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Report on the evaluation of canine olfactory as a tool to detect *Xylella fastidiosa* at inspection ports, nursery, and field scale

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1. BACKGROUND

BeXyl has explored canine olfactory detection as a complementary tool for the surveillance and early detection of *Xylella fastidiosa* at inspection points, nurseries and field scale. The experimental programme followed the general approach described by Gottwald et al. (2020)[1] for canine olfactory detection of a vector-borne bacterial plant pathogen, adapting the training and validation procedures to Xf-infected plant material.

The Italian National Canine Association (ENCI) was responsible for training two dogs to recognize *X. fastidiosa*-infected plants of olives and other plant species:

- Ellis, an English Springer Spaniel (pedigree no. LO15104180)
- Paco, a Labrador Retriever (pedigree no. LO19111311).

Training was conducted at Azienda Agricola Melcarne in Gagliano del Capo, Lecce, Apulia, Italy, a third party of the consortium (P5-CNR) located within the *X. fastidiosa*-demarcated area. This setting provided a large outdoor space and conditions representative of potential operational environments, such as nurseries and plant consignments. It also allowed the safe use of potted olive plants infected with *X. fastidiosa* subsp. pauca ST53, the bacterial lineage naturally occurring in the area.

Ethical and animal-welfare considerations were integrated throughout the programme, in accordance with the principles of Directive 2010/63/EU and the procedures reported in Deliverables D11.1 and D11.2. Welfare in dog training has been ensured together with the Italian National Canine Association (ENCI).

Training relied exclusively on positive reinforcement and was adapted to each dog. No punishment, aversive stimulus or technique capable of causing fear, pain or distress was used. The dogs were taught a passive indication response and were rewarded with food, toys, play and social interaction. Working periods were short, rest periods were provided between sessions, water was continuously available, and the dogs' behaviour and recovery were monitored by their trainers before, during and after training. These measures ensured that the scientific objectives were pursued without compromising animal welfare.

[1] Proceedings of the National Academy of Sciences, 117 (7) 3492-3501; DOI:10.1073/pnas.1914296117

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2. METHODOLOGY AND TRAINING PHASES

The experimental programme followed a stepwise workflow designed to move progressively from the *in vitro* bacterial cultures to blind discrimination of infected plants under increasingly realistic operational scenarios. Each phase had a defined purpose and informed the design of the subsequent phase.

The training activities with plants were carried out in open space, not indoor (i.e. inside greenhouses or grow chambers) due to the following reasons:

- The training conditions could be comparable with the real working conditions (i.e. in nurseries, lots of consignments, etc.),
- The training materials could be easily managed (i.e. olive potted plants)
- Space and environment would be more favourable for the dogs.

Before starting each session, the dogs were allowed to move for few minutes to allow them to perform their physiological needs. Then each session consisted of 1 to 4 passages on the lines of boxes containing cultures, plants or soil. Between sessions the dogs were allowed to rest for at least 40 minutes, during which the dogs were allowed to rest in their kennel, with water available.

The sessions were mainly carried out in the morning, given an appropriate time lapse between meals and working sessions. Training sessions were performed between 2023-2025, with the exception of August and September when temperatures were too high for performing the training sessions. In each month, from 6 to 20 training sessions were carried out. The dogs performed daily moderate physical activity (walks) and free play sessions.
When the dog correctly identified the target, he was rewarded with food, toy (ball, tug) and a play session with their trainers. Through training, the dogs learnt passive indication behaviour, stopping and sitting near the target potted plant, without coming into deep contact with the soil or the plant foliage.

2.1. Phase 1. Dog sensitization using bacterial cultures grown *in vitro*

The first phase aimed to verify if the dog were able to recognizes volatiles specifically produced by the actively growing bacterial cells onto artificial media. To this end pure *Xylella fastidiosa* cultures on BCYE and PD3 agar plates were used, along with plates containing a non-target bacterium. Agar plates containing pure cultures of *X. fastidiosa* were placed inside closed scent boxes that allowed volatile compounds to be perceived while preventing direct contact with the cultures. Plates containing a non-target bacterium (*Pseudomonas* spp.) and plates containing only the media were included to verify that the positive response of the dogs was not related to any chemical or microbiological component of the media nor to non-specific conserved bacterial metabolites (**Figure 1**). The position of the target box was changed between runs and the dog trainer was unaware of the location of the *Xylella*-plates, reducing the risk of unintentional cueing. A correct response was reinforced immediately, allowing the dogs to develop a consistent passive indication close to the target source.

This stage also served to verify motivation, search behaviour and the reliability of the indication response before introducing the greater chemical and biological complexity of plant material. Sessions were kept short and were adapted to the performance and welfare of each dog.

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Figure 1. Pictures of the plates used for the training session and the scent boxes in which the plates were located and used for the blind search by the trained dog "Paco" during an explorative training session.

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2.2. Phase 2. Small-scale discrimination of infected and non-infected olive plants

After the successful completion of the sensitization of the dog using the agar plates, training was transferred to potted olive plants to determine whether the dogs could recognise the volatile profiles associated with *X. fastidiosa* infections in the plant-soil system.

The need to work in open space implied the organization of the working sessions in the infected area of Apulia (Italy), where there are no mandatory actions for the removal of the infected plant materials. Potted infected plants were therefore used and moved outside the greenhouse for the time being the training session. However, under this condition we could only work with the plants infected by the same strain occurring in this demarcated area of Apulia; i.e., plants infected by bacterial isolates belonging to *X. fastidiosa* subsp. *pauca* ST53.

The training materials included potted olive plants that were not threatened/sprayed with insecticides/pesticides for the whole cycle of cultivation. Sessions included lines of 5 to maximum 10 plants, including one or more plants infected with *X. fastidiosa* subsp. *pauca* ST53, among non-infected controls (**Figure 2**). The location and number of target plants varied between passages. One trainer arranged the plants and recorded the position of the infected ones, while a second trainer guided the dog without knowing the positions of those infected; thereby, maintaining blind-test conditions.

The dogs were allowed to inspect both the aerial parts and the surface of the pots; upon recognizing the infected plant, they stopped and sat near the target pot without touching the foliage or digging into the substrate. The use of non-infected potted plants reduced the likelihood that other components of the plants could have an effect on the positive recognition of the plants. These small-scale trials proved strong evidence that the dogs were able, by progressive training sessions, to effectively recognise Xylella-infected plants.

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Figure 2. Pictures of the potted plants used for the training session and 'Ellis' the trained dog sniffing the soil in the pot during the passage on the five lined up plants.

Generally, only one training session (corresponding to several passages on the row of plants) was organized per day. However, in some cases, two sessions were performed: one in the morning the second in the afternoon after a break for the resting. After the session, the dog was moved in its kennel and relaxing environment watched by the dog's trainer, who observes how the dog behaves (seeks cuddles or quiet play eats or drinks calmly) and offers a gentle walk, chewing (chew toys, deer antlers, Kongs with food) and calming touch or massage.

Examples of the training sessions are available in the links provided on Annex 1.

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2.3. Phase 3. Large-scale blind testing with infected and non-infected plants and assessment of the diagnostic sensitivity

The third phase consisted in the use of a larger number of plants to be inspected and for searching the infected plants. The experimental design took into account the operational screening that could be performed to inspect nurseries or consignments. Specifically, blocks of 50 potted olive plants were arranged in five rows of ten plants, with three to five infected plants randomly distributed within each block (**Figure 3**). Ten passages were completed, corresponding to 500 plant inspections. Plant positions were re-randomised as required, and the dog trainer remained blind regarding the location of the Xylella-infected plants.



Figure 3. Pictures of the randomized potted plants in large scale tests: 5 rows x 10 plants each were screened for each passage of the dogs. In the upper picture the trained dog 'Paco' during an explorative training session.

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This step was essential for assessing whether the learned response remained stable when the target prevalence was low, the search time was longer and non-target plants prevalent.

To assess the diagnostic sensitivity, records from the small-scale and large-scale blind tests were classified as positive agreement, negative agreement, false-positive response or false-negative response. Accuracy was calculated as the proportion of all plants correctly classified (both infected/non-infected), sensitivity as the proportion of infected plants correctly indicated, and specificity as the proportion of non-infected plants correctly assigned as non-infected (lack of positive response from the dog) (**Table 1**). The results were evaluated separately for the two dogs to identify individual differences and to determine where additional training could improve performance.

The high values recorded for the accuracy indicate good diagnostic performances, even if the values of sensitivity were below 90% (**Table 1**). The performance yielded by both dogs provides preliminary evidence that canine olfactory detection can be used as a screening method to support inspections in the nursery and in consignments (Figure 4).

Table 1. Results of diagnostic-performance assessment for the two dogs participating in the study

PARAMETERS*	DOG 1	DOG 2
ACCURACY [AC=(N_{PA}+N_{NA})/N]	0,982	0,944
SENSITIVITY [SE=N_{PA}/N]	0,739	0,885
SPECIFICITY [SP=N_{NA}/N-]	0,994	0,947

* N = Total number of samples. N+= Total number of samples positive for *X. fastidiosa*. N-= Total number of samples negative for *X. fastidiosa*. N_{PA}= Total number of samples with positive agreement. N_{NA}= Total number of samples with negative agreement.

Overall the results from small-scale tests (10 plants x 20 passages) and large-scale tests (50 plants x 10 passages) produced diagnostic performance values within an acceptable range (Table 2).

Table 2. Results of diagnostic-performance assessment in different scale tests

PARAMETERS*	Small scale test	Large scale test
ACCURACY [AC=(N_{PA}+N_{NA})/N]	0.930	0.963
SENSITIVITY [SE=N_{PA}/N]	0.821	0.812
SPECIFICITY [SP=N_{NA}/N-]	0.972	0.971

* N = Total number of samples. N+= Total number of samples positive for *X. fastidiosa*. N-= Total number of samples negative for *X. fastidiosa*. N_{PA}= Total number of samples with positive agreement. N_{NA}= Total number of samples with negative agreement.

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Figure 4. The trained dog 'Paco' during an explorative training session in a nursery.

2.4. Phase 4. Assessment of the contribution of soil and cultivation substrate

The observations made during the training sessions demonstrated that dogs use to sniff primarily the surface of the pots, so the substrate of cultivation. Taken this into consideration, we did an additional set of training sessions using an aliquot (approx. 40gr) of the soil from the potted plants. The soil was closed in scent boxes under blind conditions (**Figure 5**), following the same training sessions as for the agar plates. The results, although only on a small scale, gathered similar accuracy levels as previously reported with the whole plants. As such this finding prompted for additional investigations focussing on the soils of the infected vs non-infected potted plants, supported by a complementary project ongoing at P5-CNR and tackling *Xylella fastidiosa* (REACH-XY Project funded by the Italian Ministry of University and Reseach – 2023-2027). Two research experimental approaches have been pursued: (i) characterization of the microbial-community and (ii) profiling of the volatilome of the same soil samples.

2.4.1. Characterization of the microbial-community

Genomic DNA was extracted from approximately 70 soil samples (collected from pots where infected and non-infected plants were grown, accounting for at least 3 biological replicates for each treatment) using the FastDNA™ SPIN Kit for Soil (MP Biomedicals). Shotgun metagenomic sequencing was performed on the Illumina NovaSeq 6000 platform (Novogene Co., Ltd), generating 2×150-bp paired-end reads. Data were processed using parallel bioinformatic pipelines. Filtered reads were analyzed using the SqueezeMeta v1.7.0 pipeline in co-assembly mode for open reading frame (ORF) prediction and functional annotation against KEGG, COG, Pfam, CAZy, MEROPS, and TCDB databases. More than 90% of the identified taxa were bacteria, with Proteobacteria, Actinobacteriota, Bacteroidota,

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Thermoproteota, and Halobacteriota representing the most abundant phyla. Microbial abundance and diversity differed among plant species and olive cultivars. Infected samples were enriched in metabolically flexible, stress-responsive, toxin-producing, and predatory microbes. This pattern is plausibly linked to the release of specific plant exudates. Non-metric multidimensional scaling (NMDS) demonstrated that microbial communities clustered distinctly within treatment groups, and infected samples exhibited substantial internal variability. Pathogen stress led to a significant enrichment of the genus *Halangium*, whereas *Lentzea* was underrepresented, and *Bradyrhizobium* abundance remained stable.

Infected samples exhibited a biological shift toward stimulus response and environmental survival. This is supported by the enrichment of two-component systems, which likely regulate virulence and biofilm formation, as well as aromatic compound degradation pathways that enable microbes to neutralize host phenolic defenses, potentially released in response to infection. The enrichment of antibiotic synthesis pathways underscores a highly competitive microbial niche during infection. In contrast, control samples exhibited active organic matter degradation, nitrogen fixation, plant-microbe interactions, and robust metabolic pathways within a stable microbial community.

2.4.2. Profiling of the soil volatilome

From the same set of samples the analysis of the volatilomic profile generated a preliminary GC-MS dataset that revealed clear qualitative differences between the two groups. Chromatogram elaboration highlighted the presence of several exclusive VOCs in infected soils, including oxime-, methoxy-phenyl derivatives, 2,5-dimethyl-3-hexanol acetate, 1,3-dioxane derivatives, decane and other branched alkanes. Soils from infected plants displayed an enrichment in oxygenated and branched volatile compounds, which appeared either exclusively or at markedly higher abundance. Conversely, soils from non-infected plants showed a distinct set of exclusive VOCs, such as 2-methyl-hexane, ethanol derivatives, undecane and small alcohols, typically associated with non-stress rhizosphere conditions. These preliminary differences already allow a clear separation between infected and non-infected profiles and identify promising candidate biomarkers for further validation.

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Figure 5. Pictures of the soil samples used for the currently ongoing training sessions, scent box are used for holding the soil samples for the blind test with 'Ellis'.

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3. CONCLUSIONS

- The training programme demonstrated that dogs are able to sense specific volatiles compounds produced by the bacterial cultures in vitro and by the infected olive plants.
- The results support the feasibility of canine olfactory detection as a rapid, non-destructive screening tool for *X. fastidiosa*-infected plant materials.
- The successful identification of soils collected from infected plants indicates that the cultivation substrate contributes substantially to the volatile signature detected by the dogs. This finding may simplify future training by reducing the need to train dogs separately on the full range of *X. fastidiosa* host species, although this hypothesis requires broader validation across substrates and environmental conditions.
- Microbial community profiling provided insights into the potential use of the rhizosphere microbiome as a biomarker for plant health, facilitating a better understanding of infection-induced responses in plants under stress.
- In agreement with this results, analysis of the soil volatile profiles indicated that infection processes are associated with measurable shifts in emission patterns, suggesting that pathogen presence alters the complexity of the soil microbiota and the surrounding microbial ecosystem.

Overall, this work established a promising proof of concept and a methodological basis for further operational validation in nurseries, inspection points and field environments. The changes in volatile profiles detected from the dogs have been corroborated by ongoing studies on factors implicated in such changes: the microbial composition of the soil communities and the related volatile profiles. Even if these latter studies are not yet conclusive, it should be noted that the activities performed in the framework of this project have not only successfully explored the use of dogs for rapid screening of Xylella-infected plants in nurseries and consignments, but have opened the route to new research lines which could contribute not only to improve the surveillance strategies but also with potential applications in disease management by modulating the soil microbiota toward more resilient conditions.

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ANNEX 1

Videos demonstrative of dog performance can be visualized at these links:

- BeXyl website:

https://bexylproject.org/updates/news/bexyls-ellis-and-paco-anti-xylella-dogs-starring-in-un-fao-video/

- BeXyl Instagram:

https://www.instagram.com/reel/C2FyIRtNpZT/?utm_source=ig_web_copy_link&igsh=MzRlODBiNWFlZA%3D%3D

https://www.instagram.com/p/C3KTrv3I-nA/

- BeXyl X:

https://x.com/BexylP/status/1755529782219911503

- BeXyl YouTube:

https://www.youtube.com/watch?v=-5Hj_BHtoMI

- BeXyl Facebook:

https://www.facebook.com/share/YsZDPrWpf6Ghi6bQ/

https://fb.watch/uf_R0XIT-B/

- BeXyl LinkedIn:

https://www.linkedin.com/posts/bexyl-project_beyondxylella-activity-7152364414732513280-1E87?utm_source=share&utm_medium=member_desktop

https://www.linkedin.com/posts/bexyl-project_the-sniffer-dogs-saving-olive-trees-from-activity-7198605512454631424-B5Ie?utm_source=share&utm_medium=member_desktop

- BeXyl Project repository:

https://drive.google.com/file/d/1oX5m2DijbkRwyZv7OzRZG-RcwmkSPLbj/view?usp=sharing

https://saco.csic.es/s/H9JpXSfo5tfqBD5

https://saco.csic.es/s/occQcgjJiajb8Z6

https://saco.csic.es/s/W5MDtsAmnT4NtHe

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