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SHORT COMMUNICATION / *COMUNICAÇÃO*

Alternaria species associated with 'moldy heart' on peaches in Argentina

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ABSTRACT

'Moldy heart' caused by *Alternaria* spp has been previously reported in apples in Argentina. However, according to our knowledge this is the first report of *Alternaria* spp in 'moldy heart' affected peaches in this country. Direct isolation of the pathogen was performed on Dichloran-Chloramphenicol-Malt Extract-Agar. Seven strains were isolated and identified as *A. alternata* and one as *A. tenuissima*. Their toxicogenic potential (alternariol (AOH), alternariol methyl ether (AME) and tenuazonic acid (TA) production) was determined. The toxins were extracted with chloroform and determined by TLC. All strains produced AOH and AME while six of them were TA producers. The fungicides tested on agar were captan and mancozeb. At 2.5g/L (commercial concentration) both fungicides inhibited the germination of spores. Captan completely inhibited mycelial growth. The effect of the fungicide on toxin production was variable according to fungicide type, doses, strains and toxins considered.
Keywords: *Alternaria alternata*, *A. tenuissima*, alternariol, alternariol methyl ether, tenuazonic acid.

RESUMO

Espécies de *Alternaria* associadas com 'podridão do coração' de pêssego na Argentina

O podridão do coração por *Alternaria* foi previamente reportada em maçãs na Argentina mas segundo nosso conhecimento este é o primeiro relato de *Alternaria* spp. causando esta doença em pêssego neste país. O isolamento direto de espécies de *Alternaria* foi feito sobre agar Dichloran-Chloramphenicol-Malt Extract-Agar (DRBC). Sete isolados foram obtidos e identificados, seis como *A. alternata* e um como *A. tenuissima.* Sua capacidade para produzir toxinas (AOH, AME e AT) foi determinada. As toxinas foram extraídas com clorofórmio e determinadas por TLC. Todos os isolados produziram AOH e AME e seis deles produziram TA. Os fungicidas avaliados foram Captan e Mancozeb. A 2,5 g/L (dose comercial recomendada) ambos fungicidas inibiram completamente a germinação de esporos. Captam inibiu completamente o desenvolvimento micelial do fungo. O efeito dos fungicidas sobre a produção de toxinas foi variável em relação ao tipo de fungicida, dose, isolado e toxina considerada.
Palavras-chave: *Alternaria alternata*, *A. tenuissima*, alternariol, alternariol metil eter, ácido tenuazonico.

Fruits are naturally contaminated with fungi. Molds, such as *Alternaria* spp., *Botrytis* and *Penicillium* spp., can develop on and spoil a wide variety of fruits (Tournas & Stack, 2001). The genus *Alternaria* includes both pathogenic and saprophytic species, capable of damaging the fruit in the field as well as of causing important spoilage during storage and transport (Bottalico & Logrieco, 1992). Since *Alternaria* species grow at low temperatures, they are also involved in damage during refrigerated storage (Tournas & Stack, 2001). *Alternaria* species can cause two different diseases on fruits: (i) lesions that affect the surface, e.g. "Black mold" of tomatoes, peppers, melons and blueberries, and (ii) lesions that cause rot or moldiness of the fruit heart, e.g. "Heart rot" of mandarins, oranges and lemons and 'moldy heart' of apples (Logrieco *et al*., 1990; Bottalico & Logrieco, 1992; Robiglio & López, 1995).

"Black mold" is a disease characterized by flattened or slightly sunken lesions on the affected fruit surface. A dense and felty olive-green or black mass of spores is formed on the surface and frequently the mycelia develop into the inner tissues (Bottalico & Logrieco, 1998). In the first stages of 'heart rot' and 'moldy heart', the fruits affected do not show any external symptoms, but develop a black or gray mycelium into the pulp or stone of the fruit, respectively. Later, the fruit surface turns brown, starting from the pedicel, and soon after, the fruit falls to the ground (Logrieco *et al*, 2003). The special feature of this disease is that the infected fruits do not show apparent external symptoms when found in the field and are thus harvested. Growth of the mold continues post-harvest and is favored by long periods of storage and low temperatures (Robiglio & López, 1995).

Species of *Alternaria* produce approximately 70 secondary metabolites, 30 of which have been recognized as potentially toxic (Bottalico & Logrieco, 1992). For this reason, the studies performed in the last decade focused on these fungi as toxicogenic agents rather than simply fruit rot fungi (Robiglio & López, 1995). The

most important *Alternaria* toxins are alternariol (AOH), alternariol mono methyl ether (AME), tenuazonic acid (TA), altenuene and altertoxins (Bottalico & Logrieco, 1992). These toxins have been proved to be harmful to animals. Consumption of infected fruits can represent a serious risk to human health. AOH and AME cause weakly acute toxic effects in mice (LD_{50} higher than 400 mg/kg b.w.) and show synergistic effects. AME is cytotoxic, carcinogenic and mutagenic. AOH is lethal to unborn mice at levels of 100 mg/kg b.w. TA is toxic to chicken embryos and can cause hemorrhage and death in mice (Da Motta & Valente Soares, 2000a; Da Motta & Valente Soares 2000b). In addition, a possible role of TA in the etiology of Onylai, a human hematology disorder occurring in Africa, has been suggested (Scott & Kanhere, 1980). *Alternaria* toxins have been found in fruits such as apples, blueberries, cherries and several citrus fruits. AOH, AME and TA have been found both in tomato paste and naturally infected tomatoes as well as in olives and mandarins (Bottalico & Logrieco, 1992; Robiglio & López, 1995; Tournas & Stack, 2001).

Fungal invasion of the core in apples by *A. alternata* is particularly prevalent. However, there are no data related to the incidence of the core rot diseases caused by *Alternaria* spp. in peach. Fruit core rot of the peaches caused by *A. alternata* only was reported in Greece (Thomidis et al., 2007). 'Moldy heart' disease caused by *Alternaria* spp. is frequently observed in Red delicious apples in Argentina (Robiglio & López, 1995), but there are no previous reports about this disease in peaches. Although pesticides are applied for crop protection during growth and ripening, these compounds have been demonstrated to have different effects on growth and mycotoxin production of toxicogenic fungi (Dalcero et al., 1995). The aim of this study was to determine the species of *Alternaria* causing the moldy heart of peaches collected in an experimental field in San Pedro, Buenos Aires province, Argentina, identify their ability to produce AOH, AME and TA and evaluate the effects of fungicides on toxin production, spore germination and fungal growth.

From 32 peaches collected, seven were affected by moldy heart. All the peaches were externally healthy. Infected tissue fragments from peaches were plated on Dichloran-Chloramphenicol-Malt Extract- Agar (DCMA) (Pitt & Hocking, 1997). Plates were incubated for 7 days at 25ºC. The *Alternaria* isolates were identified from single-conidial cultures using the classification scheme of Ellis (1971). The strains were cultured on autoclaved rice to determine the production of AOH, AME and TA because this medium has been described as one of the most suitable for toxin production (Schade & King, 1984). The rice was inoculated using a sterile cork borer with a 6-mm culture disk cut out from the colony margin of a one-week-old single spore culture on PDA. For each strain, two cultures on rice were performed. Each culture was incubated in the dark, one at 25ºC during 3 weeks (Visconti et al., 1992; Chulze et al., 1994) and the other at 8ºC during 4 weeks (refrigeration condition).

Chloroform was used for toxin extraction (Visconti et al., 1992; Combina et al., 1999). The analysis was performed by Thin Layer Chromatography (TLC) on pre-coated silica gel plates without fluorescence indicator (Merck G-60). The development solvent system for AOH and AME was chloroform:acetone (88:12) (Visconti *et al*., 1992). The spots were observed under UV light (260 nm). The solvent system for TA was toluene:ethyl acetate:formic acid (60:30:10) (25ºC). Plates were visualized by spraying them with 2% $FeCl_3$ in ethanol. The toxin concentration was determined by visual comparison with standard solutions of AOH, AME and TA purchased from Sigma (St. Louis, MO.).

The effect of the fungicides captan (N-(tricholomethylthio) cyclohex-4-ene-1,2-dicarboximide) (Commercial name: Captan Tomen, Chemisplant) and mancozeb (manganese ethylene bis (dithiocarbamate) - polymeric complex with zinc salt) (Commercial name: Chemispor, Chemiplant) was evaluated on the strains IMA 128, IMA 129, IMA 133. Appropriate amounts of stock solutions were added to the culture media Modified Czapeck Dox Agar (MCD) to provide two doses which were 2.5 g/l (recommended commercial dose) and 1 g/l (suboptimal) of each fungicide. This MCD medium has been proved to be suitable for *Alternaria* mycotoxin production (Chulze et al., 1994; Dalcero et al., 1995).

In this study, we isolated seven strains of *Alternaria* from peaches affected by 'moldy heart'. Six of them were identified as *A. alternata* and one as *A. tenuissima*. In Greece, this disease caused significant pre- and post-harvest damage in the cultivar 'Fayette' and the responsible fungus was identified as *A. alternata* (Thomidis et al., 2007). The toxicogenic profile for all the strains tested is shown in Table 1. All the strains isolated were able to produce AOH and AME at 25°C on rice. All strains produced AOH in high

TABLE 1 - Mycotoxin production by *Alternaria* strains isolated from "Mouldy Heart" affected peaches in rice at 25°C

Strain	Specie	TA	AOH	AME
IMA128	*A. tenuissima*	+++	+++	++
IMA129	*A. alternata*	++	+	+
IMA130	*A. alternata*	+	+++	+
IMA131	*A. alternata*	++	+++	+++
IMA132	*A. alternata*	nd	+++	+
IMA133	*A. alternata*	+	+++	++
IMA134	*A. alternata*	+	+++	++

TA (tenuazonic acid): + 0.01-0.03 mg/g, ++ 0.04-0.07 mg/g, +++ over 0.08 mg/g.
AOH (alternariol): + 0.04-0.15 mg/g, ++ 0.16-0.27 mg/g, +++ over 0.28 mg/g.
AME (alternariol methyl ether): + 0.07-0.24 mg/g, ++ 0.25-0.41 mg/g, +++ over 0.42 mg/g.
+ Low, ++ Moderate, +++Stronger, nd= no detected

quantities with the exception of IMA129. With respect to AME, the strains showed variable production pattern. Out of the seven strains obtained, three of them were considered low producers of this toxin, three of them produced moderate quantities and IMA131 produced the highest amount of AME. Only the *A. tenuissima* strain produced high amounts of TA toxin. Considering *A. alternata* strains, one did not produce, and all the others were moderate or poor producers. A high percentage of the strains (85.7%) produced this metabolite. In a previous study performed on tomato fruits, only 64% of the tested strains produced TA (Pose et al., 2004). All the strains were able to retain their toxicogenic capacity under refrigeration (8°C).

Captan completely inhibited fungal development *in vitro*, while mancozeb reduced it bt 57% on average at a concentration of 2.5 g/l. At a concentration of 1 g/l, growth was observed in both cases but captan inhibited fungal development by 20% and mancozeb by 22%. The germination of spores was totally inhibited in both cases at both doses. When the effect of fungicides on AOH, AME and TA production was evaluated, it was observed that the chemical compounds assayed showed variable results depending on the doses employed, the strain and the toxin under analysis (Table 2). This variation with the chemical compound, strain and metabolite in the effect of the fungicides on toxin production has also been observed by other authors (Dalcero et al., 1995; Ramirez et al., 2004). Thomidis et al. (2007) demonstrated *in vitro* that Folicur 25WG (tebuconazole 25%; Bayer), Switch 25/37.5 WP (cyprodimil 375 g/kg, fludioxonil 250 g/kg; Syngenta) and Rovral 50WP (iprodione 50%; BASF) completely inhibited the mycelial growth of *A. alternata* at the rates recommended by the manufacturer (0.33 mL/L, 0.8 g/L and 1.33 mL/L, respectively). On fruits, Folicur 25WG and Switch 25/37.5 WP were again most effective against *A. alternata*, though there was not complete inhibition of the mycelial growth in this case. Rovral 50WP also inhibited growth but was less effective than Folicur 25WG. Other fungicides were also evaluated. Dithane M-45 80WP (Mancozeb 80%; BASF) showed moderated effectiveness. Thiophanate methyl 70WP (Thiophanate methyl 70%; Inagro) and PIΛAZIN 60WP (carbendazine 60%; Hallafarm) showed no effect on development of *A. alternata* (Thomidis et al., 2007). With respect to our results obtained from experiments *in vitro*, those which, at appropriate doses, totally inhibit growth and therefore toxin production would be recommended. Further investigations under field conditions are needed to validate *in vitro* experiments.

Interestingly, from the peaches examined, we determined that although none of them showed external symptoms of disease, those affected by 'moldy heart' exhibited a crack between the pedicel and the fruit. This feature provides a method for distinguishing infected fruits from the non-affected ones before harvest. This is an important characteristic that the producer should keep in mind during harvest in order to discard infected fruits. Fruits exhibiting core rot disease have the potential to damage the reputation of products in domestic and overseas markets because core rot infection cannot be detected before consumption (Thomidis et al., 2007). Likewise, the determination that *Alternaria* isolates from 'moldy heart'-affected peaches produce toxic metabolites indicates a potential risk to consumers. Although the pathogen attacks the pit, the toxins potentially can spread to the pulp, as has been demonstrated in apples (Robiglio & López, 1995). For this reason the elimination of infected fruits by manufacturers is advisable. Whether Alternaria toxins are a problem in commercial peach products remains to be investigated. More research is clearly needed in order to increase the quality and safety of those products.

TABLE 2 - Effect of fungicides on growth *Alternaria* ssp., TA, AOH and AME production after 28 days of incubation at 25ºC on MCD

Strain	Growth*					Toxin productio n (µg/g)														
						TA production					AOH production					AME production				
	Co	C 1g/L	C 2.5g/L	M 1g/L	M 2.5g/L	Co	C 1g/L	C 2.5g/L	M 1g/L	M 2.5g/L	Co	C 1g/L	C 2.5g/L	M 1g/L	M 2.5g/L	Co	C 1g/L	C 2.5g/L	M 1g/L	M 2.5g/L
IMA128 *A. tenuissima*	90[a]	83[ab]	<1[c]	69[ab]	44[d]	54	54	<0.01	54	>200	12	5	<0.5	2	7	7	2	<0.5	<0.5	7
IMA129 *A. alternata*	90[a]	63[ab]	<1[c]	70[ab]	40[d]	120	80	<0.01	80	80	5	7	<0.5	<0.5	<0.5	2	4	<0.5	<0.5	<0.5
IMA133 *A. alternata*	90[a]	71[ab]	<1[c]	71[ab]	33[d]	>200	5	<0.01	<0.01	<0.01	20	8	<0.5	5	<0.5	12	3	<0.5	2	<0.5

* Colony diameter in mm
TA= tenuazonic acid, AOH= alternariol, AME= alternariol Methyl Ether
Co=Control, C=captan, M=mancozeb
All results are mean value of two replicates. Values followed by the same letter within the row are not significantly different ($p<0.05$) by the LSD test.

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