

Morpho-anatomical and microbiological analysis of kiwifruit roots with KVDS symptoms

I. D'Ippolito^{1,a}, S.M. Mang², H.S. Elshafie², I. Camele², G. Scillitani³, M. Mastrodonato³, A. Sofo¹, A.N. Mininni¹ and E. Xylogiannis⁴

¹Università degli Studi della Basilicata, DiCEM, via Lanera, 20 Matera 75100, Italy; ²Università degli Studi della Basilicata, SAFE, Viale dell'Ateneo 85100 Lucano, 10 Potenza, Italy; ³Università degli Studi di Bari, Dipartimento di Biologia, via E. Orabona, 4 Bari 70126, Italy; ⁴Zespri Fresh Produce Italy Srl., Via delle Margherite 121, 04011 Aprilia (LT), Italy.

Abstract

Italy, one of the largest producers of kiwifruit in the world, has lost 10% of its production in recent years because of the spread of the kiwifruit vine decline syndrome (KVDS). Although the aetiology of KVDS has not been characterized, root rot symptoms are often associated with water stagnation and root asphyxia. To investigate causal factors and potential solutions to counter this syndrome, an experimental trial was undertaken in a kiwifruit orchard affected by KVDS in Latina (central Italy) in 2020. Root samples from healthy plants were collected and compared with samples taken from plants affected by KVDS. Macroscopically, the roots affected by KVDS were rotting, showing a loss of rhizodermis and cortical parenchyma. Microscopic analysis revealed damage to the root system with tissue breakdown and decomposition, flaking of the rhizodermis, cortical area with a clear loss of cell turgor, initial decay of the stele and evident detachment of the cortex from the central conducting tissues. Light microscopy, morphological and molecular analyses were carried out on the rhizodermis of roots showing decay and death symptoms. Total DNA extracted from the pure fungal colonies was amplified by PCR with ITS primers, amplicons directly sequenced, and the obtained nucleotide sequences were compared with those present in the GenBank database (NCBI) through BLAST analysis. Genomic analysis allowed the identification of three abundant fungi namely *Ilyonectria vredenhoekensis*, *Fusarium oxysporum* and *Paraphaeosphaeria michotii*. Further investigation is required to determine the role of these fungi in KVDS, whether they are species favoured by water stagnation and root asphyxia; their abundance and presence in other regions, orchards, and kiwifruit species; if they compromise roots functionality individually or conjunction with other microbial pathogens or abiotic factors; and if they contribute to plant death associated with KVDS.

Keywords: antagonistic activity, kiwifruit roots, kiwifruit vine decline syndrome (KVDS), microscopy, molecular identification, waterlogging

INTRODUCTION

Kiwifruit is a crop with a significant water requirement but also can be extremely sensitive to water stagnation, even if transitory. If the soil does not rapidly drain excess water this can result in anoxic soil conditions (Reid et al., 1992). The production of kiwifruit is of great commercial importance in Italy but, in recent years, the kiwifruit industry (both for *Actinidia chinensis* var. *chinensis* and *A. chinensis* var. *deliciosa*) has been threatened by the emergence of a new syndrome which causes a severe and rapid decline, kiwifruit vine decline syndrome (KVDS), which can cause collapse of the vine within a few years of the appearance of first symptoms. The symptoms associated with KVDS include root browning, reduction of fine roots, and root morpho-anatomical changes, such as the rupture and decomposition of cortical tissues with loss of cell turgor and flaking of the rhizodermis, followed by leaf chlorosis and subsequent necrosis, wilting of the shoots and, finally, plant death. There is also

^aE-mail: dippolito.ilaria@libero.it



sufficient evidence that damage to the foliage in kiwifruit plants subject to root asphyxia is associated with the increase of ethylene, abscisic acid and the decrease of cytokinins from the damaged roots (Jackson and Drew, 1984). Unfortunately, the causes of KVDS are still unknown. As part of the Zespri Innovation project “Water and Soil management of Gold3 in Italy” in collaboration with the University of Basilicata (UNIBAS), in 2020 a trial was launched in a kiwifruit orchard affected by KVDS in Latina (Lazio, ETo = 732 mm – Italy), in order to investigate root symptoms and the possible causes of this syndrome.

MATERIALS AND METHODS

For this study, a commercial kiwifruit orchard (*Actinidia chinensis* var. *chinensis* ‘Zesy002’) located in Latina was used. The orchard had areas showing KVDS and areas that did not show disease symptoms, which were used as a control. The irrigation volume applied was approximately 6.500-7.000 m³ ha⁻¹ year⁻¹ which was about 20% higher compared to the effective irrigation requirement. In the site with KVDS symptoms, an impermeable layer at 1 m depth of the soil was revealed, the beginning of soil compaction started from a depth of 40 cm and the groundwater surface was detected at a depth of 40-50 cm (Sofa et al., 2022). In October 2020, root samples were taken in two areas (control/KVDS) from 3 plants at soil depths of 10-20 and 40-50 cm.

Light microscopy

After excavating for root harvesting (Figure 1), root samples were collected at depths 10-20 and 40-50 cm, from healthy and KVDS-affected plants for comparison. In particular, roots (from 2 to 5 mm) were collected from 3 healthy plants (control) and 3 plants affected by KVDS. For microscopic analysis, roots were fixed in 10% (v/v) formalin to preserve the morphology and chemical composition of the tissues, dehydrated to an alcohol scale to replace the tissue water with a miscible agent with the cleared paraffin solvents for impregnating tissues with a paraffin solvent, and embedded in paraffin, which penetrates inside the cells and intercellular spaces making the sample more resistant to the sectioning phase. Each individual sample was sectioned by means of a rotating microtome into sections with a thickness of 5 µm. The staining performed was safranin-fast green, which contrasts the different constituents of the sample to be observed under the optical microscope.



Figure 1. Excavation of roots from kiwifruit plants (*Actinidia chinensis* var. *chinensis*) in Latina, Italy at a depth of 1m. Roots were selected from a healthy plants (left) and plants affected by kiwifruit vine decline syndrome (right).

Fungal isolation and identification

Small pieces of kiwifruit roots, from plants showing KVDS were surface sterilized, placed on Petri dishes containing potato dextrose agar (PDA) media amended with kanamycin (1 mg L⁻¹) and streptomycin (1 mg L⁻¹) and incubated at 25°C in dark until colony growth was visible. Pure fungal cultures were obtained before morphological and molecular

identification.

DNA isolation and amplification

Total fungal genomic DNA (gDNA) was extracted from 100 mg of pure culture following a protocol fully described by Mang et al. (2020). Briefly, fungal mycelium was scrapped off the surface of a growing colony, powdered in liquid nitrogen and then gDNA was extracted using the Macherey-Nagel GmbH & Co. KG (Dürren, Germany) kit following manufacturer's instructions with some modifications. Quantity and quality of the recovered gDNAs was checked by readings at Nanodrop ND-1000 spectrophotometer (Thermoscientific Inc., Whatman, MA, USA). Total gDNAs were stored at -20°C until further analyses. Subsequently, all gDNAs were amplified by polymerase chain reaction (PCR) using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990) which amplify a fragment of the internal transcribed spacer (ITS) region of the rRNA gene as described by Mang et al. (2020). All amplicons were directly sequenced using Illumina technology and the same primers as for the PCR, by the BMR Genomics s.r.l. (Padua, Italy). Nucleotide sequences obtained were compared to those already existing in the GenBank (NCBI) public database for similar genes/regions using the Basic Local Alignment Search Tool (BLASTn) program (Altschul et al., 1990).

RESULTS AND DISCUSSION

Light microscopy

The root system of plants showing symptoms of KVDS were characterized by the scarcity or absence of fine roots; macroscopically, the structural roots were found to be rotting, with diffuse and decaying brown areas, showing a loss of rhizodermis (Figure 2). Microscopic analysis revealed damage to the root system, with tissue breakdown and decomposition (Figure 3), flaking of the rhizodermis, cortical area with clear loss of cellular turgor, initial decay of the stele and evident detachment of the cortex from the conducting tissues. In the control sample, the roots showed a rhizodermis with an average thickness of 13 µm and an average cell size of the parenchyma of 44.5 µm, unlike the KVDS sample, where the average thickness of the rhizodermis was 8.3 µm and the mean cell size 34.7 µm.



Figure 2. Kiwifruit roots from a health plant (left) and roots affected by kiwifruit vine decline syndrome (right).

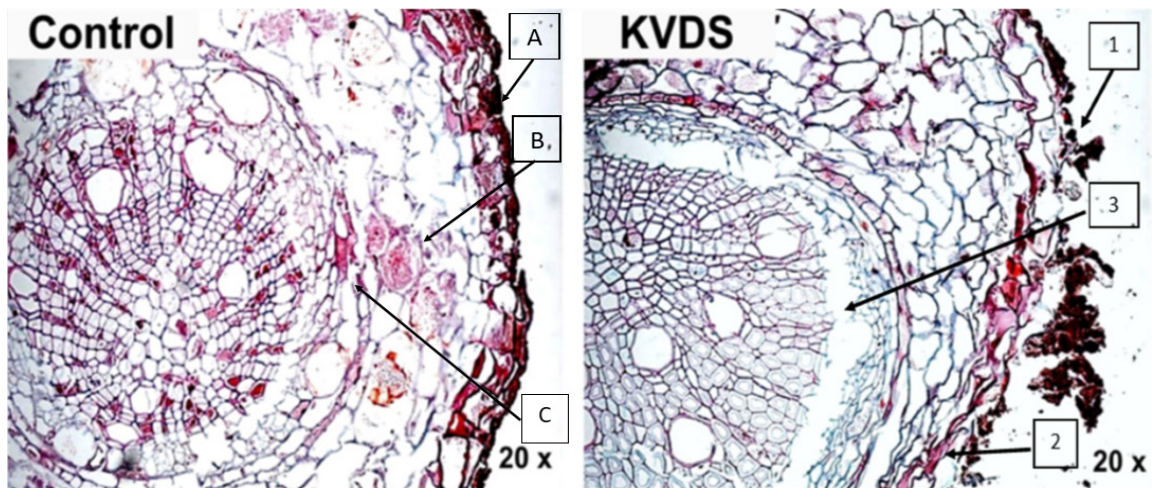


Figure 3. Cross section from a healthy kiwifruit root (left) showing an intact rhizodermis (A), cellular turgor in the cortical area (B) and intact stele (C). Cross section of a root affected by kiwifruit vine decline syndrome (right) showing a flaking rhizodermis (1), a cortical area with evident loss of cellular turgor (2) and an initial disruption of the stele (3); the detachment of the cortex from the central conducting tissues is evident.

Kiwifruit roots have high oxygen consumption, even though the space between the root cells intended for the circulation of gases is rather limited (about 2% of the entire volume) (Smith et al., 1989). For this reason, the species is counted among those highly sensitive (empty space between root cells less than 5% of the total root volume) under anoxic conditions in the rhizosphere (Jackson and Drew, 1984). Low oxygen concentrations could also be related to changes in the metabolism of root tissues affected by KVDS, likely due to the induction of certain enzymes, such as cellulase, pectinase and xylanase (Smith et al., 1990; Bailey-Serres and Voesenek, 2008). Meristems are the most sensitive tissues to anoxia, being characterized by high cell density with and high aerobic metabolic activity. Therefore, they are often the first part of the root system to be damaged (Smith et al., 1990; Bailey-Serres and Voesenek, 2008). This could explain the stunted growth of new fibrous roots, a typical symptom in kiwifruit plants affected by KVDS, and the non-reactivation of the suberized roots. Furthermore, asphyxiated soil conditions can activate the fermentative metabolism induced by the lack of oxygen, resulting in cell death when the starch reserves run out (Bailey-Serres and Voesenek, 2008). Indeed, starch depletion has been observed in the roots of kiwifruit showing symptoms of decline (Smith et al., 1990). The low oxygen concentration could also induce the loss of cortical tissue stiffness observed in plant roots affected by KVDS due to the induction of certain enzymes, such as cellulase, pectinase and xylanase (Smith et al., 1990; Bailey-Serres and Voesenek, 2008). For avoiding KVDS symptoms, we suggest to improve irrigation management avoiding the excess of the irrigation water and improving the horizontal and vertical movement of water in the soil by installing underground drainage systems.

Fungal isolation and identification

The most abundant fungi isolated from symptomatic kiwifruit roots were preliminary identified based on morphological features (colony characteristics on PDA media and light microscopy observations). They belonged to the following genera: *Paraphaeosphaeria*, *Fusarium* and *Ilyonectria*.

All isolated fungi from the kiwi roots and those utilized in the antagonistic assay, investigated in this study, have been also identified by and molecular methods. Total gDNA was successfully obtained for all studied fungi. PCR amplicons of the expected size (approximately 700 bp) were produced for all above described taxa and their nucleotide

sequences identified by BLAST analysis were deposited into the EMBL GenBank nucleotide archive. Fungal nucleotide sequences obtained had shown high similarity (about 99%) with sequences already present in the nucleotide archive (NCBI) of *Paraphaeosphaeria michotii*, *Fusarium oxysporum* and *Ilyonectria vredenhoekensis*. A possible implication of the three isolated fungi from symptomatic kiwi roots for causing KVDS should be considered in the future studies.

CONCLUSIONS

KVDS occurs mainly in compacted and waterlogged soils, two predisposing characteristics for root asphyxiation. Microscopy analysis of roots displaying symptoms of KVDS revealed damage to the root system with tissue breakdown and decomposition, flaking of the rhizodermis, cortical area with a clear loss of cell turgor, initial decay of the stele and evident detachment of the cortex from the central conducting tissues. Potential solutions for KVDS could involve improving the physical quality to minimize root asphyxiation and improve root growth. This could provide the kiwifruit roots with the oxygen necessary to mitigate the effects of potentially pathogenic microorganisms, many of which proliferate under certain conditions. Three fungal species, *Paraphaeosphaeria michotii*, *Fusarium oxysporum* and *Ilyonectria vredenhoekensis* were abundant in kiwifruit roots displaying symptoms of KVDS; further research is required to understand any role they may play in the development of KVDS.

ACKNOWLEDGEMENTS

With the support of the Zespri Innovation project “G3 water and soil management in Italy” GI21020.

Literature cited

- Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. *J Mol Biol* 215 (3), 403–410 [https://doi.org/10.1016/S0022-2836\(05\)80360-2](https://doi.org/10.1016/S0022-2836(05)80360-2). PubMed
- Bailey-Serres, J., and Voesenek, L.A.C.J. (2008). Flooding stress: acclimations and genetic diversity. *Annu Rev Plant Biol* 59 (1), 313–339 <https://doi.org/10.1146/annurev.arplant.59.032607.092752>. PubMed
- Jackson, M.B., and Drew, M.C. (1984). Effects of flooding on growth and metabolism of herbaceous plants. In *Flooding and Plant Growth*, T.T. Kozlowski, ed. (Academic Press), p.47–128.
- Mang, S.M., Scrano, L., and Camele, I. (2020). Preliminary studies on fungal contamination of two Rupestrian churches from Matera (southern Italy). *Sustainability* 12 (17), 6988 <https://doi.org/10.3390/su12176988>.
- Reid, J.B., Tate, K.G., and Brown, N.S. (1992). Effects of flooding and alluvium deposition on kiwifruit (*Actinidia deliciosa*). *N. Z. J. Crop Hortic. Sci.* 20 (3), 283–288 <https://doi.org/10.1080/01140671.1992.10421769>.
- Smith, G.S., Buwalda, J.G., Green, T.G.A., and Clark, C.J. (1989). Effect of oxygen supply and temperature at the root on the physiology of kiwifruit vines. *New Phytol.* 113 (4), 431–437 <https://doi.org/10.1111/j.1469-8137.1989.tb00354.x>.
- Smith, G.S., Judd, M.J., Miller, S.A., and Buwalda, J.G. (1990). Recovery of kiwifruit vines from transient waterlogging of the root system. *New Phytol* 115 (2), 325–333 <https://doi.org/10.1111/j.1469-8137.1990.tb00459.x>. PubMed
- Sofo, A., Mininni, A.N., Dichio, B., Mastroleo, M., and Xylogiannis, E. (2022). Physical structure and chemical quality of waterlogged soils in an Italian kiwifruit orchard. *Acta Hort.* 1332, 195-202 <https://doi.org/10.17660/ActaHortic.2022.1332.26>.
- White, T.J., Bruns, T., Lee, S., and Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protoc*, M.A. Innis, T.J. White, J.J. Sninsky, and D.H. Gelfand, eds. (New York, NY, USA: Academic Press Inc.), p.315–322.

