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Protection of apple and pear flowers against fire blight infections using biocontrol organisms applied via bumblebees

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Aim of the project

Limited tools are available to growers to protect apple and pear flowers against *Erwinia amylovora* infection of which the risk increases with increasing temperature making the secondary bloom later in the season more vulnerable than the primary bloom in early spring. Apart from regular inspections and removal of the infected plant parts preventive sprayings with plant defense enhancer molecules (e,g, fosethyl aluminium, laminarin) and heavy metals to reduce the host susceptibility in the orchard are practiced in Belgium, but not during bloom to avoid phytotoxicity. Spraying with the antagonistic yeast *Aureobasidium pullulans* (Blossom Protect™) that can block flower colonization by *E. amylovora* is another option in Belgium to cover the fire blight susceptible blooming periods, but requires at least 2 to 3 applications to protect all flowers and causes fruit russeting. In this joint research project we aim at the continuous application of the biocontrol organism (BCO) at the sites of flower infection by bumblebee (*Bombus terrestris*) vectoring.

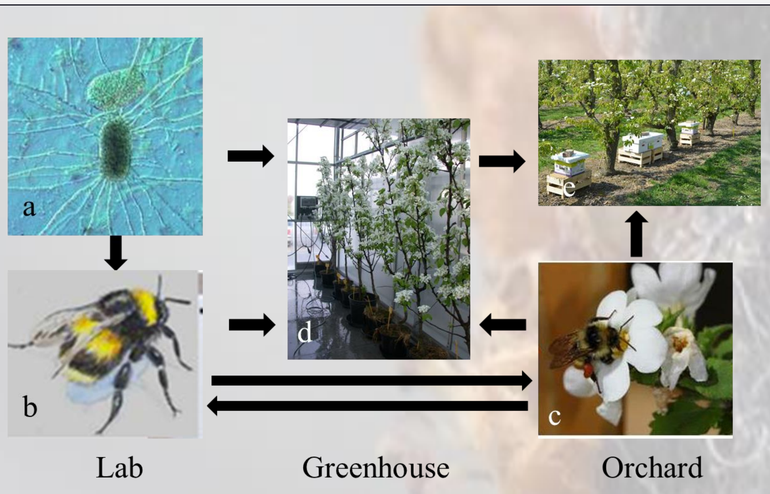

Figure 1. Workflow of the project. At the laboratory the microbiome of apple and pear flowers is studied (a), while the acquisition of BCO's by bumblebees is also assessed (b). Flower visitation by insects including bumblebees is measured at orchard level (c). Infection experiments with prior BCO application are done in the greenhouse (d). The selected BCO is finally applied in the orchard (e).

Screening of candidate BCO's against fire blight

With the majority of Belgian apples and pears being exported, the absence of fire blight in the orchards is crucial. In the absence of a curative treatment BCO's might be able to control fire blight infection during primary and secondary bloom, but they are applied by spraying and not well targeted to the flower stigma and hypanthium where *E. amylovora* bacteria multiply and penetrate, respectively. Bumblebees carrying the BCO on their body and legs should ensure BCO dissemination onto these flower parts when gathering pollen and nectar. Moreover, bumblebees are the preferred vector since they remain active under bad weather conditions and under hail nets unlike honey bees. At the onset of this project a shortlist of available BCO's used for fire blight control in other countries was prepared and the selected antagonistic bacterial (*Bacillus subtilis* QST 713, *Pantoea agglomerans* 01) and yeast (*A. pullulans* DSM14940+DSM14941) strains together with a new *Paenibacillus* sp. LMG27872 strain are tested under different conditions for several parameters. However, these BCO strains do not originate from flowers making them not well adapted to the flower habitat. We therefore are also searching for new candidate BCO's in the apple and pear flower microbiomes. An overview of the workflow is given in Figure 1, while initial results are shown in Figures 2-5.

Microbiome analysis

We aim to explore the flower for candidate BCO's that protect against *E. amylovora* infection. Flowers were sampled at different stages of primary bloom of apple ('Jonagold') and pear ('Conference'). Next to isolations on growth media, we analyzed the flower-microbiome by V5-V6 metabarcoding. Results show (Figure 2): (i) an important difference in microbiome composition between apple and pear flower, (ii) a considerable shift in microbiome composition during flowering.

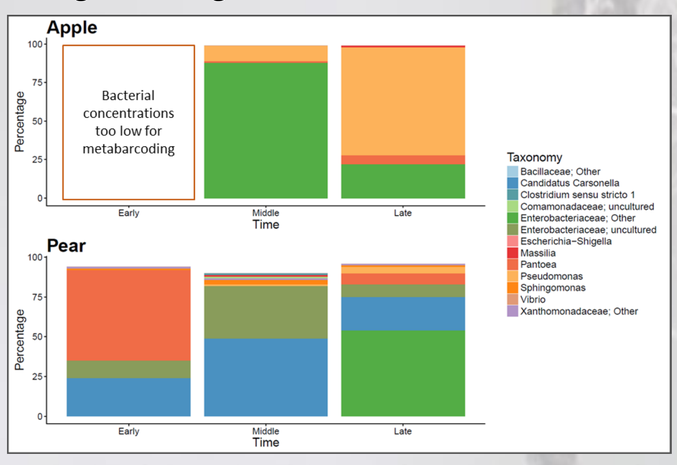

Figure 2. Analysis of the microbiome of apple and pear flowers.

Bumblebee assays

In a first step to assess and optimize the distribution of the BCO's to the apple and pear flowers the acquisition of the different BCO's onto the bumblebees was measured. After walking through a dispenser of 20 cm length filled with 1-2 mm BCO powder, the bumblebees were captured and washed with PBS buffer. A dilution series was plated and the average number of colony forming units (CFU) per bumblebee was calculated (Figure 3). Whereas *A. pullulans* reached only $6{,}58 \times 10^3$ CFU per bumblebee, up to$1{,}05 \times 10^8$ CFU of *B. subtilis* per bumblebee were counted .

BCO	Average CFU per bumblebee	SE
Aureobasidium pullulans	6585	2655
Paenibacillus sp.	45125	9921
Pantoea agglomerans	75474500	2411637
Bacillus subtilis	105700000	59611311

Figure 3. Acquisition of different BCO's by bumblebees when leaving the nest.

Greenhouse assays

To assess their potential for flower colonization and subsequent control of fire blight, flowers of potted 'Conference' trees were first sprayed by the selected available BCO's and 24 h later infected by *E. amylovora* in the quarantine greenhouse under optimal conditions. Initial results revealed that the tested bacterial and yeast BCO strains reach up to 10^4-10^6 and 10^3-10^4 CFU per flower stigma within 24 h, respectively (Figure 5a). Despite these differences all tested BCO's were able to significantly suppress ooze formation 10 days after infection (Figure 5b).

Flower visitation

Pollinator activity during primary bloom of 'Jonagold ' apple was monitored by standardized transect walks of 105 (2015) and 150 (2016) min in total (Figure 4). In 2015 bumblebees and honey bees were evenly abundant, while in 2016 honey bees were present in higher numbers. In 2016 transect walks were also done in a 'Conference' orchard (primary bloom, 75 min), but pollinator activity was nearly absent.

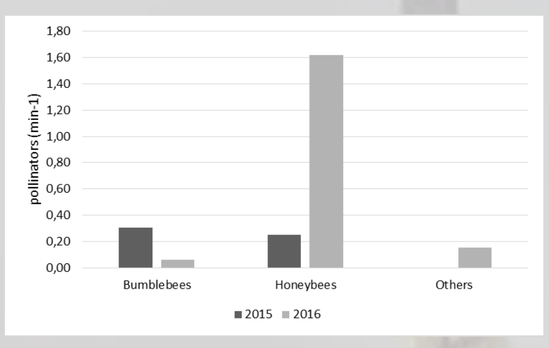

Figure 4. Pollinator activity observed during primary bloom of 'Jonagold' by transect walks. In 2015 the pollinator group "others" was not monitored.

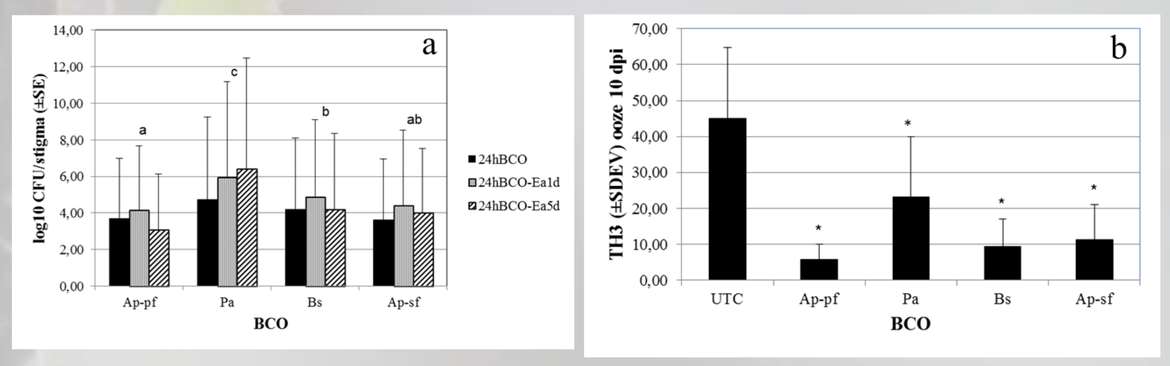

Figure 5. The average number of CFU per flower stigma expressed in a logarithmic scale (a) and the average Townsend-Heuberger TH3 value for ooze formation 10 days after *E. amylovora* (10^8 CFU/ml) flower infection (b) of potted 'Conference' trees. BCO's were sprayed, 24 h later infected and flowers harvested at different time points to count the CFU by plating on the appropriate media. Ap-pf, *A. pullulans* powder formulation (no citric acid; 1.5 kg/ha); Pa, *P. agglomerans* (0.25 kg/ha); Bs, *B. subtilis* (2.0 kg/ha); Ap-sf, *A. pullulans* soluble formulation (10.5 kg/ha citric acid + 1.5 kg/ha yeast strains); UTC, untreated control. Significant differences ($p<0.05$) among BCO's (a) and between the UTC and BCO (b) are indicated.

Conclusion: Flower microbiomes differ and their composition changes during flowering. The next challenge is to select one or more of the identified microorganisms compatible in apple and pear flowers and test for activity against *E. amylovora*. Acquisition of BCO's by bumblebees is higher for bacterial BCO's than yeast BCO. The effect of a carrier on BCO acquisition will be investigated. Insect flower visitation was absent during primary bloom of pear, whereas during primary apple bloom (and secondary bloom of apple and pear) limited flower visitation was observed. To ensure a proper transfer of the BCO to the flowers, the visitation rate of the bumblebees needs to be improved. All tested BCO's can colonize the flowers and significantly reduce fire blight disease progression in the greenhouse. Assays with bumble bee dispersal of BCO's are ongoing.

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