Crop Protection 79 (2016) 105–116

Contents lists available at ScienceDirect

Crop Protection

journal homepage: www.elsevier.com/locate/cropro

Evaluation of chemical disinfestants in reducing *Verticillium dahliae* conidia in irrigation water

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A R T I C L E I N F O

Article history:
Received 21 May 2015
Received in revised form
15 October 2015
Accepted 18 October 2015

Keywords:
Water disinfestation
Verticillium wilt
Defoliating pathotype
Hydrogen peroxide
Olive

A B S T R A C T

Irrigation water disinfestation is an unexplored option for reducing *Verticillium dahliae* inoculum in water and consequently for more efficiently managing Verticillium wilts in Andalusia. We assessed Suppressive Efficacy (SE; water was infested and subsequently treated) and Preventive Efficacy (PE; previously treated water was subsequently inoculated) of OX-VIRIN®, OX-AGUA AL 25® and Deccoklor® in reducing water infestations by *V. dahliae* conidia. Five concentrations of each disinfestant, the lowest three being recommended by the manufacturer, were tested *in vitro* against six *V. dahliae* isolates. Validation assays were carried out in experiments under natural environmental conditions in spring. The four highest concentrations of OX-VIRIN® (0.8–51.2 mL L^{-1}), the three highest of OX-AGUA AL 25® (46.4–417.5 μL L^{-1}) and the two highest of Deccoklor® (0.375 and 3.75 mL L^{-1}), showed an *in vitro*-efficacy (SE and PE) of 96.2, 80.0 and 100.0% after 30, 5 to 30 and 15 days respectively. Therefore, recommended concentrations for OX-VIRIN® and OX-AGUA AL 25® showed a greater *in vitro*-effectiveness than those recommended for Deccoklor®. Assays in natural environmental conditions proved that OX-VIRIN® at the recommended concentration of 3.2-mL L^{-1}, applied every 28 days to water, was the most effective treatment (SE and PE), with a 100% reduction of the average relative viability after 56 days. Other chemical treatments showing high *in vitro*-efficacy, such as OX-VIRIN® at 0.8 mL L^{-1} and OX-AGUA AL 25® at 46.4 μL L^{-1} showed an SE of 99.9% after 14 and 28 days when applied every 28 and 14 days, respectively. However, PE of OX-AGUA AL 25® at 46.4 μL L^{-1} was only 59 and 38% after 28 and 14 days respectively, depending on the experiment.

1. Introduction

Verticillium dahliae Kleb. is a soilborne fungus which causes vascular wilts and losses of varying intensity in many herbaceous and perennial crops (Pegg and Brady, 2002). In Andalusia, southern Spain, the disease mainly affects olive plantations and cotton crops, but Verticillium Wilt of Olive (VWO) is nowadays considered to be the most serious disease and a major economic problem (Jiménez-Díaz et al., 2012; López-Escudero and Mercado-Blanco, 2011). The disease has become even more problematic in Andalusia with the spread of the highly virulent defoliating *V. dahliae* pathotype (Jiménez-Díaz et al., 2011; López-Escudero et al., 2010).

Irrigated olive orchards in Andalusia represent 37.3% of total Andalusian olive orchards (Anonymous, 2014), and they are irrigated with surface and subterranean waters generally stored in ponds. Irrigation water is pumped from ponds into olive orchards, and the same water is usually pumped for irrigating neighbouring crops such as cotton, sugar beet, vegetables and corn. It has been shown that irrigation water from ponds, wells and pumped water in Andalusia are a source of *V. dahliae* (García-Cabello et al., 2012; Jiménez-Díaz et al., 2011; Rodríguez-Jurado and Bejarano-Alcázar, 2007; Rodríguez-Jurado et al., 2008). Irrigation water surveys of seven and 14 olive orchards in Jaen and Seville provinces, respectively, demonstrated that 87.5% of the orchards were irrigated with water infested with *V. dahliae* in 2005 and that propagules $<$20 and $\geq$1 μm in size were numerous in water compared to propagules $\geq$20 μm in size (Rodríguez-Jurado and Bejarano-Alcázar, 2007). Moreover, irrigation waters harboured non-defoliating and defoliating *V. dahliae* pathotypes differing in virulence to cotton and olive plants (Rodríguez-Jurado et al. 2008; Moraño-Moreno et al. 2011). Hence, water is one of the factors implicated in the dispersal of the highly virulent defoliating *V. dahliae* pathotype, which frequently

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http://dx.doi.org/10.1016/j.cropro.2015.10.016

causes the death of the most implanted olive cultivar in Andalusia, Picual (Moraño-Moreno et al. 2011).

There are no available cultivars resistant to infection, or highly effective soil- or root-applied methods to control the Verticillium wilts in Andalusia (Spain). For a more efficient integrated management of Verticillium wilts it is imperative to reduce the inoculum level of *V. dahliae* in irrigation water in Andalusia, to prevent the introduction and increase of pathogen inoculum density in soil through water and to avoid pathogen dispersal and the spread of the Verticillium wilts.

Among the very limited number of studies that have addressed the eradication of *V. dahliae* in water, it was found that the slow sand filtration of recirculating nutrient solution in a closed soilless growing system did not remove *V. dahliae* propagules (Martínez et al., 2009). It is also known that the sand filtration used in the irrigation system of some olive orchards retains only some microsclerotia of *V. dahliae* (García-Cabello et al., 2012). Exploring more expensive techniques, Runia (1994) found that injecting 20 g of ozone in recirculation water of glasshouses irregularly reduced the infectivity of *Verticillium* spp. over time (25.0–2.5%), and suggested that conidia and not microsclerotia were the fungal structures eliminated. To the best of our knowledge, no other water treatment has been studied to combat *V. dahliae* infestations.

Several disinfestation techniques have been assayed for their efficacy in minimising the spread of other plant pathogens in irrigation water (Hong and Moorman, 2005; Runia, 1995). Among these techniques, chemical disinfestation is a relatively inexpensive method. Different disinfestant substances can be used alone or in combination in a variety of chemical disinfestants. Water chlorination has been successfully applied to control post-harvest diseases of fruits and vegetables, and to treat water in nursery and greenhouse irrigation systems (Cayanan et al., 2009; Hong et al., 2003; Poncet et al., 2001). Aqueous solutions of hypochlorite and chlorine gas are more commonly used although chlorine gas requires tight pH and concentration control and is highly corrosive if used improperly (Newman, 2004). Other oxidative agents such as hydrogen peroxide, peracetic acid and acetic acid are applied in the food and feed industries (Wessels and Ingmer, 2013). Hydrogen peroxide is more environmentally friendly than chlorine and peracetic acid has a higher oxidation potential than that of chlorine or chlorine dioxide (Kitis, 2004). Other widely-used disinfestant substances are quaternary ammonium compounds, present in hygiene products for domestic, personal and public buildings (hospitals and other institutions) use. They are also used in the food and feed industries. Quaternary ammonium compounds differ from oxidizing agents with regards their modes of action on microorganisms, mainly acting by damaging the cell to permeate the membrane disrupting its physical and chemical integrity (Wessels and Ingmer, 2013).

The effectiveness of a disinfestant substance can be influenced by many factors, such as concentration and contact time with the target microorganism, the nature and even the type of propagules of the target microorganism, the chemical properties of the disinfestant, such as pH and stability according to the temperature (Cayanan et al., 2009; Combrink et al., 1980; Hong et al., 2003; Ioannou et al., 2007; James et al. 2012; Kitis, 2004; Newman, 2004; Segall, 1968).

In this study, a range of chemical disinfestants based on oxidizing and nonoxidizing agents were tested to: i) evaluate suppressive (SE; infested water was treated) and preventive (PE; water was previously treated and subsequently inoculated) *in vitro*-efficacy against water infestations by conidia of *V. dahliae* isolates differing in virulence and, ii) validate SE and PE under natural environmental conditions when disinfestant concentrations showed a high *in vitro*-efficacy.

2. Materials and methods

2.1. Verticillium dahliae isolates and inoculum

Six monoconidial *V. dahliae* isolates stored at the culture collection of the Area of Crop Protection, IFAPA “Alameda del Obispo” Centre (Cordoba, Spain), were used in this study. *V. dahliae* isolates obtained from irrigation water of olive orchards in southern Spain, were previously classified in our laboratory into six virulence groups on olive plants (Moraño-Moreno et al., 2011). Isolates VO188-ND, VO175-ND, VO153-ND, VO138-D, VO145-D and VO161-D ordered in increasing virulence group were assigned to the non-defoliating (ND) and defoliating (D) *V. dahliae* pathotypes (Moraño-Moreno et al., 2011). Fungal cultures, stored on plum lactose yeast extract agar covered with liquid paraffin at 4 °C in the dark, were refreshed on water agar amended with chlorotetracycline (20 g of agar and 0.3 g of chlorotetracycline per L of distilled water) (CWA), subcultured on potato dextrose agar (250 g of peeled potato, 20 g of agar and 20 g of glucose per L of distilled water) (PDA) and multiplied on potato dextrose broth (250 g of peeled potato and 20 g of glucose per litre of distilled water) (PDB) to obtain the conidia suspensions. The inoculum was prepared by placing colonized PDA plugs (3×3 mm) in 100 mL of PDB and incubated at 24° C in the dark on an orbital shaker for 6–7 days. The suspension was filtered through double cheesecloth, counted with a hemocytometer and adjusted to 10^5 conidia mL^{-1} for *in vitro* experiments. Simulating possible field situations for natural environmental experiments, the conidial suspension of ND (VO175-ND) and D (VO145-D and VO161-D) isolates were mixed in equal amounts to obtain 3 L of inoculum suspension. Among all isolates, VO175-ND and VO145-D were the most extended in water and VO161-D the most virulent on olive (Moraño-Moreno et al., 2011). Otherwise the conidial suspension was increased in the mentioned experiments (7.5×10^5 conidia mL^{-1}), preventing higher natural reduction than *in vitro* conditions as was demonstrated in previous tests carried out under those conditions (Rodriguez-Jurado, unpublished data).

2.2. Tested chemical disinfestants

The tested chemicals were OX-VIRIN® Agriculture special (Grupo OX-CTA, Compañía de Tratamiento de Aguas, S.L., Huesca, Spain, registered as 08-20/40/90-02518-HA disinfestant for Food Industry by the Ministry of Public Health, Social Services and Equal Rights, Spain, currently under revision); OX-AGUA AL 25® (Grupo OX-CTA, Huesca, Spain, registered as 05-70-2804 alguicide by the Ministry of Public Health and Consumption, Spain; currently not commercialized) and Deccoklor® (DECCO, Ibérica Post Cosecha, S.A.U., Valencia, Spain, disinfestant commercialized as a chlorinated product with Health Registry Nº 3700009/V, Spain). The active ingredients of these chemicals are as follows: 25% hydrogen peroxide plus 5% peracetic acid and 8% acetic acid for OX-VIRIN®; 5% hydrogen peroxide plus 25% alkyl dimethyl benzyl ammonium chloride for OX-AGUA AL 25® and sodium hypochlorite (NaOCl at 8% active chlorine) for Deccoklor®. According to the manufacturer's instructions, OX-VIRIN® is recommended for disinfestation of surfaces in contact with food, equipment and environments in food industry, and for irrigation circuit cleaning, whereas the manufacturer's label for OX-AGUA AL 25® describes it as an algaecide suitable for the maintenance of irrigation systems, tanks, ponds and large water reservoirs. Finally, Deccoklor® is primarily recommended for disinfesting surfaces and drinking water. Based on the manufacturer's instructions, five concentrations of each chemical were chosen for the *in vitro* experiments: 0.2, 0.8, 3.2, 12.8 and 51.2 mL L^{-1} of OX-VIRIN®; 5.2, 15.5, 46.4, 139.2 and 417.5 μL L^{-1} of OX-AGUA AL 25® and 0.000375, 0.00375, 0.0375, 0.375 and