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Detection of *Verticillium dahliae* in Olive Groves Using Canine Detection Units

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Abstract

Verticillium wilt is one of the most significant agricultural diseases in the world, in view of the fact that not only does it affec olive groves but also a wide variety of fruit, vegetable and ornamental plants. Currently, the most efficient and economical method of control involves the use of plant species that are resistant to the disease. However, as there are few varieties of olive tree with this characteristic, early diagnosis and the replacement of affected trees are key strategies to combat the disease. The present research proposes the use of a canine unit that is specifically trained to detect *Verticillium dahliae* as an early detection method for the fungus in olive groves. For the odorous samples are produced in the laboratory from *V. dahliae*, it is calculated that the dog displays a level of sensitivity of 97% and specificity of 95%, and an initial field assay shows that the sensitivity and specificity are constant when working with infected olive trees. In view of the fact that there is currently no curative treatment for this disease and the affected plants are completely lost, fast on-site detection provides significant advantages in terms of preventing the transmission of the disease. Moreover, it also enables focalized treatment to be provided on plots of land and plants and, in combination with the latest prevention methods, real-time detection of affected olive trees enables a reduction in the costs involved in vaccination, thereby contributing overall to a reduction in the financial losses caused by verticillium wilt.

Keywords

1. Introduction

Despite being a particularly resistant crop variety, the olive tree (*Olea europaea*) is not exempt in terms of suf-

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fering diseases caused by phytopathogenic fungi and bacteria. Controlling these diseases is a key factor in obtaining a healthy, quality harvest.

Verticillium wilt is a widespread disease throughout all of the countries of the Mediterranean Basin. It was discovered and diagnosed for the first time in olive trees in Italy in 1946. In Spain, the first provinces to be affected were Córdoba, Jaén and Seville in 1975. Since then, the disease has gradually spread throughout the rest of Spain's Autonomous Communities due to crop intensification and the establishment of new plantations. As a result, the disease can now be found in practically all of the olive groves in Spain [1].

The causal agent of the disease is *Verticillium dahliae*, a phytopathogenic fungus that can infect a broad range of woody and herbaceous crops. As it can feed on organic matter present on agricultural land, *V. dahliae* can remain for up to 15 years in the soil and cause recurring infections that are difficult to cure. It is mainly found in the topsoil, although it has been detected at a depth of up to a metre on certain occasions. The root exudates of the olive tree and other plants trigger the germination of the fungus, even in the case of plants immune to infection. The fungus then infects the plant through wounds caused by insects, farm machinery or directly by capillary action. Once inside the plant, it can reach the xylem without causing any apparent damage until it starts to reproduce and cause symptoms [2].

The importance of early detection is based on the fact that, by the time symptoms are exhibited, the plant is already seriously infected and there is no possible curative treatment. The presentation of these symptoms depends on the variety of *V. dahliae* that has infected the plant and, although they may coexist in the same soil, it is possible to differentiate between a defoliant and a non-defoliant variety, with the former being more virulent and resulting in a higher mortality rate [3].

Fast and early detection of the infection is one of the key factors in terms of successfully implementing a control plan for verticillium wilt. In the event of observing trees with symptoms, tissue samples must be sent to the laboratory to be processed in order to prepare a traditional microbiological culture grown in PDA (Potato Dextrose Agar) or using PCR analysis (polymerase chain reaction). Determination of the fungus using traditional microbiological techniques takes at least five days, processing a low volume of samples but achieving a high degree of sensitivity and specificity. In contrast, techniques based on molecular biology, such as PCR, are faster and enable a larger volume of samples to be processed, even these techniques can be less specific than the traditional tests [4].

The domestic dog (*Canis familiaris*) is able to detect substances at a much lower concentration than humans are able to, thanks to the olfactory epithelium covering a larger area and having a greater number of receptors in the olfactory mucosa. This great olfactory capacity has enabled dogs to be used to search for and locate a large number of substances, both biological and non-biological [5].

The use of working Canine Units (CUs) to detect the presence of microorganisms is a recent specialization within the field of detection and security CUs, and they are currently being developed for the detection of fungi [6] [7] and bacteria [8]. One of the main tools required for the specific training of a dog specializing in this work is a strong source of odour coming from the microorganism to be detected.

Microorganisms are able to produce a broad range of metabolites, including volatile organic compounds (VOC), which sometimes go undetected due to a lack of standardized methods. Many of these molecules, the majority of which are smaller than 300 Da, have a low boiling point and a lipophilic profile, which gives them their volatile nature [9]. VOCs are produced by a range of fungi and bacteria genera, and they can mostly be detected in specific genera. This means that they can be identified and, when a detection method is available, they can be located in the environment [8].

In terms of detection methods, electronic detectors have been developed that are able to classify genera of fungi, such as *Penicillium*, *Cladosporium*, *Stachybotrys* and *Aspergillus*, among others, by analysing the VOC emitted by the crops [10]. As an alternative detection method to electronic detectors, microorganism biodetection CUs can be used. Trained to detect, locate and indicate the presence of specific genera and/or species of fungus, this type of dog provides a fast, economical and new method for screening environments and substrates that are susceptible to contamination. Such CUs are currently being used successfully to detect fungi that damage buildings (*Serpula lacrymans*, *Coniophora puteana* and *Antrodia sinuosa*, among others) [11] and it may be possible to extend their use to many other areas, particularly in the agricultural sector.

For the detection of fungi with CUs, certain authors, such as Kauhanen *et al.* (2002), have used live cultures on Petri plates (for instance, *Cladosporium herbarum* and *Aspergillus niger* on Malt Extract Agar) or sowed the fungus in the detection substrate (for example, *Serpula lacrymans* and *Antrodia sinuosa* directly inoculated in

chunks of pine wood). Despite providing good results, these methods involve a health risk, both in the case of the dog, instructor and trainer, and for the environment, with a key risk being the possibility of suffering hypersensitivity reactions and/or mycosis.

Having identified the current needs in terms of controlling verticillium wilt, the potential of using CUs and the health requirements of the odorous sample, the aim of the present research is to determine the capacity of a CU to detect the fungus in controlled environments.

2. Materials and Methods

2.1. Production of Odorous Samples

The process of producing the samples is based on obtaining a pure culture of *Verticillium dahliae* on Sabouraud Agar and extracting the volatile organic compounds with sterile distilled water that is stored in sterile vials The odour profile of the samples must match the profile of the pure culture, so the suitability of each batch of samples is verified using gas chromatography and mass spectrometry.

2.2. Training the CU

The dog used in this study is an 18-month-old crossbreed from a dog pound. It has been trained by a professional instructor to sit when it detects the distinctive odour of *V. dahliae* and to stare at its source. The training system used is based on operant conditioning using positive reinforcement, whereby the dog learns to search on its own initiative with the aim of receiving a reward. This ensures that false negatives are avoided by working to enhance its motivation. In order to prevent false positives, a systematic desensitization process is carried out with respect to other microorganisms and elements related to the samples, such as the filter paper and glass. After two months of training, the dog was formally tested to ascertain its capacity to detect the samples.

2.3. Formal Testing and Statistical Analysis of the Canine Unit

To conduct the statistical analysis, a blind search was carried out (in which the instructor does not know which samples are positive) with 100 positive odorous samples and 100 negative. A total of 20 sessions were held on 20 consecutive days with the dogs being presented with 10 samples, with a combination taken from the whole set of positive and negative samples.

Once the sensitivity and specificity of detecting the odorous samples was ascertained, a series of 1:2 dilutions were made of the samples in order to determine to which level of dilution of the original sample the dog is capable of detecting.

Lastly, taking the necessary precautions, 5 search sessions were conducted with a pure culture of *V. dahliae* in a controlled space, and 10 search sessions in a real plot with 20 infected trees previously diagnosticated.

Inconclusive responses were taken as negatives. These include all of the responses in which, when coming across the odour, the dog did not sit and stare.

2.4. Safety Precautions

The samples are used under aseptic conditions, using nitrile gloves and material that is disposed after a single use. This ensures that the samples are not contaminated by other microorganisms or exogenous odours. After use, the samples are sterilized in an autoclave to prevent environmental contamination.

The dog is inspected by a vet on a quarterly basis and, as part of the general physical inspection, a sample of nasal mucus is taken in order to detect any possible changes in its microbiota.

3. Results

The dog gave a clearly positive response for 97 of the 100 positive odorous samples and an inconclusive response in the remaining 3 (staring without sitting, which is considered to be a negative response). In the case of the 100 negative samples, it gave no response in 92 cases, an inconclusive response in 3 cases (considered to be negatives), and a positive response in 5 cases.

Based on these results, for the odorous samples produced in the laboratory from *V. dahliae*, it is calculated that the dog displays a level of sensitivity of 97% and specificity of 95%.

With respect to the series of 1:2 dilutions, the dog correctly detected the samples up to a dilution of 1:16. The Petri plates with the pure culture were found in 3 minutes in an enclosed area of 100 m^2 with no air currents.

In the preliminary assay with real infected trees, the dog offered a positive response for 19 of the 20 olive trees affected by verticillium wilt (initial field effectiveness of 95%).

4. Discussion

The use of a reliable, real and safe odour source provides significant advantages in comparison to using the live microorganism. Key benefits include: 1) safety for the dog, instructor, trainer and the environment; 2) homogeneity of the odour over time and between sample batches; 3) long useful lifecycle and simple storage, with the option of industrial scale-up; 4) reduced risk of contamination by other odours and microorganisms; 5) the possibility of using the samples to develop electronic detectors, if prior determination of the VOCs is performed by HPLC-MS.

The positive results obtained in the research indicates that the use of biodetection CUs applied to olive groves could be extended to the detection of other infectious diseases, such as olive peacock spot caused by *Cycloconium oleaginum*, the rust and blackening caused by *Capnodium* spp. and *Alternaria* spp., or olive tree tuberculosis due to infection by *Pseudomonas syringae*. The fast and early detection of these diseases enables a reduction in the financial losses resulting from the treatment and the lower quality of the end product.

In the same way that diseases caused by microorganisms can be detected, other authors have demonstrated that it is possible to detect insects that cause blights and infestations, such as the flies the *Calliphoridae* genus [12]. Some of the most significant blights affecting olive groves that can be controlled using CUs are pests that cause the fruit to fall, such as olive fruit fly (*Dacus oleae*) and olive moth (*Prays oleae*), as well as pests that that suck the sap and dry the plant out, such as olive scale (*Saissetia oleae*) and olive psyllid (*Euphyllura olivina*). Moreover, detection would also be possible in the case of certain genera of nematode plant parasites, such as *Helicotylenchus* [13].

With respect to verticillium wilt, preventive measures are the most effective strategy to combat the disease, with key measures including the use of plants that are free from the pathogen, establishing plantations in unaffected soils, vaccination and the use of resistant varieties such as Escarabajillo, Racimal and Verdial de Badajoz [14].

Further measures must be taken to prevent the introduction of the pathogen, which may be caused by soil particles being carried by floods, wind or ground movements. In the event of resorting to chemical treatments, only authorized active ingredients must be used. Whichever type of prevention measures are used, they can be combined with soil screening using CUs in order to ascertain their efficacy and enhance the safety measures implemented.

With respect to plantations infected by the fungus, the traditional measures that help to prevent it from spreading include: removing and burning the infected tissues; avoiding the interspersed planting of susceptible cultivars; removing weeds; balanced fertilization (preventing an excess of nitrogen and lack of potassium); adequate irrigation management; using tolerant or resistant varieties; using organic fertilizers; and solarizing the affected soil. The use of CUs in such cases would enable, for instance, the detection of affected plants and tissues, both in the case of olive trees and weeds, as well as the identification of the soil quality with on-site sampling and the determination of the presence of *V. dahliae* in the irrigation water.

In this research, the capacity of a CU to detect the fungus in controlled working environment (laboratory) has been identified. In terms of detection in real environments, a study is being conducted to determine the effectiveness of the use of CUs for the detection of a large range of substrates, including principally: soil samples, olive tree shoots, weeds, irrigation water, farm machinery and other plants susceptible to infection (for instance, ornamental plants such as *Petunia* spp., aromatic plants such as *Mentha* spp. and fruit and vegetable plants such as *Persea americana*).

5. Conclusion

Canine units represent an economical and reliable tool for the detection of infectious diseases, blights and invasive plant species in olive groves, which is simple to apply and offers a broad range of possibilities. Canine units provide significant benefits in terms of detection time and diagnosis. They enable focalized treatments to be applied with a smaller financial investment and are a good complement to preventive methods. Lastly, it should be

noted that it is possible to perform the same tasks using an electronic detection system, after having determined and standardized the odour profile of the substance or living being to be detected.

Acknowledgements

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