Postharvest Biology and Technology 77 (2013) 43–49

Contents lists available at SciVerse ScienceDirect

Postharvest Biology and Technology

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

Imazalil residue loading and green mould control on citrus fruit as affected by formulation, solution pH and exposure time in aqueous dip treatments

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ARTICLE INFO

Article history:
Received 21 July 2012
Accepted 4 November 2012

Keywords:
Imazalil sulphate
Fungicide resistance
Penicillium digitatum
Citrus

ABSTRACT

Green mould, caused by *Penicillium digitatum*, is responsible for major postharvest fruit losses on the South African fresh citrus export market. Some of these losses as well as fungicide resistance development can be attributed to sub-optimal imazalil (IMZ) residue loading on citrus fruit (<2 $\mu g\,g^{-1}$), which is commonly the case in South African packhouses. This will result in loss of control and sporulation inhibition on decayed fruit. IMZ formulation [IMZ sulphate and emulsifiable concentrate (EC)], solution pH (IMZ sulphate at 500 $\mu g\,mL^{-1}$ buffered with $NaHCO_3$ or NaOH to pH 6 and 8) and exposure time (15–540 s) were investigated in order to improve IMZ residue loading and the green mould control on Clementine mandarin, 'Eureka' lemon, and navel and Valencia orange fruit. Exposure time had no significant effect on residue loading in the unbuffered IMZ sulphate solution (pH 3). No differences were observed between the pH buffers used, but residue loading improved with increase in pH. The maximum residue limit (MRL) of 5.0 $\mu g\,g^{-1}$ was exceeded following dip treatment in the IMZ EC (after 75 s exposure time), and IMZ sulphate at pH 8 using $NaHCO_3$ (77 s) or NaOH (89 s) as buffer. The MRL was exceeded after 161 s in IMZ sulphate solutions buffered at pH 6 with either $NaHCO_3$ or NaOH. An IMZ residue-loading curve was prepared from which residue levels can be predicted for the control of IMZ-sensitive and IMZ-resistant isolates of *P. digitatum*. From this model the benchmark residue level for 95% control of an IMZ-sensitive isolate and of an IMZ-resistant isolate were predicted to be 0.81 and 2.64 $\mu g\,g^{-1}$, respectively. Residue loading can be improved by adjusting the pH level of an IMZ sulphate solution to 6 or by using the IMZ EC formulation, but exposure time should be restricted to 45 s so as not to exceed the MRL. Conversely, sufficient exposure time of ≈90 s in an unbuffered IMZ sulphate solution (pH 3) will result to improved green mould control, but with residue loading below 2 $\mu g\,g^{-1}$. The resistant isolate could not be controlled adequately with residue levels below the MRL, therewith indicating the practical relevance of IMZ resistance.

1. Introduction

South African fresh citrus is exported to northern hemisphere destinations in the USA, Asia, the Middle and Far East, Europe, and Africa. Time from harvest to market is generally 4 weeks. Every year losses occur due to decay of which green mould, caused by the wound pathogen *Penicillium digitatum* (Pers.:Fr.) Sacc. (Smith, 1897), is usually the main contributor (Eckert and Eaks, 1989). Apart from sanitation and careful handling to prevent wounds, control relies primarily on postharvest fungicide applications.

Residue loading can give an indication of the effectiveness of fungicide application, but control of green mould can differ from one application method to another regardless of the specific residue level loaded (Erasmus et al., 2011). Although alternative application methods such as drench and sprays have shown promise in terms of residue loading, the fungicide dip tank applications gave better curative control and sporulation inhibition of green mould infections (Kaplan and Dave, 1979; Erasmus et al., 2011). In work done mainly with the IMZ EC formulation, exposure time (Brown and Dezman, 1990; Smilanick et al., 1997; Cabras et al., 1999), solution temperature (Smilanick et al., 1997; Cabras et al., 1999; Dore et al., 2009) and solution pH (Holmes and Eckert, 1999; Smilanick et al., 2005; Cunningham and Taverner, 2006; Dore et al., 2010) have all been shown to have an effect on IMZ residue loading on citrus fruits by means of a fungicide dip tank. Increasing the IMZ solution temperature alone can be used to control and increase residue loading, but the cost of heating may force packhouses to look at alternative ways to improve IMZ residue loading. Sodium bicarbonate ($NaHCO_3$) can improve the effectiveness of IMZ and

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http://dx.doi.org/10.1016/j.postharvbio.2012.11.001

residues loaded better at a higher pH (7–9) than at a lower pH (3–5) (Smilanick et al., 2005).

In South Africa, Pelser and La Grange (1981) recommended the IMZ sulphate formulation for dip application with a fruit exposure time of 1–3 min and at a concentration of 250–500 $\mu g\,mL^{-1}$. This recommendation was followed widely and IMZ sulphate is currently applied extensively by 78% of South African citrus packhouses in a fungicide dip tank at 500 $\mu g\,mL^{-1}$ (Erasmus et al., 2011). The emulsifiable concentrate (EC) formulation is applied primarily with wax in South Africa, but internationally it is also applied in aqueous dip or inline drench-type applications. A residue of 2 $\mu g\,g^{-1}$ or higher is reported to give effective control or at least sporulation inhibition of green mould (Kaplan and Dave, 1979; Brown et al., 1983; Brown and Dezman, 1990; Smilanick et al., 1997). Erasmus et al. (2011) reported that during the 2007–2009 seasons the majority of South African packhouses loaded suboptimal residue levels of $\approx$1 $\mu g\,g^{-1}$.

While dip exposure time in an IMZ sulphate solution had no significant effect on residue loading, increasing the solution pH with 2% $NaHCO_3$ from 3 to 8 increased IMZ residue loading by more than three-fold (Erasmus et al., 2011). These increased residue levels improved control of green mould caused by sensitive and resistant isolates. IMZ sulphate molecules dissolved in water are protonated and hydrated; as the pH is increased above the pK_a of IMZ (6.5), the proportion of unhydrated (water insoluble) IMZ base molecules will increase. At a pH level of 8 the majority of IMZ molecules will precipitate over time, and can therefore not be recommended. The effect of pH levels lower than the pKa level of 6.5 had to be investigated in terms of residue loading and green mould control.

Due to the inadequate residue loading in South African packhouses, which often leads to inadequate control and loss of sporulation inhibition, the risk for resistance development is ever increasing. This and the extensive use of IMZ sulphate in fungicide dip tank applications highlight the need to optimise IMZ residue loading through this specific application. Increasing the pH level showed promise in earlier studies (Erasmus et al., 2011), but the effect of different buffers and pH levels needed to be investigated further to support a recommendation that would ensure optimal IMZ residue loading of >2.0 $\mu g\,g^{-1}$, without exceeding the maximum residue limit (MRL) of 5.0 $\mu g\,g^{-1}$.

IMZ residue loading on various citrus types was studied in IMZ solutions with different pH levels and exposure times. The IMZ sulphate formulation was also compared to the EC formulation in terms of residue loading. This work also presents a first attempt at determining the benchmark residue level required to successfully control IMZ sensitive and resistant isolates of *P. digitatum*.

2. Materials and methods

2.1. Isolates, IMZ sensitivity and storage

An IMZ sensitive (S) and a resistant (R) isolate of *P. digitatum* were used as described previously (Erasmus et al., 2011). The S isolate was sourced from the Welgevallen experimental farm of the University of Stellenbosch (Stellenbosch, South Africa) and the R isolate sourced from a South African packhouse by Citrus Research International (Nelspruit, South Africa). In order to obtain inoculum for biological efficacy tests, the isolates were grown at ambient temperature on potato dextrose agar (PDA; Biolab, Merck, Wadeville, Gauteng, South Africa) medium in Petri dishes and were re-plated in 2-week cycles. Conidia were harvested from 10- to 14-day-old cultures approximately 1 h before trials commenced. The surface of a culture was washed with sterile deionised water amended with Tween 20 (Sigma–Aldrich, St. Louis, MO, USA) at a concentration of 0.01 $mL\,L^{-1}$. The conidial suspensions were amended to a concentration of 1×10^6 spores mL^{-1} by means of a haemocytometer. The conidial suspensions were placed on magnetic stirrers to maintain a uniform suspension of spores.

2.2. Fruit

Untreated export quality mandarin (cv. Nules Clementine), lemon (cv. Eureka), navel orange (cv. Washington) and Valencia sweet orange (cv. Midknight) fruit were obtained from various citrus packhouses in the Western Cape province of South Africa during the season of 2009 as the specific citrus types became available. Before trials commenced, fruit were stored at 3.5–7 °C for ±3 days. A day before a trial, fruit was transferred from cold storage to ambient (±20 °C) in order for fruit temperature to reach ambient and to allow any condensation to evaporate.

2.3. Imazalil and residue analysis

Fruit were treated with an IMZ sulphate formulation (Imazacure, 750 $g\,kg^{-1}$ SG, ICA International Chemicals, Stellenbosch, South Africa) or an IMZ EC (emulsifiable concentrate; Imazacure 500 EC, ICA International Chemicals, Stellenbosch, South Africa) formulation. For IMZ residue analyses, six fruit from two separate replicate treatments were sampled from the specific treatments described below, left to dry and frozen (−20 °C) until prepared for IMZ residue analysis. The fruit were defrosted, weighed and then macerated to a fine pulp by using a blender (Salton Elite Blender, Almalgamated Appliance Holdings Limited, Reuven, South Africa) and re-frozen. Sub-samples of the macerated fruit were submitted for IMZ (chloramizol) residue analyses by Hearshaw and Kinnes Analytical Laboratory, Cape Town, South Africa. The samples were extracted using acetonitrile followed by a matrix solid phase dispersion extraction. The extracts were analysed using liquid chromatography mass spectrometry mass spectrometry (LCMS/MS; Agilent 6410, Agilent Technologies Inc., Santa Clara, California, USA). A fresh treatment solution was prepared for each treatment.

2.4. Effect of exposure time and pH on IMZ residue loading

Fruit were immersed for 15, 45, 90, 180 and 540 s in a 500 $\mu g\,mL^{-1}$ solution of either IMZ sulphate or IMZ EC. The pH of the IMZ sulphate solutions was adjusted to a pH of 6 or 8 by using either sodium bicarbonate ($NaHCO_3$; Alkalinity Plus, Pool Perfect, Bellville, South Africa) or sodium hydroxide (NaOH; Saarchem, Wadeville, South Africa). For $NaHCO_3$, 300 and 20,000 mg L^{-1} was added for pH6 and 8, respectively. For NaOH, ±90 and ±100 mg L^{-1} was added for pH 6 and 8, respectively. A temperature-controlled stainless steel warm water bath (Unitemp, Baird and Tatlock Ltd., Essex, UK) was used as IMZ dip tank (24 L) and all solutions were kept at 35 °C during treatments. Fruit were left to dry at ambient after treatment. For each treatment, samples of six fruit each per citrus kind were collected from two separate treatment repetitions for residue analysis.

Residue data following the different treatment combinations were subjected to linear regression statistics using statistical software (SAS version 9.2, SAS Institute Inc. Cary, NC, USA); intercepts of all lines were set at 0 (*i.e.* 0 $\mu g\,g^{-1}$ at 0 s dip). Analysis of variance was conducted and Student's *t*-least significant difference was calculated at the 5% significance level to compare the slopes.

2.5. IMZ residue benchmarks for effective control of P. digitatum

Fruit were treated curatively and therefore inoculated 4–6 h before IMZ dip-treatment with either the S or R isolates of *P. digitatum*. Fruit were wounded with a triple wound inducer, which consisted of three insect needles placed in a needle clamp to create