally, the mechanisms of action of EOs is studied to select the appropriate co-formulants and consider potential EOs combinations.

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