

Postharvest control of soft-rot fungi on grape berries by fungicidal treatment and *Trichoderma*

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Abstract

The present research deals with the effect of postharvest treatment of grape berries with four commonly used fungicides and two forms of *Trichoderma harzianum* on the infection with soft-rot fungi- *Rhizopus stolonifer* and *Mucor piriformis*. This effect was evaluated by comparison of the external diameter of rot-lesion in treated and untreated berries, in addition to comparison of percent reduction in external rot-lesion diameter relative to control. Results indicated that the infection with *R. stolonifer* and *M. piriformis* was significantly reduced ($P < 0.05$) in all treated berries in comparison with untreated control berries. The highest reduction in mean external rot-lesion diameter was obtained for both *R. stolonifer* and *M. piriformis* when inoculated berries were treated with Score® (difenoconazole) applied at 0.35%(v/v) or Switch® (cyprodinil + fludioxonil) applied at 0.20%(w/v) or formulated *T. harzianum* conidia in invert emulsion applied at 9.6×10^8 conidia/ml of formulation (13.5, 13.2, and 19.3 mm, respectively for *R. stolonifer*; 7.2, 7.5, and 19.2mm, respectively for *M. piriformis*). The greatest decrease in percent reduction in external rot-lesion diameter relative to control was also obtained for both the fungal species when inoculated berries were treated with the same type of fungicides (Score® and Switch®) and *Trichoderma* (formulated *T. harzianum* in invert emulsion) (60.9, 61.7, and 44.1%, respectively for *R. stolonifer*; 74.5, 73.4, and 31.9%, respectively for *M. piriformis*). Overall results indicate that the most effective treatment obtained on grape berries could be integrated with other control measures being usually used in grape berry-rot management plans by alternating fungicidal treatment (e.g. Score® or Switch®) with application of formulated *T. harzianum* conidia in invert emulsion.

Key words: Grape, *Rhizopus stolonifer*, *Mucor piriformis*, *Trichoderma harzianum*, difenoconazole, captan, cyprodinil + fludioxonil, metalaxyl + mancozeb, postharvest.

Introduction

The rapid and extensive deterioration of the table grapes is mainly caused by the fungal decay. The major organisms that are involved in this decay are: *Botrytis cinerea*, *Aspergillus niger*, *Rhizopus stolonifer* and *Mucor piriformis* (Nelson, 1979). Of these organisms, *R. stolonifer* and *M. piriformis* are the two known fungal species which cause severe losses to table grapes during marketing and export in many countries (Lisker *et al.*, 1996). The reason for these losses is the incidence of soft-rot symptoms on the infected berries during storage at temperatures $> 8^\circ\text{C}$. Mechanical wounding dramatically increases the susceptibility of berries to soft-rot fungi and facilitates their penetration into the attacked berries (Lichter *et al.*, 2002).

The conventional method used to avoid the fungal decay in table grapes is by fumigating SO_2 or releasing it from generator pads containing a metabisulfite salt, and packaging of the fruit in polyethylene liners (Lichter *et al.*, 2002). Immersion of the detached berries in 70% ethanol also eliminates most of the fungal and bacterial population on the berry surface. This immersion results in inhibition of berry decay that is equivalent to or better than that achieved by SO_2 released from generator pads (Lichter *et al.*, 2002). High levels of SO_2 can also result in fruit damage, unpleasant aftertaste and allergies (Lisker *et al.*, 1996). Other methods such as acetic acid fumigation (Sholberg *et al.*, 1996), acetaldehyde vapors (Avisar and Pesis, 1991), and modified atmosphere packaging and generating chlorine gas (Zoffoli *et al.*,

1999) can be used instead of SO_2 fumigation. Benomyl was one of the most widely used fungicides to control postharvest fungal decay especially Mucor rot on grape berries and other fruits in packinghouse (Spotts and Cervantes, 1986). Two other chemicals, orthophenyl phanate as fog and calcium hypochlorite as chlorine vapor that were applied to the surrounding atmosphere, significantly decreased postharvest decay in artificially inoculated berries (Lisker *et al.*, 1996). Vinclozolin (Ronilan 50 WP) can be used effectively against *R. stolonifer* and *B. cinerea* on table grapes, strawberries and kiwifruit (Lima *et al.*, 1997). Finally, Ozone treatment can be considered a possible substitute for SO_2 fumigation for the control of *R. stolonifer* on table grapes (Sarig *et al.*, 1996). The subatmospheric pressures (0.25, 0.50, and 0.75 atmos) for different times (1 to 24h) were used to reduce *Rhizopus* rot lesion on table grapes and other fruit types in comparison with the control treatment (1 atmos) (Romanazzi *et al.*, 2001).

Biological control as a substitute to chemical control or as practice that could be integrated with other control practices may result in acceptable levels of fruit decay with reduced levels of pesticide use such as using the following antagonistic yeast species: *Pichia membranefaciens* to control postharvest *Rhizopus* rot on nectarine fruit (Fan and Tian, 2000), *Kloeckera apiculata* and *Candida guilliermondii* to control postharvest diseases of many fruits including table grape (McLaughline *et al.*, 1992), *Cryptococcus laurentii*, *C. flavus*, *C. albicus* to control Mucor rot on pear fruit (Roberts, 1990), *Aureobasidium pullulans* and *Candida oleophila* to control *Botrytis cinerea* and *Rhizopus*

stolonifer on strawberry, table grape and kiwifruit (Lima *et al.*, 1997). Also, using the following antagonistic bacterial species: *Pseudomonas cepacia* to control Mucor rot on apple (Janisiewicz and Roitman, 1987), *Pantoea agglomerans* to control *Rhizopus stolonifer* and *Monilinia laxa* on peach, apricot and nectarine (Bonaterra *et al.*, 2003), and *Enterobacter cloacae* to control *Rhizopus* rot on peach (Wilson *et al.*, 1987).

Although antagonistic fungus, *T. harzianum* is in widespread use against many fungal plant pathogens such as root rot fungi (*e.g.* *Pythium*, *Sclerotinia*, *Rhizoctonia* and *Fusarium*) (Fravel, 1998), and postharvest fungal pathogens on different types of fruit (*e.g.* *Botrytis cinerea*, *Alternaria alternata*, *Penicillium expansum*) (Batta, 1999, 2000, 2001, 2004a, and 2004b), no previous study was carried out to control soft-rot fungi of table grapes using formulated or unformulated *T. harzianum*. Therefore, the objectives of the present research were: i) to test the efficacy of postharvest treatment of grape berries with four types of commonly used fungicides and two forms of *Trichoderma* (unformulated and formulated *T. harzianum*) on the infection with soft-rot fungi: *R. stolonifer* and *M. piriformis*, ii) to compare the efficacy of this treatment with fungicides and *T. harzianum* forms.

Materials and methods

Grape berries used in the experiments: Healthy mature grape berries (table grape cultivar: Zaini) were used for bioassay of the treatment effect of fungicides and *T. harzianum* against *Rhizopus* and *Mucor* soft-rot.

Fungal strains used in the inoculation and treatment: The following fungal strains were used in the experiments: strain RS1 of *Rhizopus stolonifer*, strain MP3 of *Mucor piriformis*, and strain Th₂ of *T. harzianum*. The first two strains were isolated from infected grape berries (cultivar: Beirut) then subcultured on plates with potato dextrose agar (PDA) medium for production of sporangia and sporangiospores to be used in the inoculation tests. The third strain which belongs to the antagonistic fungus *T. harzianum* was obtained from Faculty of Agriculture in Gembloux (Belgium) and subcultured on plates with oat meal agar (OMA). Young cultures of 14-day old of the above-mentioned strains were used to carry out the various tests of bioassay. The concentration of the conidia or sporangiospores in the suspensions prepared from these cultures was 11.7x10⁶ sporangiospores of strain RS1 per ml, 5.5x10⁵ sporangiospores of strain MP3 per ml, and 9.6x10⁸ conidia of strain Th₂ per ml.

Treatments with fungicides and *T. harzianum* used in the experiments: Four types of treatments with fungicides were applied against *Rhizopus* and *Mucor* soft-rot inoculations on grape berries. They were: 0.30% (W/V) metalaxyl + mancozeb (sold as Ridomil® MZ 63.5 WP, concentration of a.i.=7.5% metalaxyl + 56% mancozeb); 0.35% (V/V) difenoconazole (sold as Score® 250 EC); 0.35% (W/V) captan (sold as Merpan® 50 WP) and 0.20% (W/V) cyprodinil + fludioxonil (sold as Switch® 62.5 WG, a.i.=375g/kg cyprodinil + 250 g/kg fludioxonil).

Two types of treatments with *T. harzianum* (strain Th₂) were applied against *Rhizopus* and *Mucor* soft-rot inoculations on grape berries *viz.*, conidial suspension in sterile distilled water; and formulated conidia in invert emulsion. Concentration of the conidia in both the treatments was 9.6x10⁸ conidia/ml.

Ingredients of the invert emulsion (water-in-oil type) used in the experiments were identical to those used by Batta (2004a). The conidia of *T. harzianum* (strain Th₂) harvested from 14-day old culture, were introduced into the invert emulsion according to the technique developed by Batta (2004a). Two additional non-treated control treatments were included in the tests: one with blank formulation of invert emulsion and the other with sterile distilled water only (water control treatment).

Rhizopus and Mucor inoculation and treatment effect assessment: Inoculation of *R. stolonifer* and *M. piriformis* was accomplished by depositing 25- μ l droplet of spore suspension containing 292,500 sporangiospores of *R. stolonifer* and 13,750 sporangiospores of *M. piriformis* (original suspensions contained 11.7x10⁶ and 5.5x10⁵ sporangiospores/ml, respectively) on the fruit surface after being superficially wounded using sterile scalpel. All fruits used in the tests were disinfected with 0.025% sodium hypochlorite, and then rinsed with sterile distilled water 3 times before inoculation.

For the assessment of treatment effect with fungicides and *T. harzianum* on the rot-lesion development caused by the above-mentioned fungi, the preventive effect (treatment application at the same time of the inoculation) was studied. The same droplet size (25 μ l) of fungicides' solution (mentioned earlier) or *T. harzianum* conidia formulated in invert emulsion or suspended in sterile distilled water was used in the treatments. The droplet was deposited at the same site of inoculation with sporangiospores of *R. stolonifer* or *M. piriformis* on the fruit surface immediately after the inoculation. Inoculated fruits were incubated in closed plastic containers (9.5cm diameter 6.5cm deep) with 6 fruits per container for 3 days at 20 \pm 1°C under humid conditions.

Evaluation of the treatment effect with fungicides and *T. harzianum* on the soft-rot development: Such effect was evaluated by measuring the ability of each type of fungicides or *T. harzianum* form to reduce the development of rot-lesion caused by *R. stolonifer* or *M. piriformis* on grape berries. The external rot-lesion diameter was measured in all replicates 3 days after the treatment at 20 \pm 1°C. Mean of external rot-lesion diameter in each treatment type was calculated to be used in the comparison of treatment effect. Mean percent reduction in the external rot-lesion diameter relative to non-treated water control was also calculated in each treatment for comparison of their effect.

Statistical analyses: Data were analyzed using completely randomized design (CRD) with 6 replicates representing 6 fruits per treatment. Analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) were used to test the significant differences between treatments representing the means of external rot-lesion diameter or the means of percent reduction in external rot-lesion diameter relative to non-treated water control.

Results and discussion

Effect of fungicides and *T. harzianum* on *Rhizopus* soft-rot development on grape berries: Significant differences (at $P < 0.05$) were obtained between treatments with four types of fungicides or two forms of *T. harzianum* when used against *Rhizopus* soft-rot on grape berries in comparison with the non-treated control (Table 1). Treatments with Switch® (cyprodinil + fludioxonil) and Score® (difenoconazole) resulted in the

least values of external rot-lesion diameter (13.2 and 13.5mm, respectively), followed by the treatment with formulated conidia of *T. harzianum* in invert emulsion (19.3mm). The other treatments used in the experiment resulted in significant higher values of means of external rot-lesion diameter ranging from 24.2 to 34.5mm. The treatment efficacy, in a descending order, was as follows: Switch® or Score®, formulated conidia of *T. harzianum* in invert emulsion, conidial suspension of *T. harzianum* in sterile distilled water, Merpan® or Ridomil®, blank formulation of invert emulsion or sterile distilled water as non-treated control treatments. Similarly, significant differences (at $P<0.05$) were obtained between means of percent reduction in the external rot-lesion diameter relative to control with sterile distilled water (Table 1). Therefore, the highest means of the percent reduction (60.9 and 61.7) were recorded with Score® and Switch®, respectively, followed by formulated *T. harzianum* conidia in invert emulsion (44.1). The rest of the treatments significantly caused lower means of the percent reduction ranging between 29.8 and 3.5. The treatment efficacy, in a descending order, was similar to the above-mentioned trend.

Effect of fungicides and *T. harzianum* on Mucor soft-rot development on grape berries: Significant differences ($P<0.05$) were obtained between treatments with four types of fungicides or two forms of *T. harzianum* when used against Mucor soft-rot on grape berries in comparison with the non-treated control (Table 2). Treatment with Score® (difenoconazole) and Switch® (cyprodinil + fludioxonil) gave the least values of external rot-lesion diameter (7.2 and 7.5mm, respectively), followed by the treatment with Merpan® (captan) (15.7mm), formulated conidia of *T. harzianum* in invert emulsion (19.2mm). The rest of the treatments used in the experiment gave significantly higher values of means of rot-lesion diameter ranging from 24.7 to 28.2mm. The treatment efficacy in a descending order,

was as follows: Score® or Switch®, Merpan®, formulated conidia of *T. harzianum* in invert emulsion, conidial suspension of *T. harzianum* in sterile distilled water or Ridomil®, blank formulation of invert emulsion, and sterile distilled water as non-treated control treatment. Significant differences ($P<0.05$) were also obtained between means of percent reduction in the external rot-lesion diameter relative to control with sterile distilled water (Table 2). The highest means of the percent reduction (74.5 and 73.4) were thus caused by the treatment with Score® and Switch®, respectively, followed by Merpan® (44.3) and formulated conidia of *T. harzianum* in invert emulsion (31.9). The rest of the treatments significantly caused lower means of the percent reduction ranging between 12.4 and 1.8 and similar to above mentioned trend of the treatment efficacy.

Overall results obtained in the present investigation indicate that the postharvest treatment of grape berries with Switch® (cyprodinil + fludioxonil) or Score® (difenoconazole) gave the highest percent reduction in the external rot-lesion diameter caused by *R. stolonifer* and *M. piriformis*. This effectiveness of treatment is comparable with that obtained by SO₂ fumigation, conventionally practiced to prevent fungal decay in table grape berries (Lisker *et al.*, 1996; Lichter *et al.*, 2002). SO₂ treatment remains effective as long as SO₂ level is sufficiently high, but the high levels can result in fruit damage as fruit bleaching, unpleasant aftertaste and allergies (Lisker *et al.*, 1996; Lichter *et al.*, 2002). To avoid the undesirable side-effects of chemical treatment against fungal decay of harvested fruit especially in table grape berries, treatment with biocontrol agents is recommended by many investigators (Elad, 1994; Harmann *et al.*, 1996). In the present study, the postharvest treatment of grape berries with *T. harzianum* formulated in invert emulsion also gave a high percent reduction in external rot-lesion diameter caused by *R. stolonifer* and *M. piriformis*. However, this treatment is less

Table 1. Effect of treatment with four types of fungicides and two forms of *T. harzianum* (strain Th₂) on the soft-rot development caused by *Rhizopus stolonifer* (strain RS1) on grape berries 3 days after inoculation and treatment at 20±1°C under humid conditions

Fungicides and <i>T. harzianum</i> treatments	External rot-lesion diameter (mm) developed on berries (Mean±SE)	Reduction(%) in external rot-lesion diameter relative to water (control)
Ridomil® (metalaxyl + mancozeb)	30.2±2.8 d ¹⁾	12.5 b ¹⁾
Score® (difenoconazole)	13.5±1.9 a	60.9 e
Merpan® (captan)	28.5±3.1 d	17.4 b
Switch® (cyprodinil + fludioxonil)	13.2±1.8 a	61.7 e
Formulated conidia of <i>T. harzianum</i> in invert emulsion	19.3±2.4 b	44.1 d
Blank formulation of invert emulsion as control treatment ²⁾	33.3±3.3 e	3.5 a
Conidial suspension of <i>T. harzianum</i> in sterile distilled water	24.2±2.9 c	29.8 c
Sterile distilled water as control treatment	34.5±3.1 e	

Table 2. Effect of treatment with four types of fungicides and two forms of *T. harzianum* (strain Th₂) on the soft-rot development caused by *Mucor piriformis* (strain MP3) on grape berries 3 days after inoculation and treatment at 20±1°C under humid conditions

Fungicides and <i>T. harzianum</i> treatments	External rot-lesion diameter (mm) developed on berries (Mean±SE)	Reduction(%) in external rot-lesion diameter relative to water (control)
Ridomil® (metalaxyl + mancozeb)	24.8±2.5 d ¹⁾	12.1 b ¹⁾
Score® (difenoconazole)	7.2±1.3 a	74.5 e
Merpan® (captan)	15.7±2.1 b	44.3 d
Switch® (cyprodinil + fludioxonil)	7.5±1.2 a	73.4 e
Formulated conidia of <i>T. harzianum</i> in invert emulsion	19.2±1.9 c	31.9 c
Blank formulation of invert emulsion as control treatment ²⁾	27.7±3.1 de	1.8 a
Conidial suspension of <i>T. harzianum</i> in sterile distilled water	24.7±2.8 d	12.4 b
Sterile distilled water as control treatment	28.2±3.7 e	

¹⁾ Means of external rot-lesion diameter within each column followed by different letters are significantly different at $P<0.05$ according to ANOVA and Duncan's multiple range test (DMRT).

²⁾ Invert emulsion is composed of the following ingredients (W/W): sterile distilled water (45.25%), glycerine (4.00%), water-soluble wax or Dehymuls K® (0.75%), Tween 20 (2.50%), and a mixture of 19.0% coconut oil + 28.50% soybean oil.

effective than the fungicidal treatment with Switch® or Score®, it has no undesirable side-effects e.g. leaving toxic residues or bad flavors or smells in the treated berries. To overcome the problem of harmful side-effects that appear in association with the chemical treatment of grape berries, many investigators have recommended alternation of chemical and biological treatment applications keeping meanwhile the same level of treatment efficacy. The applications of biological treatment could be also integrated with other control measures practiced within the decay-management program of grape berries.

For the other types of grape berry decay (e.g. *Botrytis* bunch rot caused by *Botrytis cinerea*), the decay-management program was performed successfully using both chemical and biological means such as 0.5-1.0 g L⁻¹ of *T. harzianum* dry conidia + 0.5 g L⁻¹ of vinclozolin or iprodione or 0.25 g L⁻¹ of diethofencarb plus carbendazim resulted in up to 78% disease reduction in grape berries (Elad, 1994). Also, treatment with *T. harzianum* dry conidia could replace some applications of iprodione or vinclozolin with little reduction in efficacy against *B. cinerea* on grape berries (Harmann *et al.*, 1996).

The effectiveness of postharvest treatment with *T. harzianum* formulated in invert emulsion was proved by us in controlling other types of fruit decay on various types of fruits at postharvest stage (e.g. gray mold on strawberry and apple; blue mold on apple; black fruit spot of persimmon) (Batta, 1999, 2001, 2004a and 2004b). We have also proved in our previous research that this type of postharvest biocontrol treatment has no harmful side-effects since the ingredients used for the invert emulsion are especially oils and emulsifiers which are safe and have no phytotoxic effect on treated fruits. These ingredients are also likely to be non-toxic to humans as they are also used as food additives and in the manufacture of cosmetics.

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