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STUDY OF THE EFFECT OF 2 BIOFONGICIDES ON APPLE POSTHARVEST FUNGAL PATHOGENS

W. ROUISSI [(1)], I. FARHAT.[(2)]

[(1)] INRAT, Rue Hedi Karray, 2080, Tunis + TUNISIE + wafa.r24@hotmail.com
[(2)] INAT, 43 Avenue Charles Nicolle, 1082, Tunis + Tunisie + farhat_imene@yahoo.fr

RÉSUMÉ

Les inquiétudes croissantes concernant la présence de résidus chimiques dans les pommes, ainsi que le développement de souches résistantes aux fongicides de *Penicillium expansum* en particulier, ont suscité un grand intérêt à adopter des moyens de lutte alternatifs plus sûrs. Le traitement post-récolte des fruits Golden par immersion dans la solution de prev-am à 1%, un biofongicide, était aussi efficace que le fongicide thiabendazole réduisant de manière significative le pourcentage de fruits infectés par *P.expansum* et *Alternaria sp.* après 8 mois dans la chambre froide. Tandis qu' aucune réduction significative n'a été signalée pour le traitement par la vapor Gard 0,7%. Par conséquent, le premier biofongicide peut être considéré comme le moyen de lutte alternatif le plus efficace.

Mots-clés : Pomme, fongicide, resistance, *P.expansum,* biofongicide.

ABSTRACT

The growing concerns over the presence of chemical residues in apple fruit, together with the development of fungicide-resistant strains of *Penicillium expansum* particularly, have generated a great interest to adopt safer alternative control means. Post-harvest treatment of Golden fruits through immersion onto prev-am solution 1%, a biofungicide, was as effective as the fungicide Thiabendazole reducing significantly the percentage of infected fruits by *P. expansum* and *Alternaria sp.* after 8 months at cold room. while no significant reduction was reported for treatment with Vapor Gard 0,7%. Consequently, the former can be considered as an effective alternative mean of control.

Keywords: Apple fruit, fungicide, resistance, *P.expansum,* biofungicide.

INTRODUCTION

Apple is one of the most popular fruit favored by health conscious, fitness freaks who believe in "health is wealth." However, growing apples profitably for today's market is a challenge involving problems turnover on the long way from the orchard to the consumer's table. Spoilage of fruits can occur before or after harvest during subsequent handling and it takes on great economic importance the closer it is to the sale of the fruit. Worldwide, these postharvest losses may reach as much as 50% during the shelf life of the fruit (El-Ghaouth, 1997). Postharvest losses in apple are mainly caused by fungal pathogens that limit the extension of storage life of fruit. Blue mould, caused by *Penicillium expansum* Link and grey mould, caused by *Botrytis cinerea* Pers.: Fr (Naqvi, 2004) are common diseases of apple even in production areas where the most advanced storage technologies are available. Both of them are wound invading pathogens that cause decay on stored fruits damaged by insects, early splits and mechanical harvesting. The former is widely known as a pathogen of stored pome fruits (Murphy *et al*., 2006). Aside from direct economic considerations, some postharvest pathogens pose a potential health risk. A number of fungal genera such as *Penicillium*, *Alternaria* and *Fusarium* are known to produce toxic metabolites (mycotoxins) under certain conditions. Generally speaking, the greatest risk of mycotoxin contamination occurs when diseased product is used in the production of processed food or animal feed (Morales *et al.,* 2008). Consequently, the control of apple fruit losses in the postharvest chain is decisive. The management of postharvest pathogens carried out with synthetic fungicides actually has undergone a strong reduction because of the public awareness of pesticide residues, environmental contaminations and the concomitant development of fungicide-resistant fungal strains du to the prolonged and excessive use of the same fungicides within the packinghouses. Hence, a significant interest was given to the development of alternative non chemical methods for disease control. According to Eckert (1988), rotation with non benzimidazoles fungicides alone or in a combination with products that have different modes of action are two possible fungicide-resistance management strategies to control benzimidazole-resistant populations. In an urgent attempt to find alternate fungicides to TBZ, two biofungicides, Vapor Gard and prev-am were evaluated for their efficacy against blue mould rot caused by *P expansum* and the other rots detected on apple fruits, aim of this work.

MATÉRIEL ET MÉTHODE

Field and packinghouse surveys and identification of the main pathogens attacking Apple fruit in Tunisia

Field and packinghouse surveys were conducted in the major Apple production areas of Tunisia including Ben Arous(Mornag) and Kasserine (Sbiba). Farms were selected according to their location and/or production importance. In total 10 farms and 2 packinghouses were chosen. During each survey, infected fruits showing different symptoms of rot (black spots, brown spots, blue spores, etc.) were picked from the soil, the trees and the boxes of non treated fruits and brought to the laboratory for isolations. The spores of *Penicillium* spp. obtained from infected fruits were cultured on PDA. Fruits showing symptoms other than *Penicillium* were washed with water then disinfected by sodium chloride and small pieces of the rot tissue were put on PDA dishes. Cultures were purified and maintained on Potato Dextrose Agar (PDA) slants at 4°C for further use, with periodic transfers through apples. Finally, the isolated pathogens were identified under microscope basing on their morphological features.

In vitro Study of the resistance to TBZ

The resistance to commercial fungicide TBZ [2-(thiazol-4yl) benzimidazole] as Tecto, Syngenta, (France) was investigated on all *Penicillium sp.* collection containing 16 strains. MEA was amended with commercial TBZ at the commercial dose of 400 µg/mL, selected as discriminatory dose for

resistance or sensitivity to fungicide. Control was represented by non amended MEA. Assays were performed on conidial germination and mycelium growth. In the first case, aliquot (0.1 mL) of a conidial suspension (10^3 conidia/ mL) was spread on MEA Petri dishes. After incubation for 3 days at 20 °C, CFU/plate were counted and the inhibition of conidial germination respect to control was calculated.
The same assay was performed for mycelium growth, by putting a plug of 6 mm diameter of each strain in the center of MEA plates in order to assess the colony diameter (mm). The experiment was replicated 5 times (dishes) per each strain and repeated three times.

In vivo assay with biofungicides: Postharvest treatment

The experiment was conducted on "Golden" fruit harvested from apple orchard located in the region of Ben Arous (Mornag) of Tunisia. Fruits selected for uniformity of size and absence of diseases and disorders were immersed for 30 seconds in each bio-fongicide solution and TBZ and air dried for half an hour at room temperature. Control fruits were treated with water. For each treatment, fruits were divided into 3 boxes of 30 fruits each, placed in commercial boxes and stored immediately at 0 °C and 90-95% RH. Postharvest treatments were based on two biofungicides: Prev-am and Vapor Gard, which were applied at the rate of 0, 5 % and 0, 7 % respectively and TBZ at 400 µg/mL.
Prev-am solution was prepared dissolving 50 ml of Prev-am in 10 litres of tap water. The Vapor Gard solution was prepared dissolving 70 ml of Vapor Gard emulsion in 10 l of tap water. The number of decayed fruit was evaluated 3 times: after 25, 28 and 31 weeks by visual inspection during storage and expressed as incidence of decay (percentage of infected fruit). Finally, in order to estimate the incidence of each kind of rot, pathogens were isolated from infected fruits and preliminary identified under microscope by mean of their morphological features.

Statistical analyses

All data were subjected to a one-way analysis of variance (ANOVA) using a commercial statistical software (statgraphics plus 5.1, Manugustics, Inc., Rockville, M. d., USA). Separation of means was performed using LSD test at $P<0.05$.

RÉSULTATS

The main pathogens attacking Apple fruit in Tunisia

A large number of pathogenic and saprophytic microorganisms were isolated from Apple fruits collected from different fields and packinghouses. The majority of these microorganisms were represented by filamentous fungi particularly *P. expansum* and *Alternaria sp.* with only a small percentage of other pathogens such as *Rhizopus sp.* and *stephylium sp*. However, the most representative fungal pathogen was *P. expansum*.

In vitro study of Thiabendazole resistance of *P. expansum* strains

A screening of fungicide resistance of thirteen *P. expansum* strains (derived from infected fruit) was performed *in vitro*, following common traditional methods: mycelium growth (colony diameter) and spore germination (CFU) on medium amended with TBZ (400 µg/mL) with respect to control. *P. expansum* strains studied split into two discrete distributions, one sensitive and the other resistant. The strains classified as sensitive (S) did not grow on TBZ amended medium or grew with a significantly lower ($P<0.05$) rate with respect to the control. Among the resistant strains, those that showed, on TBZ amended medium, a conidial germination or a mycelium growth similar to that observed on non TBZ-amended medium (control) are considered resistant (R); whereas, strains for which TBZ induces a significantly higher ($P<0.05$) percentage of conidial germination or mycelium growth are classified as highly resistant (RR).

Commercial TBZ reduced the mycelial growth and conidial germination of 10 (77%) out of 13 *P. expansum* strains. In the absence of fungicide, the colony diameter for *P. expansum* strains on MEA after 3 days at 20°C showed an average of 50 mm and the rate of conidial germination was 99%. In the presence of TBZ (400 µg/mL), the mycelial growth as well as the conidial germination were almost completely inhibited for 9 strains, these strains were classified as sensitive (S). Four strains (P3, P6, P12 and P13), were considered TBZ-resistant (R) since they showed a low mycelial growth inhibition ranging from 5% (P6) to 31% (P12) with respect to the control (Table.I). Basing on conidia germination results, two strains P6 and P11 were classified as resistant since they were able to germinate on TBZ-amended medium, while the 2 strains P12 and P13, for which TBZ showed a stimulatory effect on conidial germination was classified as highly resistant (RR) (Table. I).
In conclusion, an agreement was noticed between the results of the effect of TBZ on conidial germination and mycelium growth for all 11 *P. expansum* strains screened for sensitivity to TBZ except 2 strains P3 and P11 which behaved differently in confront of TBZ. A little effect on spore germination of P11 strain was observed, while mycelium growth was completely inhibited. In the contrary, P3 showed a significant reduction of conidial germination (99% inhibition) and was considered as a sensitive strain based on this parameter, but a low mycelium growth inhibition of 23% was reported and thus the strain was classified as resistant (R).

Table I. Effects of commercial Thiabendazole (TBZ) on mycelium growth and conidia germination (CFU) of thirteen *Penicillium expansum* isolates derived from apple fruit. Inhibition percentage was calculated relating to a control without TBZ addition. Isolates sensible (S) o resistant (R) following the classification reported in table 1. Data represent the mean of five repetitions ± dev st.
Tableau I. L'effet du Thiabendazole (TBZ) sur la croissance mycélienne et la germination des conidies (UFC) de treize isolats de *Penicillium expansum* dérivant des pommes. Le pourcentage d'inhibition a été calculé relativement au control non additionné de TBZ. Les isolats classifiés sensibles ou resistants sont rapportés dans le tableau 1. Les donnés représentent la moyenne de cinq répétitions ± dev st.

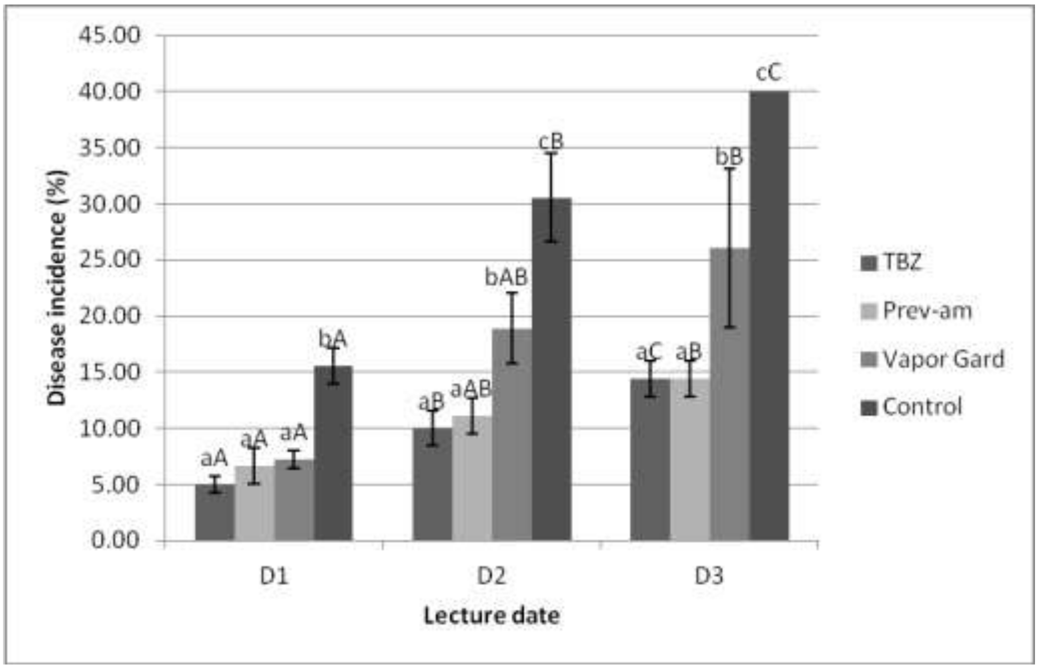
Isolates	TBZ sensitivity	Inhibition (%)	
		Mycelial growth	CFU
P1	S	100±0,28	100±3,97
P2	S	100±0,23	100±4,98
P3	S	23±0,1	99±8,62
P4	S	100±0,35	100±7,06
P5	S	100±0,22	100±5,92
P6	R	5±0,23	26±12,7
P7	S	100±0,09	100±13,4
P8	S	100±0,25	100±8,62
P9	S	100±0,4	100±5,22
P10	S	100±0,22	100±3,27
P11	S	100±0,26	9±9,02
P12	R	31±0,19	-24±15,4
P13	R	14±0,24	-5±6,65

Effect of postharvest application of biofungicides on Apple fruits rot

-Disease Incidence

The effect of postharvest application of Prev-am (1%) and Vapor Gard (7%) solutions on rot incidence on Golden fruits was examined after 25, 28 and 31 weeks of cold storage (0°C and 90% RH) (Fig.1). Decay among fruits was mainly due to *Penicillium,* but other agents that do not frequently cause commercially significant losses were also present.

Figure 1. Treatments effects on the disease incidence of 'Golden delicious' apples after 25, 28 and 31 weeks.
Figure 1. les effets des traitements sur l'incidence de la maladie dans les pommes 'Golden delicious' après 25, 28 et 31 semaines.



Means for the same treatment and different lecture dates with different capital letters and means for the same lecture date and different treatments with different lowercase letters are significantly different by LSD test ($P<0.05$) applied after an ANOVA.

As it is evident from Fig. 1, Prev-am was as effective as the fungicide TBZ in reducing significantly the disease incidence while Vapor Gard was less efficient with respect to the control.
On the first assessment, the percentages of reduction of disease incidence on apple fruits for TBZ, Prev-am and Vapor Gard treatments were 95, 93.33 and 92.78% respectively and there were no significant difference between them ($P<0.05$). On the second evaluation, an increase of the rot incidence from 5 to 10% was reported on TBZ-treated fruits, from 6.67 to 11.11% on Prev-am treated fruits and from 7.22 to 18.89% on Vapor Gard treated fruits. The same disease incidence was recorded on the last assessment for both TBZ and Prev-am treatments (14.44%) contrarly to Vapor Gard which induced a significant increase compared to the other treatments (26.11%).
Over time, the increase of the rot incidence on the chemically treated apples was significantly different ($P<0.05$) contrarly to the other ones immersed in the biological products where the increase was not significantly different ($P<0.05$).

-Fungal identification

The identification of the fungal pathogens isolated from treated apples showed the presence of four wound invading pathogens (*Penicillium spp., Alternaria spp., Rhizopus spp.* and *Stemphylium spp.*) with different combinations. These fungi were present in different proportions depending on the treatment and the date of assessment.

Table II. The fungal development after 25, 28 and 31 weeks on 'Golden Delicious' apples treated with Thiabendazole and biological products.

Tableau II. le dévelopment fongique après 25, 28 et 31 semaines dans les pommes 'Golden Delicious' traitées avec le Thiabendazole et les produits biologiques.

Date	Treatment	% Pen	% Alt	% Rhi	% Stem
D1	TBZ	2,78 ± 0,79 aA	3,89 ± 2,36 aA	0,56 ± 0,79 aA	1,11 ± 0 abA
	Prev-am	5 ± 2,36 aA	3,89 ± 0,79 aA	0 ± 0 aA	0 ± 0 aA
	Vapor Gard	2,78 ± 0,79 aA	3,33 ± 3,14 aA	1,67 ± 0,79 aA	1,67 ± 0,79 bA
	Control	10 ± 1,57 bA	10 ± 1,57 bA	1,67 ± 0,79 aA	1,67 ± 0,79 bA
D2	TBZ	3,89 ± 2,36 aA	7,78 ± 0 bA	0,56 ± 0,79 aA	1,11 ± 0 aA
	Prev-am	7,22 ± 0,79 aAB	3,89 ± 0,79 aA	0,56 ± 0,79 aA	2,22 ± 0 abB
	Vapor Gard	8,33 ± 2,36 aAB	11,67 ± 0,79 cAB	2,78 ± 0,79 abA	2,78 ± 0,79 bA
	Control	20,56 ± 2,36 bB	20,56 ± 0,79 dB	1,67 ± 0,79 bA	2,78 ± 0,79 bA
D3	TBZ	11,11 ± 0 aB	9,44 ± 2,36 bA	2,22 ± 0 abA	1,11 ± 1,57 aA
	Prev-am	11,67 ± 2,36 aB	4,44 ± 0 aA	0,56 ± 0,79 aA	2,22 ± 0 aB
	Vapor Gard	15 ± 0,79 aB	16,67 ± 6,29 cB	3,33 ± 1,57 bA	2,78 ± 0,79 aA
	Control	23,89 ± 2,36 bB	33,33 ± 0 dC	1,67 ± 0,79 abA	6,11 ± 0,79 bB

[a] Pen = *Penicillium*, Alt = *Alternaria*, Rhi = *Rhizopus*, Stem = *Stemphylium*.

[b] Means for the same treatment and different assessement dates with different capital letters and means for the same assessment date and different treatments with different lowercase letters are significantly different by LSD test ($P<0.05$) applied after an ANOVA.

According to the results summarized in the table above, the percentage of the fungal pathogens increased along the cold storage period.

In the case of *Penicillium*, Thiabendazole treatment, normally recommended to prevent the dissemination of the fungus, did not inhibit its proliferation and the amount of attacked fruit significantly increased ($P<0.05$) from 2.78 % to 11.11% between the 25th and the 31st week. The two other treatments with Prev-am and Vapor Gard gave similar results to the chemical one and the percentage of attacked fruits with *Penicillium* significantly increased ($P<0.05$) from 5 to 11.67% and from 2.78 to 15% respectively. However, there were no significant treatment effect on the three assessment dates.

Similarly, the three treatments did not show a significant difference ($P<0.05$) with *Stemphylium* (1.11, 2.22 and 2.78% the 31st week), and there were no significant time effect with Thiabendazole and Vapor Gard too.

Whereas, the treatment effect was significantlty different ($P<0.05$) with *Alternaria*. Indeed, Prev-am gave the lowest percentage of infected fruits (4.44%) followed by Thiabendazole (9.44%) and Vapor Gard (16.67%). Besides, the percentage of Prev-am treated fruits attacked by *Alternaria* did not significantly increase during the storage period (3.89%, 3.89% and 4.44% the 25th, 28th and 31st week respectively).

Finally, there was no treatment effect on *Rhizopus* with regard to the control (2.22, 0.56, 3.33 %).

DISCUSSION

Field and packinghouse prospections in the main Tunisian Apple areas and identification of the most frequent apple pathogens on fruits revealed the presence of a large number of different microbial species. The majority of these microorganisms were represented by *Penicillium* spp. and *Alternaria* spp. A small percentage of other pathogens such as *Rhizopus* spp. and *stephylium* spp. were also present. However, the most representative genus was *Penicillium* spp., particularly *P. expansum*, the causal agent of blue mould. The latter is the most aggressive species and the most frequently associated with blue mould during apples storage worldwide (Pianzzola *et al.,* 2004; Sholberg *et al.,* 2005). In addition to *P. expansum*, *P. solitum has* been recognized as an agent of blue mould on apple in Uruguay (Pianzolla *et al.,* 2004) and Serbia (Vico *et al.,* 2014). Both were recovered most frequently from pear and apple dump tank water, however the majority of fruit were infected with *P. expansum* followed in order of decreasing frequency by *P. solitum, P. commune* and *P. aurantiogriseum* (Sanderson and Spotts, 1995).

The management of blue mould rot relies mainly on the use of synthetic fungicides, TBZ, belonging to benzimidazole class. Its repeated application in the past has led to the emergence of resistance among the *P. expansum* strains, considered to be the cause of ineffective disease control. The limited success of benzimidazoles in the control of postharvest fungal pathogens was reported by Bryk (1997) on *B.cinerea* and *P. alba,* on *M. fructicola (*Cox *et al*.,2009*)* and *P. expansum* (Sanchez-Torres and Tuset, 2011).. In the present study, the preliminary *in vitro* screening has been useful to discriminate TBZ-resistant and -sensitive strains. The results of monitoring of the occurrence and the distribution of the resistant strains consisting on direct-planting of pathogen strains on MEA plates amended with commercial TBZ (400 µg/mL), revealed that the percentage of TBZ-resistant strains isolated from apples is two-times lesser (31%) than those reported by other authors (Errampalli *et al*. 2006). The low frequency of the occurrence of resistant strains can be explained by the fact that the use of TBZ was abondonned many years ago (10 years) and that the integrated management program adopted probably has reduced the risk of the fungicide resistance emergence. In addition, our resistant strains of *P. expansum* could be less fit, implying that the population might tend to return to its original state of balanced adaptation in absence of selection pressure by the fungicide as reported by Prusky *et al*., (1985). Study of the effects of TBZ at 400 µg/mL on *P. expansum* resistant strains has revealed a weak effect on conidial germination. Among the TBZ- resistant strains scored in this work, seven strains showed higher percentage of conidial germination on TBZ-amended medium (RR) than control. According to Baraldi *et al*, (2003), TBZ may have a stimulatory effect on conidial germination. Such effect has been studied earlier for metalaxyl (aphenylamidefungicide) on the vegetative growth of some isolates of *Phytophtora infestans* (Zhang *et al*., 1997). The behavior of two strains P11 and P3 in presence of TBZ observed in this work (Table.I) is not well documented. Trials on TBZ resistance of P11 strain showed a greater inhibition of mycelium growth than conidial germination, similar results were discussed also by Cabanas *et al*. (2009). TBZ seems to inhibit spore germination, but it's more effective when germination has begun. Allen and Gottlieb (1970) added that TBZ is fungicidal and causes stunting and malformation of the germ tubes once they have begun to emerge from spores. More investigations are needed in order to elucidate the effect of TBZ on P3 strain. Our results suggest that the germination assay based on the counting of CFUs on fungicide amended medium is suitable for phenotyping strains for resistance to TBZ.

In the present study, the *in vivo* assay with Thiabendazole did not inhibit *Penicillium* rot confirming thus the fact that some Tunisian strains have developed resistance to the fungicide and that alternative means of control would be welcomed. For this reason, two biological products: Prev-am and Vapor Gard, have been tested as postharvest treatments for controlling apple fruit decay.

Prev am which is composed of orange essential oil, surfactants and micronutrients is used as a fungicide and an insecticide in several cultures like grapes, strawberry, tomatos (Biondi *et al*., 2012). It acts on the fatty substances contained in the cells chitin of insects and fungi making them more fragile to desiccation (Constant, 2008). Generally, applied on the field or in greenhouses against

powdery mildew (Cerkauskas *et al*., 2011; Cunningham and Nelson, 2012), only few papers reported its effect on the storage potential of potato when applied before harvest (Kurzawinska and Mazur, 2012; Mazur *et al*., 2016) and on root vegetables (Robak and Adamicki, 2007). In fact, to our knowledge, this is the first work on the potential of Prev-am on postharvest pathogens of apple fruits.
Prev-am postharvest treatment gave similar results to TBZ (85.56% reduction of disease incidence after 31 storage weeks). Moreover, it was the most efficient against *Alternaria*. On behalf of these results, Prev-am would be a good alternative until it is as efficient as the latter in the control of *Penicillium* and more efficient against *Alternaria*, major apple fungal postharvest pathogens.
The antitranspirant Vapor Gard was less efficient compared to the other treatments. Basing on the literature, the obtained results seems to be logic since this biological product plays roles other than preventing the pathogens attacks. It aims to reduce the transpiration of cultures in the field under stress conditions (Latocha *et al*., 2009; Ouerghi *et al*., 2014) and no stress conditions (Lipe and Thomas, 1980; Schon, 1993), or to prevent the physiological disorders of apple (Byers *et al*., 1990; Ghafir *et al*., 2013), mango (Khader, 1992; Lazan *et al*., 1990). Thus, the perspective of using Vapor Gard with Prev-am in an integrated pest management could be a good alternative of control.

CONCLUSION

In a time of increased fungicide resistance development within pathogen populations and public awareness against chemical treatments, the perspective of using Prev-am with Vapor Gard could be a good alternative management strategy.

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