Full Length Research Paper

Effect of benzimidazole fungicides and calcium chloride on *Alternaria alternata* and *Penicillium expansum* rot during storage of pears

Abdelfettah Maouni, Ahmed Lamarti, Anouar Aidoun, Mustapha Khaddor and Alain Badoc

1Département de Sciences Naturelles, École Normale Supérieure, BP 202, 93150 Martil, Morocco.
2Département de Biologie, Unité de Biotechnologie et d’Amélioration des plantes, Faculté des Sciences M’hannech II, BP 212, 93002 Tétouan, Morocco.
3Laboratoire de Mycologie et Biotechnologie végétale, Faculté des Sciences Pharmaceutiques, Université Victor Segalen Bordeaux 2, 146, rue Léo Saignat, 33076 Bordeaux Cedex, France.

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The present study assayed the effect of the fungicides benomyl, methyl-thiophanate, thiabendazole and calcium chloride on fungal decay of pears caused by *Alternaria alternata* and *Penicillium expansum*. Both *in vitro* and *in vivo*, the efficacy of fungicides alone against the two fungi was shown to be weak and without any practical interest. *In vitro*, CaCl$_2$ alone was tolerated by both species at more than 4%. *In vivo* and at low temperature, CaCl$_2$ significantly reduced fungal decay when used at 4 and 6%. The association fungicides - CaCl$_2$ (4%) allowed a better control of *A. alternata*.

Key words: Pears, conservation, fungi, calcium chloride, fungicides.

INTRODUCTION

During storage of pears, certain fungi lead to problems with serious economical consequences (Palazón et al., 1984). Blue mold rot caused by *Penicillium expansum* and black spot caused by *Alternaria alternata* (Fr.) Keissler are among the main diseases found on pears and apples in storage (Amrani and Najim, 1985; Bondoux, 1969, 1992; El Hassani, 1991; Maouni, 1980; Ramdani, 1989; Selmaoui and Douira, 1999).

When they were first introduced, chemical treatments by fungicides benzimidazoles considerably reduced losses caused by these fungi. Nevertheless, the latter again began to appear during storage even after intense chemical protection and resistance phenomena developed (Bondoux, 1992; Mc Phee, 1980; Ramdani, 1989). To solve this problem of resistance and to fight against certain physiological disorders of apples and pears linked to calcium deficiency such as bitter pit, calcium chloride was used (Mason and Welsh, 1970; Perring, 1986; Raese and Stahly, 1988) and the association fungicide-CaCl$_2$ was proposed to control *Alternaria* sp. (Biggs et al., 1993).

The aim of this work was to test *in vitro* and *in vivo* the association of three benzimidazole fungicides against two of the main fungal pathogens.

MATERIALS AND METHODS

Pathogens

Isolates of *A. alternata* and *P. expansum* were selected for by their aggressiveness among several isolates found in different pears cultivars: these isolates developed well on Malt-Extract-Agar (MEA, Biolife Italiana s.r.l.) and Potato-Dextrose-Agar (PDA, Biolife Italiana s.r.l.) media and produced the largest lesions on inoculated pears. The fungi were maintained on PDA, with periodic transfers through pears.

Fungicides and calcium chloride tested

The fungicides tested in this work are largely marketed in Morocco and were used at authorized doses (AD): benomyl (B) 50% (benlite, 60 g/hl), methyl-thiophanate (MT) 70% (Pelt 44, 350 g/hl), and thia-
bendazole (TB) (Decco 20S, 150 g/hl), alone or with CaCl₂ 96% purity at 4% (w/v). The calcium chloride was tested alone at 2, 4 and 6%.

**In vitro tests**

To study conidial germination, a spore suspension \(10^5\) spores/ml of A. alternata and P. expansum was spread evenly over the surface of three petri plates containing an agar – fungicide mixture and/or CaCl₂. Germinated spores were counted on 200 spores after 24 h incubation at 25°C in darkness.

To evaluate the mycelial growth, a solution of each fungicide and/or CaCl₂ was incorporated in the MEA medium still in fusion and the mixture was placed in petri dishes. In each petri dish three inoculations points were done with mycelial discs issued from culture of 10 to 15 days. Mycelial growth was evaluated after seven and ten days of incubation at 25°C by measuring the diameter respectively of P. expansum and A. alternata colonies.

As for the sporulation, four of 5 mm discs diameter, taken at the edge of each of the 27 cultures colonies used to study mycelial growth was placed in a tube containing 1 ml of distilled water. After 30 s shaking with a vortex, the spores were counted by a Malassez cell at a rate of three counting per suspension.

The efficacy of each treatment was given according to the following formula:

\[
E(\%) = \frac{X - Xi}{X} \times 100
\]

**X:** Estimation of germination, growth or sporulation in a medium without fungicide or CaCl₂ (Control). \(Xi\): Estimation of germination, growth or sporulation in a medium with fungicide and/or CaCl₂.

**In vivo tests**

‘Williams’ pears were surface sterilised by dipping them into 1% (w/v) sodium hypochlorite for 1 min, rinsed 3 times with sterile distilled water and blotted dry on sterile filter paper. The fruits were wounded by a 1 mm diameter needle three points on their equatorial area and dipped into the solution of fungicide and/or CaCl₂. After 24 h, they were inoculated by 1 mm diameter mycelium fragments. Thereafter, the fruits were distributed separately under black plastic sheeting to maintain a relative humidity at 100% (Maouni et al., 2001) and were preserved for two months at 4°C or 10 days at 25°C.

The efficacy (inhibition percentage) of each treatment is given according to the following formula:

\[
E(\%) = \frac{D - Di}{D} \times 100
\]

\(D\): Diameter of lesion without treatment (control). \(Di\): Diameter of lesion after treatment by fungicide and/or CaCl₂.

**Statistical analysis**

Each treatment was replicate three times. All data were submitted to analysis of variance and the means were separated with Duncan’s multiple range test \((P = 0.05, n = 27)\). Before the analysis, the percentage was converted into arc sin angles to produce approximately constant variance.

**RESULTS**

**Efficacy of CaCl₂ in vitro and in vivo**

Up to 6% (Table 1), CaCl₂ had little efficacy on the three developmental stages of the two species in vitro. In most cases, the inhibition percentage did not exceed 50%. The inhibition seemed greater for sporulation and increases with CaCl₂ concentration. In vivo (Table 2), efficacy against the two species increased with the CaCl₂ concentration and was clearly greater at 4°C than 25°C. From 4% concentration, CaCl₂ was efficient for conservation at 4°C.

**Efficacy of fungicides and CaCl₂ in vitro**

The efficacy of the fungicides tested alone is weak and without any practical interest on the conidial germination of the two species (Table 3). In the presence of CaCl₂, the efficacy of B and MT increases remarkably for both species, whereas those of TB remain unchanged. The three tested fungicides (Table 3) had little action on the mycelial growth of A. alternata. On the contrary, the efficacy of TB on P. expansum was quit high (about 70%). In the presence of CaCl₂, the efficacy of B and MT significantly increases, contrary to that of TB. These associations were without practical interest against A. alternata. All the fungicides tested had little effect on the sporulation of the two studied species (Table 3) and were less active than CaCl₂. The association of B and TB with calcium chloride gave better results, especially on P. expansum. All in all, the efficacy of the fungicides alone was relatively low on the three developmental stages of the two species studied and tended to increase in the presence of calcium chloride.

**Efficacy of fungicides and CaCl₂ in vivo**

In vivo (Table 4), the fungicides had little effect against the two species whatever the conservation temperature and had less effect than CaCl₂ at 4°C. The association fungicides - CaCl₂ was significantly positive against A. alternata, whatever the conservation temperature, and gave satisfactory result even at 4°C. With regard to P. expansum, the association appeared effective only for the B on fruits stored at 25°C.

**DISCUSSION**

The results obtained in vitro and in vivo show that A. alternata and P. expansum tolerate benzimidazole fungicides at the authorised doses. Similar results were obtained with P. expansum on apples and pears (Maouni et al., 2002; Ramdani, 1989; Wicks, 1977).
Table 1. Efficacy (%) of calcium chloride on the development of *Alternaria alternata* and *Penicillium expansum* in vitro.

<table>
<thead>
<tr>
<th>CaCl$_2$ (%)</th>
<th><em>Alternaria alternata</em></th>
<th></th>
<th><em>Penicillium expansum</em></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Germination</td>
<td>Growth</td>
<td>Sporulation</td>
<td>Germination</td>
</tr>
<tr>
<td>2</td>
<td>8.2c</td>
<td>5.2c</td>
<td>17.6b</td>
<td>2.6c</td>
</tr>
<tr>
<td>4</td>
<td>12.4b</td>
<td>8.1b</td>
<td>25.4a</td>
<td>15.0b</td>
</tr>
<tr>
<td>6</td>
<td>24.8a</td>
<td>22.1a</td>
<td>26.4a</td>
<td>21.8a</td>
</tr>
</tbody>
</table>

Means within a column, followed by the same letter, are not significantly different (P= 0.05) according to Duncan’s multiple range test.

Table 2. Efficacy (%) of calcium chloride on the development of *Alternaria alternata* and *Penicillium expansum* on ‘Williams’ pears.

<table>
<thead>
<tr>
<th>CaCl$_2$ (%)</th>
<th><em>Alternaria alternata</em></th>
<th></th>
<th><em>Penicillium expansum</em></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4°C</td>
<td>25°C</td>
<td>4°C</td>
<td>25°C</td>
</tr>
<tr>
<td>2</td>
<td>57.7a</td>
<td>27.5a</td>
<td>8.2a</td>
<td>4.7a</td>
</tr>
<tr>
<td>4</td>
<td>71.5b</td>
<td>35.2b</td>
<td>68.0b</td>
<td>22.5b</td>
</tr>
<tr>
<td>6</td>
<td>92.5c</td>
<td>59.0c</td>
<td>72.5b</td>
<td>40.0c</td>
</tr>
</tbody>
</table>

Means within a column, followed by the same letter, are not significantly different (P= 0.05) according to Duncan’s multiple range test.

Table 3. *In vivo* efficacy (%) of fungicides (A.D) associated or not with calcium chloride (4%) on the development of *Alternaria alternata* and *Penicillium expansum*.

<table>
<thead>
<tr>
<th>Conditions</th>
<th><em>Alternaria alternata</em></th>
<th></th>
<th><em>Penicillium expansum</em></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Germination</td>
<td>Growth</td>
<td>Sporulation</td>
<td>Germination</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>20.5c</td>
<td>10.6a</td>
<td>43.0c</td>
<td>22.6a</td>
</tr>
<tr>
<td>B</td>
<td>29.0d</td>
<td>6.6a</td>
<td>10.0ab</td>
<td>31.6b</td>
</tr>
<tr>
<td>B + CaCl$_2$</td>
<td>71.6e</td>
<td>20.0b</td>
<td>50.6d</td>
<td>91.6c</td>
</tr>
<tr>
<td>MT</td>
<td>15.7bc</td>
<td>13.0ab</td>
<td>7.0ab</td>
<td>35.0b</td>
</tr>
<tr>
<td>MT + CaCl$_2$</td>
<td>95.0f</td>
<td>35.6c</td>
<td>14.7b</td>
<td>97.0c</td>
</tr>
<tr>
<td>TB</td>
<td>3.9a</td>
<td>30.4c</td>
<td>4.9a</td>
<td>33.5b</td>
</tr>
<tr>
<td>TB + CaCl$_2$</td>
<td>7.6ab</td>
<td>35.6c</td>
<td>44.6cd</td>
<td>37.8b</td>
</tr>
</tbody>
</table>

Means within a column, followed by the same letter, are not significantly different (P= 0.05) according to Duncan’s multiple range test.

Table 4. Efficacy (%) of fungicides (A.D) associated or not with calcium chloride (4%) on the development of *Alternaria alternata* and *Penicillium expansum* on ‘Williams’ pears.

<table>
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<tr>
<th>Conditions</th>
<th><em>Alternaria alternata</em></th>
<th></th>
<th><em>Penicillium expansum</em></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4°C</td>
<td>25°C</td>
<td>4°C</td>
<td>25°C</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>69.0d</td>
<td>34.6b</td>
<td>61.0c</td>
<td>21.6bc</td>
</tr>
<tr>
<td>B</td>
<td>15.6a</td>
<td>10.6a</td>
<td>5.9a</td>
<td>3.6a</td>
</tr>
<tr>
<td>B + CaCl$_2$</td>
<td>84.0e</td>
<td>74.0d</td>
<td>65.0c</td>
<td>60.0d</td>
</tr>
<tr>
<td>MT</td>
<td>51.6c</td>
<td>30.0b</td>
<td>27.0b</td>
<td>10.0ab</td>
</tr>
<tr>
<td>MT + CaCl$_2$</td>
<td>88.6e</td>
<td>59.6c</td>
<td>55.6c</td>
<td>32.8c</td>
</tr>
<tr>
<td>TB</td>
<td>40.0b</td>
<td>29.7b</td>
<td>14.0a</td>
<td>7.0a</td>
</tr>
<tr>
<td>TB + CaCl$_2$</td>
<td>84.0e</td>
<td>58.0c</td>
<td>64.0c</td>
<td>20.7b</td>
</tr>
</tbody>
</table>

Means within a column, followed by the same letter, are not significantly different (P= 0.05) according to Duncan’s multiple range test.
Resistance to benzimidazoles has also been reported in Botrytis cinerea (Besri and Diatta, 1985; Hmouni et al., 1996). It is very likely that the repetitive uses of these systemic fungicides and their persistence during long periods of conservation (Ben Arie, 1975; Prusky, 1985) have led to considerable selective pressure on both species. Moreover, the single site action of these fungicides could have facilitated the appearance of resistant strains (Lepoivre, 1989).

In vitro, CaCl₂ had little efficacy against the two fungi, even at 6% while in vivo and at low temperature, it was effective at 4%. Similar results were obtained with P. expansum and Alternaria sp. on apples (Biggs et al., 1993; Conway, 1982) and pears (Maouni et al., 2001), and with Leucostoma persoonii on peaches (Biggs et al., 1994). The Ca²⁺ ions might bind with intercellular pectic acids and constitute pectate chloride; which is resistant to the fungal peptolytic enzyme polygalacturonase (Bateman, 1964; Conway and Sams, 1984). At 25°C, the calcium would be insufficient to prevent this enzymatic activity.

The benzimidazoles - CaCl₂ association appears profitable in vitro, and against A. alternata in vivo. Satisfactory results were observed with other fungicides associated with CaCl₂ (Biggs et al., 1993). The increase efficacy of benzimidazole fungicides in the presence of CaCl₂ can be explained by the fact that CaCl₂ is an acid salt. Moreover, they might act by linking with a microtubule protein, thereby preventing cellular division (Davidec and Flach, 1978). It seems that calcium salts would reinforce the bonds of these fungicides with this protein.

In conclusion, the strategy of associating benzimidazoles with CaCl₂ could be used to control certain physiological and parasitic diseases in pears. The present findings show that it gives satisfactory results against A. alternata.

REFERENCES


