NEWSLETTER

N° 5 - September 2023

Periodical information bulletin for the Prometeo project co-financed by the European Union in the framework of the ENI Cross-Border Cooperation (CBC) Programme "Italy-Tunisia" 2014-2020

The ENI CBC Programme "Italy-Tunisia" 2014-2020 is a bilateral cross-border cooperation programme, co-financed by the European Union under the European Neighbourhood Instrument (ENI). With a budget of EUR 33.3 million, the programme, which joint management has been assigned to the Sicilian Region's Programming Office, aims to promote fair, equitable and sustainable economic, social and territorial development in order to foster cross-border integration and enhance the territories and resources of the two participating countries. https://www.italietunisie.eu/

Table of contents of the Newsletter:

SPECIAL EDITION: PLANT PATHOLOGY AND MATERIALS SCIENCE PROTOCOLS OF THE PROMETEO PROJECT

With this special issue, the publication of the scientific protocols of the Prometeo project is completed; in this Newsletter, space is dedicated to the other scientific protocols of Plant Pathology and Material Science.
During July, August and September, field experimentation and validation of the protocols continued, and the results achieved will provide a solid scientific basis to guide agricultural policies, strengthen phytosanitary services, increase production efficiency, competitiveness and sustainability in these sectors, and improve quality standards in food safety.

Enjoy your reading!

Blue and green mould of citrus *Penicillium digitatum* and *P. italicum*

Green and Blue Molds by *Penicillium digitatum* and *P. italicum* (family *Trichocomaceae*), stand out as the most destructive post-harvest diseases of citrus fruit. The geographical distribution of these two species includes all of the citrus-producing areas in the world and they have been also described in countries that only import but do not produce citrus (Frisvad and Samson 2004). Both *P. digitatum* and *P. italicum* are strict wound pathogens that infect the fruit through peel injuries produced in the field, in the packing house or during the fruit commercialization chain (Bautista-Baños 2014; Palou 2014*). P. digitatum* is the most serious and widespread pathogen and is considered the main cause of economic losses in citriculture, resulting in 90% of the total post-harvest losses of citrus fruit (Costa et al. 2009). *P. italicum*, instead, is more common in fruit held in cold storage during the summer and it can spread in packed cartons more readily than Green Mold, causing a so called 'nest' of decayed fruit (Ismail and Zhang 2004). Up to date knowledge on the etiology of the diseases, especially regarding aspects related to the mechanisms of infection and the environmental conditions favoring the pathogen development, could be useful to plan 'smart' management strategies. In this respect, the adoption of minimal requirements, such as the implementation of improved post-harvest handling, sanitation of equipment, transport and storage conditions (Naqvi 2006), could represent necessary and sufficient measures to safeguard yield and, at the same time, to guarantee the safety of the environment and of human health.

1. Diagnosis

1.1. *In field* - symptoms and signs

At the early stages, *P. digitatum* wound infections from which fruit spoilage starts develop a soft area on the fruit peel surrounding the wound. Warm temperatures promote the development of a white mycelium on the soft area and, shortly after, the production of green, spores. The infection proceeds quickly, and within a few days, the entire fruit results totally molded and covered by green conidia. The fruit begins to shrink and, if exposed to air, becomes a slimy shapeless mass. Initial lesions of *P. italicum* are similar to lesions caused by *P. digitatum*, but the spores are blue in color and the area of the citrus peel where they appear is typically surrounded by a narrow band of white mycelium growing on water-soaked rind. With time, the entire surface of the fruit is completely covered by spores; then, the fruit begins to shrink and, if exposed to air, becomes a slimy shapeless mass.

Fig.1 Orange fruit affected by green (*P. digitatum*) and blue (*P. italicum*) molds. Bottom left, 7-day-old cultures of *P. digitatum* isolate (left) and *P. italicum* (right) grown on PDA medium at 25°C.

1.2. *In laboratory* – isolation, characteristics of cultures and molecular identification of the pathogen

Penicillium spp. can be isolated by placing a 5 mm fragment of *Penicillium* infected citrus peel excised from the margin of the advancing growing area, disinfected with 1% NaClO for 2 min, rinsed in sterile distilled water, and plated on potato dextrose agar (PDA) amended with streptomycin sulfate at the concentration of 0.25 g/L. After 24 h of incubation at 25˚C in the dark, the growing colony are transferred on PDA. Pure cultures are obtained by single-hypha transfer. Macroscopic characteristics can be studied on Czapek yeast autolysate agar (CYA), sucrose yeast extract agar (YES), malt extract agar (MEA) and potato dextrose agar (PDA) (Samson et al., 2014; Visagie et al., 2014). These characteristics include: colony size, colony colour front and reverse, appearance and grade of sporulation. The colour, texture and shape of colonies can vary from one species to another. Colonies of *P. digitatum* are characterized in the front by an olive-green color, and in the back the colony ranges from colorless to creamy yellow or dull light brown. Instead, *P. italicum* colonies are plane, heavy sporing, blue or gray-green colored and often appear granular due to the presence of bundles of conidiophores and conidial heads. The reverse is uncolored or gray to yellow-brown, although it can turn to brownish orange or red brown. Microscopic observations are made from 7-10 old cultures using a light microscope (Leica). These observations concern the length of phialides, metules and stipes; the length and width of conidia; and the type of penicillia structure.

The identity of the pathogen is confirmed by traditional PCR by the amplification of the Internal Transcribed Spacer (ITS) region of the nuclear ribosomal DNA (rDNA), by using the universal primer pairs ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3', (White et al., 1990). The PCR products are purified using a PCR purification column (Macherey-Nagel) and sent for sequencing. Sequences were compared using the BLAST programme (http://www.ncbi.nlm.nih.gov/BLAST/).

2. Management

The traditional management of this disease involves specific actions which begin in the field and continue to the storing stage in the warehouse. Typical post-harvest measures, including drenching with sanitation compounds, such as chlorine or recently peroxyacetic acid (Ismail and Zhang, l.c.). Fruit moved internationally are further chemically treated to control *Penicillium* spp. using compounds such as imazalil and thiabendazole (La Spada et al. 2021). The employment of fungicides has detrimental side effects including the presence of substances with acute toxicity in fruit peels and the accumulation in soil, water and plants, of non-biodegradable active compounds, resulting in environmental contamination and in additional hazards for human health. An additional negative aspect of the use of synthetic pesticides is the emergence of resistance strains in pathogen populations. In accordance with the European Directive 2009/128/EC, which establishes a frame of community action for the sustainable use of pesticides in order to reduce the risks for human health while satisfying the growing request for high-quality, safe and eco-friendly products, alternative means to synthetic fungicides, have been actively identified and tested. These alternatives include the use of antagonistic microorganisms or of their bio-derivatives (La Spada et al. l.c.; Riolo et al. 2023) as well as the use of natural substances (botanicals and other organic substances) and other natural antimicrobial compounds (La Spada et al. l.c.; Yang et al. 2021).

3. References

1. Frisvad J., Samson R. (2004). Polyphasic taxonomy of *Penicillium* subgenus *Penicillium*. A guide to identification of food and air-borne terverticillate penicillia and their mycotoxins. Stud. Mycol., 49:1–174.
2. Baiyewu, R. A., Amusa, N. A., Ayoola O. A., Babalola O. (2007). Survey of the post-harvest diseases and aflatoxin contamination of marketed pawpaw fruit (Carica papaya L) in South Western Nigeria. African *Journal of Agricultural Research*. 2, 178–18. DOI/10.5897/AJAR.9000665.
3. Bautista-Baños S (2014). Postharvest Decay: Control Strategies. Elsevier Inc. 1-383.
4. Costa J.H., Bazioli J.M., de Moraes Pontes J.G., Fill TP (2019). Penicillium digitatum infection mechanisms in citrus: What do we know so far? Fungal Biol 123(8):584-593. https://doi.org/10.1016/J.FUNBIO.2019.05.004
5. La Spada F., Aloi F., Coniglione M., Pane A., Cacciola S.O. (2021). Natural biostimulants elicit plant immune system in an integrated management strategy of the postharvest Green Mold of orange fruits incited by *Penicillium digitatum*. Front Plant Sci

12:1-16. Naqvi S (2006) Diagnosis and management of pre and post-harvest diseases of citrus fruit. Dis Fruits Veg 1:339–359.
6. Naqvi.S. (2006). Diagnosis and management of pre and post-harvest diseases of citrus fruit. Dis. Fruits Veg. 1:339–359.
7. Palou L. (2014) *Penicillium digitatum, Penicillium italicum* (Green Mold, Blue Mold). In Postharvest Decay, Academic Press: Cambridge, MA, USA, 2014; pp. 45–102.
8. Riolo M., Luz C., Santilli E., Meca G., Cacciola S.O. (2023). Antifungal activity of selected lactic acid bacteria from olive drupes. Food Bioscience 52: Article 102422. https://doi.org/10.1016/j.fbio.2023.102422
9. Visagie, C. M., Houbraken, J., Frisvad, J. C., Hong, S. B., Klaassen, C. H. and Perrone, G. (2014). Identification and nomenclature of the genus *Penicillium*. Studies in Mycology. 78, 343-371. DOI: 10. 1016/j. simyco. 2014. 09. 001
10. White, T. J., Bruns, T., Lee, S., and Taylor, J. W. (1990). "Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics," in PCR Protocols: A guide to Methods and Applications. eds. M. A. Innis, D. H. Gelfand, J. J. Sninsky and T. J. White (San Diego, California, USA: Academic Press, Inc), 315–322.

Root and crown rot of almond-*Phytophthora* spp.

The root and crown rot of almond is a widespread disease associated to several *Phytophthora* species, including *Phytophthora cambivora, P. citrophthora, P. cryptogea* and *P. megasperma*, *P. cactorum*, *P. citricola*, *P. niederhauserii*, *P. plurivora*, *P. chlamydospora*. To date, the disease has been reported from Australia, Spain, California, Iran and Turkey (Wicks et al., 1986; Wicks, 1989; Browne et al., 1997; Sahragard et al., 2006; Pérez-Sierra et al., 2010; Kurbetli et al., 2011; Abad et al., 2014; Browne et al., 2015; Çiftçi et al., 2016; Kurbetli et al., 2016; Türkölmez et al., 2016; Browne et al., 2020).

Fig. 1 Symptoms observed in field and nursery samples of almond trees: (a) leaf yellowing; (b) canker and crown rot; (c) general decline; (d) gum exudates at the base of the trunk; (e) root and crown rot (image from Beluzán et al., 2022)

As for similar diseases incited by *Phytophthora* species, the cycle of Phytophthora root and collar rot of almond is complex and involves numerous sources of primary and secondary inoculum and several modes of dissemination. The primary inoculum, which survives as mycelium,

oospores and chlamydospores in infected tissues, starts epidemics when environmental conditions are favorable and the presence of a host plant stimulates spores to germinate. Conditions conducive to the development of Phytophthora root and collar rot are typically wet tropical conditions. Then, zoospores, swarming from sporangia, swim toward the growing host root and aggregate either just behind the root tip, in wounds, or in areas where roots branch off. They encyst just before infecting the root. The cyst germinates and begins to differentiate the hyphae that grow inside the host. Although soil saturation is necessary for infection, once *Phytophthora* is inside plant tissues, it can continue to colonize the root even if the soil is not saturated and grows through the root system. At the beginning, only a few fine roots are damaged. Then, the pathogen grows through the root system into larger roots until it reaches the root crown area where it kills the cambium. Consequently, the upper portions of the plant begin to wilt and die back and the root rotting is accelerated. Symptoms may develop quickly when the water demand of the plant increases during the first dry periods of summer. The success of primary infection leads to the differentiation of secondary inoculum on the surface of rotting roots. In general, *Phytophthora* depends on free water for spread and infection, and on human activity for long distance spread (Cacciola et al., 2011).

1. Diagnosis

1.1. *In field* - symptoms

Phytophthora root and collar rot of almond usually occurs in trees where long-term water-logging of the soil has taken place. Symptoms on infected trees include chlorosis and premature drop of the leaves, progressive thinning and dieback of the whole canopy (Fig. 1). Symptoms on the canopy are caused by the extensive necrosis of roots and by the consequent reduction of the active root system. Additional symptoms include wilting, cankers and profuse stem gumming.

1.2.1 *In laboratory* – isolation from infected plant material

Isolation from symptomatic tissues (roots or collar fragments). Symptomatic tissues are thoroughly washed in tap water, superficially disinfected in 1% NaClO for 2 min, then immersed in 70% EtOH for 30 s, rinsed in sterile distilled water, dipped dry, and plated on selective PARPNH V8-agar (Jung et al., 1996). After an incubation period of 24–48 h in the dark at 25° C, pure cultures are obtained by transferring outgrowing single hyphae onto V8-juice agar (V8A) (Erwin and Ribeiro, 1996). Purified cultures are finally obtained by single hyphal culture on V8-agar.

1.2.2 *In laboratory* – molecular identification of isolates

Although, in the past, identification of *Phytophthora* isolates has largely relied on synoptic keys (Waterhouse, 1963; Newhook et al., 1978; Stamps et al. 1990) which are based on morphological criteria, the overlap of a number of microstructures and characters among species and the high intraspecific variability make these approaches inadequate. (Cacciola et al., 2011). For this reason, nowadays molecular methods based on PCR represent the best approach for identifying *Phytophthora* isolates.

The prerequisite to the application of PCR is the DNA extraction from pure culture. To this aim, commercial kits can be used.

The most common conventional PCR protocol for the identification of *Phytophthora* isolates consist in the amplification, sequencing and bioinformatic analysis of the barcodes Internal Transcribed Spacer (ITS) of ribosomal DNA (rDNA) and the cytochrome oxidase (cox) 1 of mitochondrial DNA (mtDNA) (Robideau et al. 2011). The ITS region can be amplified by using primer pairs ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') / ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990) or ITS6 (5'-GAAGGTGAAGTCGTAACAAGG-3') (Cooke et al., 2000) / ITS4. COX 1 barcode can be amplified by using the oomycete-specific primers OomCoxI-Levup (5'-TCAWCWMGATGGCTTTTTTCAAC-3') and Fm85mod (5'-RRHWACKTGACTDATRATACCAAA-3') (Robideau et al. 2011).

1.2.3. *In laboratory* – detection of *Phytophthora*-infected plant material by Real-time PCR

Due to the high variability of different *Phytophthora* species involved in root and collar rot of almond, no species-specific Real-Time PCR protocols are suggested. A more successful strategy can be perceived in the detection and quantification of *Phytophthora* DNA present in plant tissue. To this aim, three TaqMan RT-PCR assays are proposed: i. Internal Transcribed Spacer

(ITS) region (amplified using All_Phy_probe and FITS_15Ph, RITS_279Ph primers); ii. trnM-trnP-trnM region (amplified using TrnM_PhyG_probe2 and primers PhyG-F2, PhyG-Rb); iii. atp9-nad9 region (amplified using ATP9_PhyG2_probeR and primers PhyG_ATP9_2FTail, PhyG_R6_Tail). Details about primers and probes are reported in Puertolas et al. (2021).

2. Management

Many studies demonstrate the crucial role of the soil-water status on both the production of sporangia and the occurrence of root infections by *Phytophthora* species. As asphyxiation due to soil water saturation predisposes the roots to the infection of these pathogens, Phytophthora root and crown rots are associated with both heavy soils and prolonged periods of rainfall (Cacciola et al., 2007). Consequently, the management of soil water is basic for the control of this disease (Cacciola et al., 2011).

Careful selection and preparation of the planting site could help in preventing soil water-logging problems. Similarly, cultural practices that prevent prolonged saturation of soil such as planting on mounds, soil drainage and proper management of irrigation may reduce root and collar rot (Cacciola et al., 2011).

Irrigation technologies involving the use of emitters that do not wet the trunks and instruments that measure the water status in soil, such as tensiometers and neutron probes, may be valuable for the development of an integrated disease management approach (Cacciola et al., 2011).

3. References

1. Abad, G.; Abad, J.; Cacciola, S.; Pane, A.; Faedda, R.; Moralejo, E.; Pérez-Sierra, A.; Abad-Campos, P.; Álvarez-Bernaola, L.; Bakonyi, J.; et al. *Phytophthora niederhauserii* sp. nov., a polyphagous species associated with ornamentals, fruit trees and native plants in 13 countries. Mycologia **2014**, 106, 431–447
2. Beluzán, F.; Miarnau, X.; Torguet, L.; Armengol, J.; Abad-Campos, P. Survey of Oomycetes Associated with Root and Crown Rot of Almond in Spain and Pathogenicity of *Phytophthora niederhauserii* and *Phytopythium vexans* to 'Garnem' Rootstock. *Agriculture* **2022**, *12*, 294. https://doi.org/10.3390/agriculture12020294
3. Browne, G.; Schmidt, L.; Brar, G. First report of *Phytophthora niederhauserii* causing crown rot of almond (*Prunus dulcis*) in California. Plant. Dis. **2015**, 99, 1863
4. Browne, G.; Viveros, M. Diverse symptoms and tree losses caused by *Phytophthora* spp. in California almonds. Acta Hortic. **1997**, 470, 570–575.
5. Browne, G.; Ott, N.; Forbes, H.; Yaghmour, M.; Milliron, L. First Report of *Phytophthora chlamydospora* causing crown and root rot on almond in California. Plant. Dis. **2020**, 104, 2033.
6. Cacciola, S.O.; Faedda, R.; Pane, A.; Scarito, G. Root and crown rot of olive caused by *Phytophthora* spp. In Olive Diseases and Disorders; Schena, L., Agosteo, G.E., Cacciola, S.O., Eds.; Transworld Research Network: Trivandrum, Kerala, India, **2011**; pp. 305–327.
7. Çiftçi, O.; Türkölmez, Ş.; Derviş, S.; Serçe, Ç. First report of canker and root rot of almond caused by Phytophthora plurivora in Turkey. Plant. Dis. **2016**, 100, 1507.
8. Cooke, D.E.L.; Drenth, A.; Duncan, J.M.; Wagels, G.; Brasier, C.M. A molecular phylogeny of Phytophthora and related oomycetes. Fungal Genet. Biol. **2000**, 30, 17–32
9. Erwin, D.C.; Ribeiro, O.K. Phytophthora Diseases Worldwide; APS Press—The American Phytopathological Society: St. Paul, MN, USA, **1996**; pp. 84–95, 96–144, 456–463.
10. Jung, T.; Blaschke, H.; Neumann, P. Isolation, identification and pathogenicity of *Phytophthora* species from declining oak stands. Eur. J. For. Pathol. **1996**; 26, 253–272.
11. Kurbetli, I.; Değirmenci, K. First report of *Phytophthora* taxon *niederhauserii* causing decline of almond in Turkey. New Dis. Rep. **2011**, 23, 14.
12. Kurbetli, İ.; Yılmaz, A. Almond decline caused by *Phytophthora megasperma* in southeastern Anatolian region of Turkey. J. Turk. Phytopathol. **2016**, 45, 13–20.
13. Newhook F.J., Waterhouse G.M., and Stamps, D.J. Tabular key to the species of *Phytophthora* de Bary. Commonw. Mycol. Inst., Kew, Surrey (UK) Mycological Papers **1978**, 143, 20.
14. Pérez-Sierra, A.; León, M.; Álvarez, L.; Alaniz, S.; Berbegal, M.; García-Jiménez, J.; Abad-Campos, P. Outbreak of a new *Phytophthora* sp. associated with severe decline of almond trees in eastern Spain. Plant. Dis. **2010**, 94, 534–541.
15. Puertolas A., Bonants P.J.M., Boa E., Woodward S. Application of Real-Time PCR for the Detection and Quantification of Oomycetes in Ornamental Nursery Stock. *Journal of Fungi*. **2021**; 7(2):87. https://doi.org/10.3390/jof702008
16. Robideau G.P., De Cock A.W.A.M., Coffey M.D., Voglmayr H., Brouwer H., Bala K., et al. DNA barcoding of oomycetes with cytochrome c oxidase subunit I and internal transcribed spacer. Mol Ecol Resour. **2011**; 11: 1002–1011. pmid:21689384
17. Sahragard, N.; Banihashemi, Z. Evaluation of resistance of some almond genotypes and cultivars to *Phytophthora cactorum*. Iran. J. Plant. Pathol. **2006**, 42, 97–99.
18. Stamps, D.J., Waterhouse, G.M., Newhook, F.J., and Hall, G.S. Revised Tabular Key to the species of *Phytophthora*, Mycological Papers. **1990**; 162, 1.
19. Türkölmez, Ş.; Derviş, S.; Çiftçi, O.; Ulubaş Serçe, Ç. First report of *Phytophthora chlamydospora* causing root and crown rot on almond (Prunus dulcis) trees in Turkey. Plant. Dis. **2016**, 100, 1796.
20. Waterhouse, G.M. Key to the species of Phytophthora De Bary. Commonwealth Mycological Institute, Kew (UK), Mycological Papers. **1963;** 92, 22.
21. White, T.J., Bruns, T., Lee, S., and Taylor, J.W. 1990. "Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics," in *PCR Protocols: a Guide to Methods and Applications*, eds M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (San Diego, CA: Academic Press Inc), 315–322.
22. Wicks, T.; Lee, T. Phytophthora crown rot of almond trees. Aust. J. Agric. Res. **1986**, 37, 277–287
23. Wicks, T. Susceptibility of almond and cherry rootstocks and scions to *Phytophthora* species. Aust. J. Exp. Agric. **1989**, 29, 103–109.

Root and collar rot of Olive-*Phytophthora* spp.

As a consequence of the expansion of plantings in new areas with heavy soils and the more intensive use of irrigation in both olive nurseries and commercial groves, during recent years, Phytophthora root and collar rot (Fig. 1) is emerging as a serious disease in several olive-growing countries, such as Australia, Italy and Spain (Cacciola et al., 2011).

The disease has been reported from most olive-growing countries and is caused by several soil-borne species belonging to *Phytophthora* genus, including *Phytophthora acerina*, *P. bilorbang*, *P. cinnamomi*, *P. citricola*, *P. cryptogea*, *P. drechsleri*, *P. gonapodyides*, *P. inundata*, *P. megasperma*, *P. nicotianae*, *P. palmivora*, *P. pini*, and *P. plurivora* (Cacciola et al., 2011; Santilli et al. 2020).

Fig. 1 Decline symptoms on a tree of olive (*Olea europaea*) cv. Nera di Gonnos incited by *Phytophthora bilorbang* in Calabria.

The disease cycle is complex as it involves numerous sources of primary and secondary inoculum and several modes of dissemination; all these features confer to this organism a high plasticity. The primary inoculum, which survives as mycelium, oospores and chlamydospores in infected tissues, starts epidemics when environmental conditions are favorable and the presence of a host plant stimulates spores to germinate; conditions conducive to the development of Phytophthora root and collar rot are typically wet tropical conditions. Then, zoospores, swarming from sporangia, swim toward the growing host root and aggregate either just behind the root tip, in wounds, or in areas where roots branch off. They encyst just before infecting the root. The cyst germinates and begins to differentiate the hyphae that grow inside the host. Although soil saturation is necessary for infection, once *Phytophthora* is inside plant tissues, it can continue to colonize the root even if the soil is not saturated and grows through the root system. At the beginning, only a few fine roots are damaged. Then, the pathogen grows through the root system into larger roots until it reaches the root crown area where it kills the cambium. Conse-

quently, the upper portions of the plant begin to wilt and die back and the root rotting is accelerated. Symptoms may develop quickly when the water demand of the plant increases during the first dry periods of summer. The success of primary infection leads to the differentiation of secondary inoculum on the surface of rotting roots. In general, *Phytophthora* depends on free water for spread and infection, and on human activity for long distance spread. Explosive epidemics are caused by the rapid increase of secondary inoculum and the slope of the disease progress curve depends upon the rate of propagation success of these propagules. In Mediterranean regions, temperatures during winter may limit the development of root infections of species with a higher optimum temperature such as *P. nicotianae* and *P. palmivora* (Cacciola et al., 2011).

1. Diagnosis

1.1. *In field* - symptoms

Phytophthora root and collar rot of olive usually occurs in young trees where long-term water-logging of the soil has taken place; only occasionally, however, this disease has been found also on trees growing in well-drained soils. Symptoms on infected trees include chlorosis and premature drop of the leaves, progressive thinning and dieback of the whole canopy (Fig. 1). Symptoms on the canopy are caused by the extensive necrosis of roots and by the consequent reduction of the active root system. When soil conditions are conducive to the infection, a crown and basal stem rot girdling the tree, which can be better noticed by unearthing the tree, may occur. Infected trees decline progressively over several years or die suddenly. Chronically infected trees appear stunted (Cacciola et al., 2011; Linaldeddu et al., 2020; Santilli et al. 2020).

1.2.1 *In laboratory* – isolation from infected plant material

Isolation from symptomatic tissues (roots or collar fragments). Symptomatic tissues are thoroughly washed in tap water, superficially disinfected in 1% NaClO for 2 min, then immersed in 70% EtOH for 30 s, rinsed in sterile distilled water, dipped dry, and plated on selective PARPNH V8-agar (Jung et al., 1996). After an incubation period of 24–48 h in the dark at 25° C, pure cultures are obtained by transferring outgrowing single hyphae onto V8-juice agar (V8A) (Erwin and Ribeiro, 1996). Purified cultures are finally obtained by single hyphal culture on V8-agar.

1.2.2 *In laboratory* – molecular identification of isolates

Although identification of *Phytophthora* isolates has largely relied on synoptic keys (Waterhouse, 1963; Newhook et al., 1978; Stamps et al. 1990) based on morphological criteria, the low number of different microstructures together with the great intra-specific variability and overlapping characters among species, make these approaches inadequate (Cacciola et al., 2011). For this reason, nowadays molecular methods based on PCR represent the best approach for identifying *Phytophthora* isolates.

The prerequisite to the application of PCR is the DNA extraction from pure culture. To this aim, commercial kits can be used.

The most common conventional PCR protocol for the identification of *Phytophthora* isolates consist in the amplification, sequencing and bioinformatic analysis of the barcodes Internal Transcribed Spacer (ITS) of ribosomal DNA (rDNA) and the cytochrome oxidase (cox) 1 of mitochondrial DNA (mtDNA) (Robideau et al. 2011). The ITS region can be amplified by using primer pairs ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) / ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990) or ITS6 (5′-GAAGGTGAAGTCGTAACAAGG-3′) (Cooke et al., 2000) / ITS4. COX 1 barcode can be amplified by using the oomycete-specific primers OomCoxI-Levup (5′-TCAWCWMGATGGCTTTTTTCAAC-3′) and Fm85mod (5′-RRHWACKTGACTDATRATACCAAA-3′) (Robideau et al. 2011).

1.2.3. *In laboratory* – detection of *Phytophthora*-infected plant material by Real-time PCR

Due to the high variability of different *Phytophthora* species involved in root and collar rot of olive, no species-specific Real-Time PCR protocols are suggested. A more successful strategy can be perceived in the detection and quantification of *Phytophthora* DNA present in plant tissue. To this aim, three TaqMan RT-PCR assays are proposed: i. Internal Transcribed Spacer

(ITS) region (amplified using All_Phy_probe and FITS_15Ph, RITS_279Ph primers); ii. trnM-trnP-trnM region (amplified using TrnM_PhyG_probe2 and primers PhyG-F2, PhyG-Rb); iii. atp9-nad9 region (amplified using ATP9_PhyG2_probeR and primers PhyG_ATP9_2FTail, PhyG_R6_Tail). Details about primers and probes are reported in Puertolas et al. (2021).

2. Management

Many studies demonstrate the crucial role of the soil-water status on both the production of sporangia and the occurrence of root infections by *Phytophthora* species. As asphyxiation due to soil water saturation predisposes the roots to the infection of these pathogens, Phytophthora root and collar rot of olive is associated with both heavy soils and prolonged periods of rainfall (Teviotdale, 2005; Cacciola et al., 2007). Consequently, the management of soil water is basic for the control of this disease (Cacciola et al., 2011).

Careful selection and preparation of the planting site could help in preventing soil water-logging problems. Similarly, cultural practices that prevent prolonged saturation of soil such as planting on mounds, soil drainage and proper management of irrigation may reduce root and collar rot (Cacciola et al., 2011).

Irrigation technologies involving the use of emitters that do not wet the trunks and instruments that measure the water status in soil, such as tensiometers and neutron probes, may be valuable for the development of an integrated disease management approach (Cacciola et al., 2011).

3. References

1. Cacciola, S.O., Scarito, G., Salamone, A., Fodale, A.S., Mulè, R., Piraino, G., and Sammarco, G. **2007**. Integrated Protection of Olive Crops, A. Kalaitzaki (Eds.), IOBC/wprs Bull., 30, 251.
2. Cacciola, S.O.; Faedda, R.; Pane, A.; Scarito, G. Root and crown rot of olive caused by *Phytophthora* spp. In Olive Diseases and Disorders; Schena, L., Agosteo, G.E., Cacciola, S.O., Eds.; Transworld Research Network: Trivandrum, Kerala, India, **2011**; pp. 305–327.
3. Cooke, D.E.L.; Drenth, A.; Duncan, J.M.; Wagels, G.; Brasier, C.M. A molecular phylogeny of Phytophthora and related oomycetes. Fungal Genet. Biol. **2000**, 30, 17–32
4. Erwin, D.C.; Ribeiro, O.K. Phytophthora Diseases Worldwide; APS Press—The American Phytopathological Society: St. Paul, MN, USA, **1996**; pp. 84–95, 96–144, 456–463.
5. Jung, T.; Blaschke, H.; Neumann, P. Isolation, identification and pathogenicity of *Phytophthora* species from declining oak stands. Eur. J. For. Pathol. **1996**; 26, 253–272.
6. Newhook F.J., Waterhouse G.M., and Stamps, D.J. Tabular key to the species of *Phytophthora* de Bary. Commonw. Mycol. Inst., Kew, Surrey (UK) Mycological Papers **1978**, 143, 20.
7. Puertolas A., Bonants P.J.M., Boa E., Woodward S. Application of Real-Time PCR for the Detection and Quantification of Oomycetes in Ornamental Nursery Stock. *Journal of Fungi*. **2021**; 7(2):87. https://doi.org/10.3390/jof702008
8. Robideau G.P., De Cock A.W.A.M., Coffey M.D., Voglmayr H., Brouwer H., Bala K., et al. DNA barcoding of oomycetes with cytochrome c oxidase subunit I and internal transcribed spacer. Mol Ecol Resour. **2011**; 11: 1002–1011. pmid:21689384
9. Santilli, E.; Riolo, M.; La Spada, F.; Pane, A.; Cacciola, S.O. First Report of Root Rot Caused by *Phytophthora bilorbang* on *Olea europaea* in Italy. *Plants* **2020**, *9*, 826. https://doi.org/10.3390/plants9070826
10. Stamps, D.J., Waterhouse, G.M., Newhook, F.J., and Hall, G.S. Revised Tabular Key to the species of *Phytophthora*, Mycological Papers. **1990**; 162, 1.
11. Teviotdale, B.E. **2005**, Diseases of olive. Olive production manual 2 nd edition, G.S. Sibbett, L. Ferguson, J.L. Coviello, and M. Lindstrand (Eds.), University of California, Division of Agriculture and Natural Resources, Oakland, Publ. N. 3353, 119
12. Waterhouse, G.M. Key to the species of Phytophthora De Bary. Commonwealth Mycological Institute, Kew (UK), Mycological Papers. **1963;** 92, 22.
13. White, T.J., Bruns, T., Lee, S., and Taylor, J.W. 1990. "Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics," in *PCR Protocols: a Guide to Methods and Applications*, eds M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (San Diego, CA: Academic Press Inc), 315–322.

Protocol for the analysis of plant tissues by means of Time-of-Flight Secondary Ion Mass Spectrometry

Introduction

Time-of-Flight Secondary Ion Mass Spectrometry is an analytical technique used to analyze the surface composition of solid materials at a molecular level. ToF-SIMS provides detailed information about the elemental and molecular composition and spatial distribution of species present on the surface of a sample[1]. In ToF-SIMS, a high-energy primary ion beam, typically composed of cesium or bismuth, is directed to the sample surface. Upon impact, the primary ions dislodge and ionize secondary ions from the surface of the sample. These secondary ions are then extracted, accelerated, and detected based on their mass-to-charge ratio (m/z) using a time-of-flight mass spectrometer. The ToF-SIMS instrument measures the flight times of the secondary ions from the sample surface to the detector. The flight time is directly proportional to the mass-to-charge ratio of the ions, allowing for their identification and quantification. By scanning the primary ion beam across the sample surface, ToF-SIMS can generate two-dimensional images that depict the spatial distribution of specific molecular or elemental species. ToF-SIMS can provide valuable information about the chemical composition of surfaces, such as organic and inorganic compounds, polymers, metals, semiconductors, and biological materials. It is widely used in various fields, including materials science, surface chemistry, thin film analysis, semiconductor research, biomedical research, and forensic science.

ToF-SIMS is a valuable tool for surface analysis in the field of plant science and can contribute to a better understanding of the chemical and structural properties of vegetal samples[2]. It can be used to study vegetal samples such as leaves or wood providing valuable insights into the surface composition and chemical characteristics of the vegetal specimen. When analyzing vegetal samples, ToF-SIMS can provide information about the distribution of various elements, organic compounds, and biomolecules present on the surface. It can detect and identify a wide range of chemical species, including lipids, sugars, proteins, pigments, lignin, cellulose, and other components that are important for understanding the composition and structure of leaves, wood, and other plant materials. By analyzing the molecular composition and spatial distribution of these compounds, ToF-SIMS can provide insights into the physiology, metabolism, and environmental interactions of plants. It can be used to study the effects of environmental factors, such as pollutants or pathogens, on the surface chemistry of leaves. It can also be useful in the characterization of wood samples, including the identification of different wood species, assessment of wood degradation, and evaluation of treatment methods[3].

Sample preparation

ToF-SIMS analysis requires the sample to be placed under vacuum conditions. The primary reason for this is because the sample is bombarded with a primary ion beam, and the resulting secondary ions are detected and analyzed. Operating the instrument under vacuum conditions ensures that the secondary ions can freely travel to the detector without significant scattering or interference from gas molecules. Vacuum conditions can potentially cause certain issues or artifacts when analyzing vegetal samples with ToF-SIMS such as:

- Sample dehydration: Vacuum conditions can cause the evaporation of volatile compounds, leading to sample dehydration. Vegetal samples, such as leaves or wood, often contain moisture and volatile components that may be lost during the vacuum process. Dehydration can affect the sample's surface composition and alter the distribution of certain compounds.

- Structural changes: Vegetal samples may undergo structural changes under vacuum due to

1 Benninghoven, A. (1994). Chemical analysis of inorganic and organic surfaces and thin films by static time-of-flight secondary ion mass spectrometry (TOF-SIMS). *Angewandte Chemie International Edition in English*, *33*(10), 1023-1043.

2 Perkins, M. C., Bell, G., Briggs, D., Davies, M. C., Friedman, A., Hart, C. A., ... & Rutten, F. J. M. (2008). The application of ToF-SIMS to the analysis of herbicide formulation penetration into and through leaf cuticles. *Colloids and Surfaces B: Biointerfaces*, *67*(1), 1-13.

3 La Spada, F., Pane, A., Licciardello, A., Debdoubi, A., Tuccitto, N., & Cacciola, S. O. (2022). A super absorbent polymer containing copper to control Plenodomus tracheiphilus the causative agent of mal secco disease of lemon. *Frontiers in Microbiology*, *13*, 987056.

the removal of moisture or changes in pressure. These structural alterations can impact the surface chemistry and overall composition of the sample, potentially affecting the results of the ToF-SIMS analysis.

To mitigate these issues, certain precautions can be taken when analyzing vegetal samples with ToF-SIMS under vacuum:

- Minimize the vacuum exposure time to reduce dehydration effects.
- Consider cryogenic techniques or alternative sample preparation methods to preserve the native state of the sample.
- Use appropriate sample handling techniques and ensure a clean vacuum environment to minimize contamination risks.

It's important to evaluate the specific requirements of the study and consult with experienced operators to determine the most suitable approach for analyzing vegetal samples while minimizing the potential negative effects of vacuum conditions.

Cryogenic techniques for sample preparation involve subjecting the sample to low temperatures using cryogens, such as liquid nitrogen. These techniques are used to preserve the native state of samples and minimize any potential changes or artifacts during analysis[4]. Here are a few cryogenic techniques used in sample preparation:

Cryofixation: Cryofixation involves rapidly freezing the vegetal sample using liquid nitrogen or other cryogens. This technique helps to immobilize the sample's structure and preserve its properties at a specific moment in time. Cryofixation is often used in electron microscopy to capture samples in their native state, providing high-resolution imaging without significant structural alterations.

Cryosectioning: Cryosectioning is a technique used to obtain thin slices (sections) of a frozen sample. Vegetal sample is embedded in a medium, such as a resin or gelatin, and rapidly frozen using liquid nitrogen. The frozen block is then sectioned using a cryostat, which allows precise cutting of the sample at low temperatures. Cryosectioning is commonly employed in various applications, including histology and biological sample preparation for imaging or analysis.

Cryomicrotomy: Cryomicrotomy is similar to cryosectioning, but it is specifically used for cutting thin sections of samples for analysis under a microscope. The sample is frozen, and a microtome equipped with a cryo-chamber is used to cut thin slices of the sample while maintaining low temperatures. Cryomicrotomy is frequently used in materials science, biology, and other fields where the preservation of sample structure is crucial.

Cryoembedding: Cryoembedding involves embedding the sample in a cryoprotective medium, such as OCT (Optimal Cutting Temperature) compound or resin, and freezing it for subsequent analysis. The cryoembedded sample can be sectioned, mounted, and subjected to various analytical techniques while maintaining the integrity of the sample structure.

By employing cryogenic techniques, samples can be preserved in their native state, minimizing dehydration, structural changes, and other artifacts that may occur during traditional sample preparation methods. Cryogenic sample preparation is particularly useful for studies involving delicate or temperature-sensitive samples, such as biological tissues, polymers, and soft materials.

Analysis conditions

Ion beam damage can occur when analyzing vegetal samples with techniques like ToF-SIMS. The high-energy primary ions in ToF-SIMS can cause sputtering, which is the physical removal of atoms or molecules from the sample surface. Sputtering can lead to the loss of surface layers, including organic compounds and biomolecules. This can affect the representation of the sample's true surface composition and alter the spatial distribution of certain components. The en-

4 Passarelli, M. K., & Winograd, N. (2011). Lipid imaging with time-of-flight secondary ion mass spectrometry (ToF-SIMS). *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, *1811*(11), 976-990.

ergy deposition from the ion beam can induce structural modifications in vegetal samples. The ions can break chemical bonds, disrupt the molecular arrangement, and cause changes in the sample's surface morphology. These structural modifications can impact the surface chemistry and alter the distribution of chemical species. The ion beam can also induce chemical transformations in the vegetal sample. The high-energy ions can break or rearrange chemical bonds, leading to the formation of new chemical species or the modification of existing ones. This can result in chemical artifacts or the production of secondary compounds that were not originally present on the sample surface.

To mitigate these effects, it is important to carefully consider the ion beam conditions used in the analysis. Optimization of ion beam energy, dose, and size can help minimize the extent of damage caused to vegetal samples. It is also important to select appropriate analysis parameters to ensure that the obtained data accurately represent the sample's surface composition. Additionally, complementary techniques can be used to corroborate and complement the results obtained from ion beam analysis. Techniques such as microscopy, spectroscopy, or imaging techniques that do not involve high-energy ion beams can provide additional insights into the structure and composition of vegetal samples, helping to validate the findings obtained from ion beam analysis.

Understanding the potential effects of ion beam damage and carefully controlling the experimental conditions are crucial for accurate interpretation of the results and obtaining meaningful insights into the surface composition of vegetal samples. Cluster ion beams can offer certain benefits when analyzing vegetal samples or other sensitive materials[5]. They consist of clusters of atoms or molecules instead of single ions, and they can provide advantages over monatomic ion beams, such as reduced sputtering and damage effects. Cluster ion beams can enhance the detection and characterization of molecular species on vegetal samples. The larger cluster size and the collective impact of multiple atoms or molecules in a cluster can promote desorption of intact molecular fragments, providing more comprehensive molecular information about the sample. Their larger size and lower energy per constituent atom results in a specific deposit energy which may induces less structural damage to vegetal samples. This can help preserve the integrity of the sample's structure, minimize chemical transformations, and provide more accurate representation of the surface composition. Cluster ion beams can improve the detection sensitivity for certain species on vegetal samples. The enhanced desorption and ionization efficiency of clusters can lead to increased signal intensities, allowing for the detection of low-abundance or trace components that may be of interest in the analysis. However, it's important to note that the choice of ion beam, whether monatomic or cluster, depends on the specific requirements of the analysis and the nature of the vegetal sample being studied. The optimal ion beam conditions should be carefully selected to balance the need for sufficient signal intensity, minimal damage, and accurate representation of the sample's surface composition.

Data treatment

ToF-SIMS analysis of vegetal samples acquired by using cluster beams likely produces very rich and complex dataset. Multivariate analysis can be highly beneficial for handling and interpreting complex datasets obtained from ToF-SIMS analysis[6]. Principal Component Analysis (PCA) is a widely used multivariate analysis technique that can be applied to ToF-SIMS data to reduce dimensionality, identify patterns, and extract meaningful information.

ToF-SIMS data often comprises a large number of variables (intensity values for each m/z ratio). PCA can condense this high-dimensional dataset into a smaller number of principal components (PCs) while retaining most of the relevant variation in the data. This reduction in dimensionality simplifies data interpretation and visualization. PCA allows for the exploration of the inherent patterns and similarities in the ToF-SIMS dataset. By plotting the samples or variables in the space of the principal components, one can identify clusters, trends, or outliers that may correspond to distinct sample groups, chemical features, or surface compositions. This aids in understanding the underlying structure of the data. PCA helps identify the most influential var-

5 Winograd, N. (2005). The magic of cluster SIMS.
6 Tuccitto, N. (2018). Automated data mining of secondary ion mass spectrometry spectra. *Journal of Chemometrics*, *32*(3), e2968.

iables (m/z ratios) contributing to the observed variation in the dataset. The loading values associated with each principal component indicate the weights of different variables. High-loading variables can indicate m/z ratios that are significant for differentiating samples or capturing key chemical differences. PCA can be used for vegetal sample classification or discrimination based on their ToF-SIMS data. By assigning samples to specific groups or categories using known reference samples, PCA can facilitate the identification of similarities or differences between sample groups, aiding in classification and characterization. Prior to PCA, appropriate data preprocessing steps, such as normalization or scaling, may be required to remove systematic variations or artifacts in the dataset. This ensures that the PCA results are not biased by factors unrelated to the underlying sample composition. It's worth noting that PCA is just one of many multivariate analysis techniques available for ToF-SIMS data arising from vegetal dataset. Other methods, such as Partial Least Squares (PLS) regression or clustering algorithms, can also be employed depending on the specific objectives and characteristics of the dataset. Multivariate analysis techniques like PCA can effectively extract meaningful information, reveal hidden patterns, and aid the interpretation of complex ToF-SIMS datasets, enabling a deeper understanding of the surface composition and chemistry of the analyzed samples.

Protocol for extraction of natural substances with biological activity from agricultural by-products

Introduction

Phenolic compounds are present in several types of nuts and offer various beneficial properties. Numerous studies have shown that these compounds have a positive impact on human health by offering cardioprotective, neuroprotective, antidiabetic, anti-inflammatory, and antioxidant effects. The valorization of almond waste and its by-products, which are rich in polyphenols, has emerged as a promising solution to meet the demand for these active compounds.

Objective

Extraction of natural and biologically active substances, which can be utilized for controlling plant diseases and/or for therapeutic purposes in human and animal diseases.

Methods

1- Methodology for isolating the teguments (envelope)

Whole almonds are shelled and the hull is removed. The almond seeds are subjected to the following experimental protocol: Freezing-thawing method to isolate the natural almond peels (Mandalari et *al*., 2010a). This step involves freezing the seeds at -20˚C and subsequently thawing them by placing them at 4˚C (Mandalari et *al*., 2010a).

The almond peels are then removed and left to air dry. They are then ground using a mortar and finally stored in darkness.

2- Extraction of total lipids (Soxhlet method)

A preliminary step of extraction using the Soxhlet reflux method is carried out to remove lipids and pigments (Milbury et *al., 2006*) and to minimize oil content in the dry extract.

To determine oil yields, the fat content was extracted using the Soxhlet reflux method, which maximizes oil removal from the dry extract. Six grams of ground almond peels are placed in a cartridge and introduced into a Soxhlet extractor, equipped with a 250 mL round bottom flask at its base, that contains 220 mL of petroleum ether. The solvent is heated to approximately 55˚C for a duration of 6 hours. Once the extraction is complete, the obtained solvent is evaporated under vacuum at 40˚C using a rotary evaporator, where any remaining solvent traces are removed from the extracted oil. The result of this process is the delipidated fraction.

3- Extraction of polyphenols

The preparation of polyphenolic extracts is carried out using the maceration method men-

tioned by Stankovi (2011) with slight modifications.

Five grams of the dried almond peels were mixed in flasks with 50 mL of ethanol (96%), a polar solvent (Mandalari et *al*., 2013). After shaking the flasks in an incubator for 1 hour at room temperature, the mixture was allowed to infuse for 24 hours in darkness at 4°C. The contents of the flasks were then filtered through Whatman filter paper N°. 1, and the residues were re-extracted with an equal volume of solvent. After 48 hours, the process was repeated, and the supernatants were combined and evaporated under vacuum at 40°C using a rotary evaporator (Babbar et *al*., 2011). The resulting extracts were dried under a nitrogen stream and stored at 4°C.

4- Total polyphenol quantification using the Folin-Ciocalteu method

In an alkaline environment, polyphenols reduce the Folin-Ciocalteu (F-C) reagent - a complex of phosphotungstic/phosphomolybdic acid (yellow color) - into tungsten and molybdenum oxide (blue color). The resulting coloration, measured by spectrophotometry at 760 nm, is proportional to the amount of polyphenols present in the reaction medium. Total polyphenols were determined using the modified method of Folin Ciocalteu par Singleton, Orthofer et Lamuela-Raventós (1999). 200 µL of extract was diluted to 1 mL with Milli-Q water, then mixed with 5 mL of Folin-Ciocalteu reagent (1:10) and 4 mL of sodium carbonate (1N). After 1 hour, the mixture was measured using a UV spectrophotometer (Shimadzu, UV-1700) at 760 nm. Samples were prepared in triplicate. Results were expressed as gallic acid equivalents (GAE) by referring to the gallic acid standard curve.

5- Total flavonoids quantification

The method used in this test was that described by Zhishen, Mengcheng, and Jianming (1999) and modified by Jahanban-Esfahlan and Jamei (2012). It is based on the reaction of aluminum ions with flavonoid molecules under basic conditions.

The test was performed with a slight modification. A total of 1.5 mL of the extract was added to 450 µL of 5.3% $NaNO_2$, 900 µL of 10% $AlCl_3$-H_2O, and 4 mL of 1 M NaOH. The mixture is stirred and allowed to stand for 5 minutes before each addition. The final volume is made up to 15 mL with Milli-Q water. The absorbance is measured at 510 nm. The results are expressed in mg of catechin equivalent per 1 g of dry mass (mg EC/g MS) (Kamtekar et *al*., 2014) determined from the catechin calibration curve.

6- Evaluation of antioxidant activity

6.1- Total antioxidant activity

This test was based on the reduction of molybdenum (VI) to molybdenum (V) in an acidic medium by the antioxidant power of the extract, which induces the formation of the green-colored phosphate/Mo complex (Prieto et al., 1999).

100 µL of almond peel extract was mixed with 1 mL of a solution composed of sulfuric acid (0.6 M), sodium phosphate (28 mM), and ammonium heptamolybdate (4 mM). The mixture was then incubated in a water bath at 95°C for 90 minutes. The absorbance was then measured at 695 nm.

The total antioxidant activity was expressed in mg of equivalent of gallic acid per gram of dry matter (mg EAG/g MS), determined from the equation of the standard calibration curve.

6.2- The inhibitory activity of the radical DPPH·

The antiradical potential of a sample is reflected by measuring the inhibitory activity of the radical DPPH· (Scherer et Godoy, 2009).

DPPH· (2,2-diphenyl-1-picrylhydrazyl) is a synthetic radical with an intense violet color and absorbs in the visible domain at a wavelength of 517 nm (Binsan et al., 2008; Brand-Williams et al., 1995). The test measures the ability of an antioxidant (AH, generally phenolic compounds) to reduce the chemical DPPH· (2,2-diphenyl-picrylhydrazyl) radical by transferring a hydrogen to the nitrogen atom of the DPPH· radical, which is manifested by a discoloration of the solution

that turns yellow (Gülçin et al., 2006). This discoloration explains the ability of almond skin extracts to trap this radical detected by a UV-visible spectrophotometer at 517 nm (λ max DPPH•). The measurement of the anti-radical power by trapping the DPPH• radical was carried out according to the protocol described by Koh et al. (2011) with modifications. 900 µL of an ethanolic solution of DPPH (60 µM), with an initial absorbance of about 0.6 at 517 nm at room temperature, were mixed with 100 µL of different concentrations of each extract to be tested (0, 10, 20, 60, 100, 140, 180, 220, 260, and 300 µg/mL). After shaking, the tubes were incubated for 30 minutes in the dark, and then the absorbances of the samples were measured at λmax = 517 nm. The control solution was prepared by adding 100 µL of ethanol to 900 µL of DPPH solution. The positive control was represented by a solution of the standard antioxidant Trolox (3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H-1-benzopyran-2-carboxylic acid), whose absorbance is measured under the same conditions as the test sample.

6.3- FRAP assay

The antioxidant capacity was measured according to Benzie and Strain (1996). A volume of FRAP reagent, prepared with 83.3% ammonium acetate (300 mM; pH = 3.6) and 16.7% of the mixture (50:50) of tripyridyltriazine (TPTZ) reagent in 40 mM HCl and 20 mM FeCl36H2O, was heated at 37°C for 30 minutes. The absorbance at 593 nm was recorded as the initial data. Then, 1 mL of antioxidant extract was added, and once the kinetic reaction is complete, the final absorbance was measured. The results were expressed in µmoles of heptahydrated ferrous sulfate (Fe2+) per 100 g of wet sample.

A volume of 0.2 mL of each of the extracts at different concentrations, 0.5 mL of phosphate buffer (0.2 M, pH 6.6), and 0.5 mL of potassium ferricyanide (K3Fe(CN)6; 1%) were mixed and incubated at 50°C for 20 minutes to reduce Fe3+(CN-)6 to Fe2+(CN-)6. The reaction was stopped by adding 0.5 mL of 10% (w/v) trichloroacetic acid, followed by centrifugation at 1000 rpm for 10 minutes. Finally, 1 mL of the upper layer is mixed with an equal volume of distilled water and 0.2 mL of iron chloride (III) (FeCl3; 0.1%). The absorbance readings are taken against a blank without the extract at 700 nm. In this experiment, ascorbic acid (AA) was used as a positive control at the same chosen concentrations and under the same operational conditions. The absorbance graphs obtained for the different concentrations used were plotted. An increase in absorbance corresponds to an increase in the reducing power of the tested fractions. The effective concentration (EC50), which is defined as the concentration providing 0.5 absorbance, is an index used to compare and express the reducing capacities of phenolic compounds (Wu et al., 2015).

7- <u>Determination of flavonoid compounds by HPLC</u>

The identification and quantification of phenolic compounds were performed by liquid chromatography using the initial extract concentrated with a nitrogen stream. The extract was filtered (Nylon, 0.45 µm, 0.25 mm) before being injected into the chromatograph.

An HPLC series 1100 with a diode array detector (DAD) and a fluorescence detector (FLD), 1200 Series (Agilent Technologies) is used. The mobile phase is prepared with Milli-Q water acidified with 1% formic acid (A = aqueous phase) and methanol (B = organic phase).

The compounds were separated according to the following gradient, expressed as a percentage of A: 0-1 min up to 95%, 1-25 min up to 80%, held for 5 min, 30-90 min up to 0%, held for 10 min. Between the two consecutive injections, a period of 40 min was necessary to equilibrate the column. The column used was an Eclipse XDB-C18 column (150 x 4.6 mm, 4 µm) at a constant flow rate of 1 mL/min and an injection volume of 100 µL. For the identification and quantification of flavonoids, DAD signals were recorded at wavelengths of 280 and 360 nm, and FLD at 230 and 310 nm, respectively for excitation and emission. The internal standard was daidzein, an isoflavone present in plant-derived foods (Bolling et al., 2010). The results were expressed in mg of phenolic compounds per 100 g of fresh weight.

References

1. **Babbar, N., Oberoi, H.S., Uppal, D.S., Patil, R.T. (2011).** Total phenolic content and antioxidant capacity of extracts obtained from six important fruit residues. *Food Research International*.44 : 391- 396.
2. **Benzie, I.F., Strain, J. (1966).** The ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant power": The FRAP assay.

Analytical Biochemistry. 239, 70–76.

3. **Binsan, W., Benjakul, S., Visessanguan, W., Roytrakul, S., Tanaka, M., and Kishimura, H. (2008).** Antioxidative activity of Mungoong, an extract paste, from the cephalothorax of white shrimp (*Litopenaeus vannamei*). *Food Chemistry*. 106 (1) :185-193.
4. **Bolling, B.W., Dolnikowski, G., Blumberg, J.B., Oliver, C.Y. (2010)** Polyphenol content and antioxidant activity of California almonds depend on cultivar and harvest year. *Food Chemistry*.122, 819–825.
5. **Brand-Williams, W., Cuvelier, M. E., Berset, C. (1995).** Use of a free radical method to evaluate antioxidant activity. *Lebensmittel-Wissenschaft Und-Technologie* .28, 25–30.
6. **Gülçin İ., Mshvildadze V., Gepdiremen A., Elias R. (2006).** The antioxidant activity of a triterpenoid glycoside isolated from the berries of Hedera colchica: 3-O-(ß-Dglucopyranosyl)-hederagenin.*Phytotherapy Research*. 20(2): 130-134.
7. **Jahanban-Esfahlan, A., Jamei, R. (2012)**. Properties of biological activity of ten wild almond (*Prunus amygdalus* L.) species. *Turkish Journal of Biology*. 36, 201–209.
8. **Kamtekar, S., Keer, V., Patil, V. (n.d.). (2014).** Estimation of Phenolic content, Flavonoid content, Antioxidant and Alpha amylase Inhibitory Activity of Marketed Polyherbal Formulation. *Journal of Applied Pharmaceutical Science*. 5.
9. **Koh, P. H., Mokhtar, R. A. M., Iqbal, M. (2011).** Antioxidant potential of Cymbopogon citratus extract: alleviation of carbon tetrachloride-induced hepatic oxidative stress and toxicity. *Human & Experimental Toxicology*. 31(1), 81–91.
10. **Mandalari, G., Faulks, R. M., Bisignano, C., Waldron, K. W., Narbad, A., & Wickham, M. S. J. (2010b).** In vitro evaluation of the prebiotic properties of almond skins (Amygdalus communis L.). FEMS Microbiology Letters, 304(2), 116–122.
11. **Mandalari, G., Tomaino, A., Arcoraci, T., Martorana, M., Lo Turco, V., Cacciola, F., Rich, G.T., Bisignano, C., Saija, A., Dugo, P., Cross, K.L., Parker, M.L., Waldron, K.W., Wickham, M.S.J. (2010a).** Characterization of polyphenols, lipids and dietary fibre from almond skins (Amygdalus communis L.). *Journal of Food Composition and Analysis*. 23: 166–174.
12. **Mandalari, G., Arcoraci, T., Martorana, M., Bisignano, C., Rizza, L., Bonina, F. P., Trombetta, D., and Tomaino, A. (2013).** Antioxidant and Photoprotective Effects of Blanch Water, a Byproduct ofthe Almond Processing Industry. *Molecules*, 18, 12426-12440.
13. **Milbury, P.E., Chen, C.Y., Dolnikowski, G.G., Blumberg, J.B. (2006).** Determination of flavonoids and phenolics and their distribution in almonds. *Journal of Agricultural and Food Chemistry*. 54, 027- 33.
14. **Prieto, P., Pineda, M., Aguilar, M. (1999).** Spectrophotometric Quantitation of antioxidant Capacity through the Formation of a Phosphomolybdenum Complex : Specific Application to The Determination of Vitamin E. Analytical Biochemistry ; 269 : 337-341.
15. **Scherer, R., et Godoy, H. T. (2009).** Antioxidant activity index (AAI) by the 2,2-di-phenyl-1- picrylhydrazyl method. *Food Chemistry* ; 112 (3) : 654-658.
16. **Stankovi, M. S. (n.d.). (2011).** Total phenolic content, flavonoid concentration and antioxidant activity of Marrubium peregrinum l. Extracts. *Kragujevac Journal of Science*. 33 : 63-72.
17. **Singleton, V.L., Orthofer, R., Lamuela-Raventós, R.M. (1999)**. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *Methods of Enzymology*. 299, 152–178.
18. **Wu, P., Ma, G., Li, N., Deng, Q., Yin, Y., Huang, R. (2015).** Investigation of In vitro and In vivo antioxidant activities of flavonoids rich extract from the berries of Rhodomyrtus tomentosa (Ait.) Hassk. *Food Chemistry*. 173:194-202.
19. **Zhishen, J., Mengcheng, T., Jianming,W. (1999).** The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chemistry*. 64, 555–559.

The final Prometeo event will be held next November 14th and 15th in Tunis, Tunisia.

Are you ready?

General information on PROMETEO

Main beneficiary
Università degli Studi di Catania (UNICT)

Partners
P2: Université de Tunis El Manar (UTM)
P3: Centre Technique des Agrumes (CTA)
P4: Institut National de la Recherche Agronomique de Tunisie (INRAT)
P5: Agence Nationale de Promotion de la Recherche scientifique (ANPR)
P6: Comune di Palazzolo Acreide (PALAZZOLO)
P7: Centro di Ricerca per l'Innovazione e Diffusione della conoscenza (CERID)
P8: Expergreen S.R.L. (EXPERGREEN)

PROMETEO PROJECT IN NUMBERS	
Duration	**24 months**
Project start-up	**29/10/2021**
Date of completion	**28/10/2023**
N. of project Partners	**8**
Overall budget	**1.459.103,08 €**
EU Contribution	**1.291.659,13 €**

PROMETEO PROJECT ACTIVITIES	
Number of dissemination events and thematic meetings	**5**
Involved participants	**450+**
Project website	**1**
Social media channels	**4**

OUR CONTACTS

Project website: https://www.prometeo-italietunisie.eu

E-mail address: info@prometeo-italietunisie.eu

Facebook: https://www.facebook.com/Prometeo.ItalieTunisie

Instagram: https://www.instagram.com/prometeo_italietunisie/

Twitter: https://twitter.com/prometeo_ItaTun

Youtube: https://www.youtube.com/@prometeoitalietunisie4919

This document has been created and maintained with the financial support of the European Union in the framework of the ENI Cross-Border Cooperation (CBC) Programme "Italy-Tunisia" 2014-2020. Its content is the sole responsibility of CERID and does not necessarily reflect the views of the European Union and/or the Managing Authority.