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Suppressive and preventive activity of chemical disinfectants against sclerotia of *Verticillium dahliae* in water

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ABSTRACT

Disinfectants have been widely assessed against conidia, zoospores and mycelial growth of fungal plant pathogens in water, however, studies against sclerotia or melanized structures are limited. The disinfectants OX-VIRIN®, OX-AGUA AL25® and Deccoklor® reduce *Verticillium dahliae* conidia in water, but their potential efficacy in reducing sclerotia of the pathogen in water remains unknown. In this study, sclerotia of six *V. dahliae* isolates differing in virulence were exposed *in vitro* to a range of concentrations of the mentioned disinfectants for 30 days to evaluate the suppressive efficacy. In addition, concentrations with higher suppressive effect were tested for their preventive efficacy by means of assays where treated water was subsequently infested. Concentration and monitoring time (1 min, and 5, 15 and 30 days post-chemical treatment; dpc) were the critical factors for the efficacy of the chemicals, whereas variations depending on isolates virulence were negligible. The three highest concentrations of OX-VIRIN® (3.2–51.2 $mL\,L^{-1}$) and the two highest concentrations of OX-AGUA AL25® (0.4175 and 1.2525 $mL\,L^{-1}$) showed an average suppressive efficacy ranging from 87.8 to 100% and 99.2–100% at the last three sampling times, respectively. Deccoklor® was ineffective at the evaluated concentrations. The three highest concentrations of OX-VIRIN® and the highest concentration of OX-AGUA AL25® maintained a preventive efficacy above 97 and 95%, respectively, at all sampling times. For OX-AGUA AL25® at 0.4175 $mL\,L^{-1}$, the preventive efficacy fluctuated over sampling time, but it was of at least 68.9% at 30 dpc in repeated experiments.

1. Introduction

Verticillium dahliae Kleb. is a world-wide soilborne fungus causing Verticillium wilt in more than 400 plant species (Pegg and Brady, 2002). Different commercial crops are affected by *V. dahliae* in Spain, but olive farming is the sector most economically threatened by the associated disease (Verticillium wilt of olive; VWO). Andalusia is an autonomous community in southern Spain recognized as the main olive-cropping region worldwide (Barranco et al., 2010). Surveys carried out in major growing areas of Andalusia have reported 20% disease incidence in affected olive orchards (López-Escudero et al., 2010). The implementation of irrigation techniques in last two decades has been one of the responsible factors for the disease and severity increase in this region (Jiménez-Díaz et al., 2011; López-Escudero and Mercado-Blanco, 2011; Rodríguez et al., 2008). The transition from dry-land to irrigated grove farming has led, among other things, the presence and dispersion of the pathogen through watering systems (García-Cabello et al., 2012; Rodríguez-Jurado and Bejarano-Alcázar, 2007). Rodríguez-Jurado and Bejarano-Alcázar (2007) conducted a survey in diverse olive orchards affected by Verticillium wilt in Andalusia and found that 85.7% of them used surface or subterranean irrigation waters infested by micropropagules (< 20 and ≥ 1.2 μm in size; conidia and mycelial fragments) and/or sclerotia (≥ 20 μm in size) of *V. dahliae*. Populations of *V. dahliae* collected from irrigation waters showed a diversity in virulence to olive (six virulence group), prevailing the defoliating pathotype in critical growing areas (Jiménez-Díaz et al., 2011; Moraño-Moreno et al., 2011; Rodríguez-Jurado et al., 2008).

Lower average levels of sclerotia (1.5 per m^3) than micropropagules (3159 per m^3) were recovered from natural infested irrigation waters in Andalusia (Rodríguez-Jurado and Bejarano-Alcázar, 2007). Other researchers found up to an average of 3.225 sclerotia per m3 in water samples collected from a pumping station in Cordoba, province of Andalusia (García-Cabello et al., 2012). Although levels of sclerotia collected from irrigation waters could seem relatively low, these structures demand special caution since they contribute to a longer-term survival of *V. dahliae* in soil compared to micropropagules and constitute the infective propagules of the pathogen (Green, 1969; Schnathorst, 1981; Santos-Rufo et al., 2017). These structures are

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formed in infected plant tissues and incorporated into the soil profile after decomposition of plant debris (Jiménez-Díaz et al., 2012). Sclerotia attached to plant debris or soil particles can survive up to 14 years in soil (Schnathorst, 1981) and be spread by diverse means (i.e. runoff water, wind, filtration or human activity) to reach surface and subterranean irrigation water sources. Effective long-distance spread of sclerotia through irrigation system, from the pumping station of infested irrigation water to the emitter exit at level of farming plots, has been corroborated (García-Cabello et al., 2012).

Control methods used to reduce sclerotia density in soil of olive orchards have been explored, more commonly through solarization and biofumigation. Solarization has a high cost that makes it financially challenging to use in practice, and biofumigation has a questionable long-term efficacy since it needs to be implemented on the between-rows space (López-Escudero and Blanco-López, 2001; Jurado-Bello et al., 2013). The use of biocontrol agents to protect the root system against infection or utilizing resistant cultivars to escape from infection are other measures under study, but their effectiveness at field conditions remains unknown (López-Escudero and Mercado-Blanco, 2011). Given the limited scope of feasible control actions and knowing that irrigation waters are a source of sclerotia and highly virulent isolates of *V. dahliae*, it would be essential to include the control of sclerotia in irrigation water as an additional measure within the integrated management of VWO.

Physical, chemical, cultural and biological control methods have been tested on major phytopathogenic species detected in irrigation waters (Hong and Moorman, 2005). Chemical treatments, including fungicides and disinfectants, are the most important components of integrated pest management practices aimed to control and limit the spread of plant pathogens such as pythiaceous species in nurseries irrigation networks (Garbelotto et al., 2009; Linderman and Davis, 2008). Some physical barriers and chemical disinfectants were assessed against *V. dahliae*. Commercial sand filters proved to be ineffective in retaining sclerotia dissemination either in recirculating solution system or irrigation system of olive orchards (García-Cabello et al., 2012; Martínez et al., 2009). In contrast, chemical disinfectants based on oxidizing active ingredients (chlorine-releasing agent and activated peroxygen; most allowed water disinfectant agents; European Chemical Agency, 2015), and non-oxidizing agents (quaternary ammonium compounds) reduced water infestations by *V. dahliae* conidia (Santos-Rufo and Rodríguez-Jurado, 2016).

Most olive orchards and nurseries in Andalusia include currently a pond for storing and regulating water taken from rivers and wells, capable of holding enough water to drip-irrigate for at least 30 days (Pastor, 2005). The implementation of disinfecting treatments on ponded water would be an affordable and user-friendly practice for farmers. The disinfectants OX-VIRIN®, OX-AGUA AL25® and Deccoklor® applied to water reduced the conidia infestations with an efficacy that varied heavily with the concentration (Santos-Rufo and Rodríguez-Jurado, 2016). The efficacy of a disinfectant differs also with the type of propagule of the target microorganism (Chaidez et al., 2007; Hong et al., 2003; James et al., 2012; Kitis, 2004; Scarlett et al., 2016). *V. dahliae* conidia are hyaline, smooth-walled, non-septate, $6.5 \pm 1.5 \times 3.0 \pm 0.5\,\mu m$ in size (long x wide) and commonly single-celled. In contrast, *V. dahliae* sclerotia are brown-pigmented clumps of thick-walled cells, measuring between 25 and 100 μm in diameter (aggregates up to 200 μm) (Inderbitzin et al., 2011). It is predictable that concentrations reducing water infestations by conidia will not reduce in the same way water infestations by sclerotia. Thus, the aim of this study was to assess the *in vitro* efficacy of the aforementioned disinfectants in suppressing (infested water was treated) water infestations by sclerotia of several *V. dahliae* isolates differing in virulence. Preventive efficacy (water was previously treated and subsequently infested) in reducing water infestations by sclerotia was also assessed at disinfectant concentrations with high suppressive efficacy.

2. Materials and methods

2.1. Fungal isolates and sclerotial inoculum production

Monosporic *Verticillium dahliae* isolates differing in virulence on olive plants were evaluated for sclerotia mortality. Isolates VO188, VO175, VO153 VO138, VO145 and VO161, ordered from low to high virulence on olive plants and belonging to non-defoliating (1st to 3rd) or defoliating (4th to 6th) pathotype of the pathogen, were used (Moraño-Moreno et al., 2011). These isolates are native from irrigation water of olive orchards in southern Spain and stay kept on plum lactose yeast extract agar covered with liquid paraffin at 4 °C in darkness in the culture collection of the Area of Sustainable Crop Protection, IFAPA "Alameda del Obispo" Centre (Cordoba, Spain).

For inoculum preparation, cultures in active growth were obtained by refreshing the stored cultures on chlortetracycline water agar (CWA, 20 g of agar and 0.03 g of chlortetracycline per litter of distilled water). Active cultures were subcultured on Potato Dextrose Agar (PDA; 250 g of peeled potato, 20 g of agar and 15 g of glucose per litter of distilled water) and grown for 14 days at 24 ± 1 °C in darkness. PDA plugs (0.5 mm in diameter) colonized by the fungus were transferred to 1 L-flasks containing 450 g of sterilized (121 °C for 75 min, twice) substrate (cornmeal, silica 2–4 mm in bead diameter, and distilled water; CSW at 1:9:2, w/w). The infested CSW substrate was incubated at 24 ± 1 °C in darkness for 40 days, shaking the flasks every 4–5 days to get a redistributed growth. Sclerotia extraction was carried out as follow: a 40 g-sample of colonized CSW substrate was comminuted with a two-speed Waring® blender at low speed in 100 mL tap water for ten 10 s pulses. The blended medium was filtered through a nested set of stainless steel and nylon Filtra® sieves of 800-, 125-, and 40-μm in opening and a diameter of 200 mm (Filtra Vibración, S.L. Badalona, Spain). The contents on the sieves were rinsed under tap water for 2 min and the residue retained in the 125-μm sieve was comminuted and filtered again through 125- and 40-μm sieves arranged in tandem; the remaining content on the 40-μm sieve (containing sclerotia) was recovered in 100 mL of distilled water with 0.05% chlortetracycline and concentrated by filtering through a Sefar-Nitex® nylon membrane (41 μm in pore size and 47 mm in diameter; Sefar Headquarter, Switzerland) utilizing a Millipore® equipment (Millipore Ibérica S.A., Spain) with a vacuum pump. Equipment was disinfested in 95% ethanol for 10 min and rinsed with sterile distilled water between isolates. The sclerotia disk obtained (nylon containing sclerotia) was air dried in an airflow cabinet for 7 days to remove mycelia fragments. Subsequent blending and filtering processes through 40-μm sieve were carried out as above to break up the dried mass of sclerotia and clean out the mycelium, resulting in a final 100 mL-suspension of mycelium-free sclerotia. Inoculum concentration was estimated with a hemocytometer (Nageotte chamber) and adjusted to 1.5×10^4 sclerotia mL^{-1} using distilled water.

2.2. Chemical disinfectants

Three disinfectants differing in composition were evaluated for sclerotia eradication: OX-VIRIN®, OX-AGUA AL25® and Deccoklor®. Table 1 provides details including active agents of the various disinfectants. The manufacturer's labels recommend OX-VIRIN® Agriculture Special for disinfestation of surfaces in contact with food, equipment and environments in food industry, and for irrigation circuit cleaning, whereas OX-AGUA AL 25® is described as an algaecide suitable for the maintenance of irrigation systems, tanks, ponds and large water reservoirs, and Deccoklor® is primarily recommended for disinfesting surfaces and drinking water. Five to six concentrations of each product were checked (Table 1), the three lowest being recommended for disinfestation according to manufacturer's instructions. Each desired concentration was always freshly prepared in sterile distilled water.