European Journal of Plant Pathology **108:** 421–427, 2002.

Sensitivity of Red Delicious apple fruit at various phenologic stages to infection by *Alternaria alternata* and moldy-core control

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Accepted 13 February 2002

Key words: disease control, *Malus sylverstris*, moldy-core disease, polyoxin B, sterol inhibitor fungicides, strobilurins

Abstract

The fungus *Alternaria alternata*, is considered to be the predominant fungus involved in moldy-core of Red Delicious strains of apple. In this paper, we report on the sensitivity of various phenologic stages to infection by *A. alternata*, and on the efficacy of various fungicides in controlling moldy-core disease in apple orchards. Artificial inoculations conducted in the orchard during 1999 and 2000 seasons revealed that the beginning of bloom (10–30%) and full bloom were the most susceptible developmental stages for infection. Natural infection with *A. alternata* in fruits was relatively high, reaching 44% and 46% of the fruits on control non-treated trees in 1999 and 2000. Four foliar applications of polyoxin B, difenoconazole and azoxystrobin, starting from the beginning of bloom until fruit set, reduced the number of infected fruits by 54–70%, 61–70% and 50–65%, respectively, compared with non-treated trees. Four or eight foliar applications of each fungicide provided similar levels of control. There were no significant differences between two, four or six foliar applications of difenoconazole, neither between two or four applications of polyoxin B. Adding $CaCl_2$, as a tank mixture with difenoconazole at full rate, did not improve efficacy. *Alternaria* was recovered from the inner part of the core region of 71–88% of the fruits of the non-treated control, but was recovered less frequently from the outside part of the core region. Fruits of difenoconazole and polyoxin B treated trees were less colonized with *A. alternata* at both the inner and outside parts of the core region, as compared with controls. Results indicate that a control programme based on spray applications of difenoconazole or polyoxin B, during bloom period, can effectively reduce *Alternaria* on Red Delicious.

Introduction

Alternaria alternata, is responsible for moldy-core in the apple cv. Red Delicious (Brown and Hendrix, 1978). Spores presumably infect young fruits through the open calyx tube and mycelia reach the seed and carpel wall during fruit development and storage (Miller, 1959). Moldy-core is characterized by the growth of the mycelia within the locules, with or without penetration into the mesoderm. The disease may become invasive and lead to a slow, dry rot confined to the flesh immediately surrounding the core (Ellis and Barrat, 1983; Spotts, 1990). External symptoms are rare, although infected fruits may colour and drop prematurely (Ellis and Barrat, 1983; Spotts, 1990). Conditions of high relative humidity, mild temperatures and tissue susceptibility are important factors that affect the natural infection in orchards. However, the optimal phenologic stage for penetration and infection of fruits by the fungus is not clearly undestood. Once inside the fruit, the fungus is protected against contact fungicides, and conditions for continued growth of the pathogen are excellent.

In recent years, levels of moldy-core in Red Delicious have caused significant losses in Israel. The incidence of fruits infected with moldy-core, according

to samplings from a packaging house in Northern Israel, was 15%, 8%, 7% and 4–5% respectively, in 1997, 1998, 1999 and 2000. In general, when more than 9% of the fruits are infected with moldy-core, they can only be sold for industry at very low prices. The Red Delicious cultivars constitute about one third of the total amount of apple trees planted in Israel. Therefore, moldy-core is a factor that reduces apple fruit quality and can become an economically important problem.

Attempts to control *Alternaria* and moldy-core by using foliar sprays of several fungicides, e.g. benomyl, captan, dodine, iprodione, mancozeb or some of their combinations, were unsuccessful (Biggs et al., 1993; Combrink et al., 1985; Ellis and Barrat, 1983). However, polyoxin B has been reported to control some species of *Altenaria* (Hori et al., 1974; Hwang and Yun, 1986), and other compounds, including azoxystrobin (Abound, Syngenta) and difenoconazole (Score, Syngenta), act on certain species of *Alternaria*. The recently introduced trifloxystrobin (Flint, Bayer), inhibits broad-spectrum fungi (Margot et al., 1998). These compounds inhibit conidial germination and/or mycelial growth of *A. alternata in vitro* and inhibit decay that develops on post-harvest inoculated fruit (Reuveni, unpublished data). However, their efficacy in controlling moldy-core in the field has not been examined.

The objectives of the present paper were: (1) to study the optimal phenologic stage of fruit development for infection in the orchard, by inoculating fruits at different stages of development with *A. alternata* spores; (2) to evaluate the efficacy of foliar sprays of polyoxin B (Polar), azoxystrobin, trifloxystrobin and difenoconazole for control of *A. alternata* in Red Delicious apple orchards. Preliminary results have been presented (Reuveni et al., 2000b).

Materials and methods

Fungicides

The control of apple moldy-core with the following fungicides was examined: difenoconazole (Score, 250 EC, Syngenta, Basel, Switzerland), the strobilurins trifloxystrobin (Flint 50 WG, Bayer, Germany), and azoxystrobin (Amistar, Abound, 250 SC, Syngenta), and polyoxin B (Polar, 50 WG, Kaken Pharmaceutical, Japan).

Fungal isolation and inoculation in the orchard

Star King Delicious apple fruits, infected with moldy-core, were disinfected with 90% ethanol. Small pieces (1–2 mm) of the mesoderm tissue external to the core region showing moldy-core symptoms were removed and placed on potato dextrose agar (PDA, Difco) at 25 °C. Single-spore colonies of *A. alternata* prepared from the resultant cultures were maintained on PDA plates for 10–12 days, until conidia were produced. Conidia were harvested from PDA plates by adding a small amount of sterile distilled water to each plate and gently rubbing the sporulating mycelial mass with a bent glass rod. Spore concentration was adjusted with the aid of a haemocytometer, to obtain a suspension containing 1×10^5 spores ml^{-1}. The conidial suspension was held on ice and used to spray-inoculate flower clusters at various phenological stages in the orchard: the beginning of bloom, full bloom, petal fall and young fruits (Table 1). At each date, inoculation was carried out on ten flower clusters from each of five replicates of the control treatment as described below. Following spray inoculation, to maintain high humidity, clusters were covered with plastic bags, which were removed in the following morning. The procedure to determine percentages of fruits infected with moldy-core is described below.

Field experiments

Field experiments with the apple cvs. Star King and Super Chief, were conducted in commercial orchards in 1999 and 2000 on the Golan Heights. These cvs. are susceptible to moldy-core, which had been prevalent in these orchards in previous years. Fertilization, irrigation and other cultural practices were as recommended to commercial growers by the Extension Service of the Ministry of Agriculture, Israel. The annual rainfall in this region during the winter (October–April) is 800–900 mm, and the average midday relative humidity (RH) and temperature in summer are 35–40% and 30 °C, respectively. The sky is cloudless during most of the summer. Night temperatures fall occasionally from the end of May until August to 14–20 °C, and dew may accumulate during some nights on the leaf surface. During the spring, moderate temperatures (10–25 °C) and favourable humidity conditions exist in this region.

Table 1. Sensitivity of various phenologic stages to artificial inoculation with conidial suspension of *A. alternata* in the orchard

Phenologic stage of inoculation[1]	Percentage infected fruits, as tested on PDA plates[2]			
	Experiment 1, 1999		Experiment 2, 2000	
	6 June	4 July	4 August	3 September
Bloom, 10–30%	45.5 a[3]	43.0 a	55.0 a	56.0 a
Full bloom	42.0 a	45.0 a	50.0 a	38.0 a
Petal fall	28.0 b	30.0 ab	42.0 ab	33.5 a
Young fruits	25.0 b	20.0 b	30.0 b	16.0 b
Control	20.0 b	20.0 b	42.0 ab	46.0 a

[1]Artificial spray inoculations with a suspension of 10^5 conidia of *A. alternata*/ml of water were conducted in each stage on ten flower clusters in each replicate (5 replicates/treatment), as described in Materials and methods. Control plants were sprayed with water. Inoculation dates were 18, 25 April, 2, 11 May 1999, and 15, 21, 27 April and 4 May 2000 in Experiments 1 and 2 at the beginning of bloom, full bloom, petal fall and young fruits, respectively.

[2]Ten fruits from each replicate were examined for the presence of *A. alternata*, as described in Materials and methods.

[3]Means within columns followed by different letters are significantly ($P = 0.05$) different according to Duncan's Multiple Range Test.

Experimental design

Application of fungicides began at the pink-cluster stage (unless otherwise stated). Sprays were applied to run-off (2500 L ha^{-1}) with a 100-L gun-sprayer (1400 kPa), or with a 1000-L speeder type sprayer (1500–2000 L ha^{-1}), at the time intervals specified for each experiment on 'calendar' and phenological basis. Spacing between trees was 5 m × 3 m or 5 m × 4 m with a buffer row between treatments. Treatments were arranged in a randomized complete block design. Plots consisted of four to six trees replicated four or five times in all experiments.

Fungicides efficacy

In 1999, three fungicides difenoconazole (0.01% v/v), polyoxin B (0.025% v/v) and azoxystrobin (0.04% v/v) were tested at El Rom on cv. Star King trees (16 years old, on MM.106 rootstock). Fungicides were applied four or eight times, starting on 16 April, at the pink-cluster stage, following by applications on 21 April (bloom), 28 April (petal fall) and 5 May, 1999 (young fruits). The intervals between the remaining four sprays (when applied) were 10–12 days thereafter. Non-treated trees were used as control (Table 2).

In 2000, the first experiment conducted in the same El Rom orchard, was undertaken to compare the efficacy of the various fungicides in controlling moldy-core and to determine the effects of number and timing of the applications. This experiment included the following treatments: fungicides difenoconazole (0.02% v/v), polyoxin B (0.025% v/v), azoxystrobin (0.04% v/v) and trifloxystrobin (0.015% v/v), each applied four times, starting at the pink-cluster (<10% bloom) stage on 14 April. Further applications were made on 18 April (full bloom), 26 April (80% petal fall) and 3 May, 2000 (young fruits, 5–8 mm) (Table 3). Two applications were tested with the fungicides difenoconazole (0.02% v/v), polyoxin B (0.025% v/v) and azoxystrobin (0.04% v/v), sprayed at the first two application dates. Three applications of difenoconazole (0.02% v/v) were sprayed on the first three dates, and when six applications of difenoconazole were made, the remaining two sprays were applied at 10 days intervals thereafter. Non-treated trees were used as control.

The second experiment was conducted at Ramat Magshimim and consisted of five treatments as follows: non-treated control, the fungicides difenoconazole (0.02% v/v), polyoxin B (0.025% v/v), trifloxystrobin (0.015% v/v) and a mixture of difenoconazole (0.02% v/v) + $CaCl_2$ (0.1% v/v), each applied four times, starting at the pink-cluster (<10% bloom) stage on 14 April to cv. Super Chief trees (10 years old, on MM.106 rootstock). Further applications were made on 18 April (full bloom), 27 April (end of petal fall) and 4 May, 2000 (young fruits) (Table 3).

Demonstration trial in a commercial orchard

To further evaluate the potential use of difenoconazole (0.02% v/v) against moldy-core, a large-scale demonstration trial was conducted in 2000 in a commercial orchard in the Golan Heights. The fungicide was sprayed with a 2000-L speeder-type sprayer (1800–2000 L ha^{-1}) starting on 14 April, at the beginning of bloom. Further applications were made on 19 and 28 April at full bloom and petal fall, respectively. Fungicide was applied to about 1000 trees of cv. 'Oregon' (9 years old trees, on MM.106 rootstock),

Table 2. The effect of fungicides on control of *A. alternata* in apple fruit

Treatment and concentration (% v/v)[1]	Percentage fruits infected with *A. alternata*[2]					
	4 July		4 August		13 September	
	4 sprays	8 sprays	4 sprays	8 sprays	4 sprays	8 sprays
Control	20.0 a		42.0 a		44.0 a	
Azoxystrobin, 0.04	10.0 a[3]	20.0 a	22.0 b	20.0 b	22.0 b	24.0 b
Difenoconazole, 0.01	20.0 a	20.0 a	16.0 b	26.0 b	16.0 b	22.0 b
Polyoxin B, 0.025	10.0 a	10.0 a	14.0 b	16.0 b	14.0 b	26.0 b

[1]Four foliar sprays of each fungicide were applied starting at the pink-cluster stage on: 16 April, 1999. Further applications were made on 21 April (bloom), 28 April (petal fall) and 5 May 1999 (young fruits). The intervals between the remaining four sprays were 10–12 days thereafter. Non-treated trees served as control.
[2]Ten fruits from each replicate were brought to the laboratory at the given dates and examined for the presence of *A. alternata*, as described in Materials and methods.
[3]Means within columns followed by different letters are significantly ($P = 0.05$) different according to Duncan's Multiple Range Test.

Table 3. The effects of fungicides and number of applications on control of *A. alternata* in apple fruit in 2000

Treatment and concentration (% v/v)[1]	Percentage infected fruits with *A. alternata*			
	Experiment 1, El Rom[1]		Experiment 2, Ramat Magshimim[2]	
	1 August	3 September	17 August	29 August
Control	42.0 a[3]	46.0 a	20.0 a	32.5 a
Azoxystrobin, 0.04	30.0 ab	16.0 b	nt	nt
Difenoconazole, 0.02	12.0 b	14.0 b	10.0 a	12.5 b
Polyoxin B, 0.025	10.0 b	20.0 b	12.5 a	15.0 b
Trifloxystrobin, 0.015	nt[4]	18.0 b	7.5 a	22.5 ab

[1]Fungicides were applied four times, starting at the pink-cluster (<10% bloom) stage on 14 April, 2000. Further applications were made on 18 April (full bloom), 26 April (80% petal fall) and 3 May, 2000 (young fruits, 5–8 mm). Non-treated trees served as control.
[2]Fungicides were applied four times, starting at the pink-cluster (<10% bloom) stage on 14 April, 2000. Further applications were made on 18 April (full bloom), 27 April (end of petal fall) and 4 May, 2000 (young fruits). Non-treated trees served as control.
[3]Means within columns followed by different letters are significantly ($P = 0.05$) different according to Duncan's Multiple Range Test.
[4]nt = not tested.

planted at 2 m × 4 m spacing between trees, with six rows of different cultivars as buffers between treatments. Five non-treated trees in the end of each row were used as controls.

Assessment of A. alternata *infected fruits*

At various stages during the growth season ten fruits were randomly collected from each replicate plot and treatment. Each fruit was cut in half longitudinally and a small part of the mesoderm tissue at a distance of about 2 mm outside the core region was asepticaly removed from each half of each fruit and plated on PDA in sterile 9-cm Petri dishes. In order to compare the recovery of *A. alternata* from the inside and the outside of the core region, tissue samples were also taken from inside the core of each of six of ten fruits of each replicate and plated on other PDA dishes. Samples for this purpose were taken from the following three treatments: non-treated control, difenoconazole and the polyoxin B, of two experiments (1 and 2 in 2000) (Table 4). Plates were incubated at 25 °C in the dark for 8–10 days and colonies and conidia of *A. alternata* were examined microscopically. The percentage of fruits infected with *A. alternata* was determined.

Statistical analysis

Analysis of variance (ANOVA) using the SAS GLM (SAS Institute, 1992) procedure was applied to arc-sin

Table 4. Recovery of *A. alternata* colonized inside or outside the core region of fungicide-treated or control non-treated apple fruit

Treatment and concentration (% v/v)[1]	Percentage fruits infected with *A. alternata*[2]			
	Experiment 1, ER		Experiment 2, RM	
	In	Out	In	Out
Control	87.5 a[3]A[4]	50.0 aB	70.8 aA	16.7 aB
Difenoconazole, 0.02	50.0 bA	0.0 cB	58.3 aA	12.5 aB
Polyoxin B, 0.025	45.8 bA	16.7 bB	54.2 aA	8.3 aB

[1]Four foliar sprays of each fungicide were applied in each experiment starting at pink-cluster stage. See footnotes 1 and 2 in Table 3 (Experiments 1 and 2) for more details.
[2]Data obtained randomly from six of ten fruits in each replicate and treatment on 3 September 2000 (Experiment 1 – El Rom) and on 17 August (Experiment 2 – Ramat Magshimim), are presented.
[3]Numbers within a column (effect of fungicides) followed by different lower case letters are significantly ($P < 0.05$) different according to Duncan's Multiple Range Test.
[4]Numbers in rows (Inside or outside) within each experiment followed by different upper case letters are significantly ($P < 0.05$) different according to the same test.

transformed data. Duncan's Multiple Range Test was applied to determine whether differences between treatments were significant.

Results

Inoculations in the orchard

Artificial inoculations conducted in the orchard during 1999 and 2000 seasons revealed that the beginning of bloom (10–30%) and full bloom, were both the most susceptible developmental stages for infection by the pathogen (Table 1). Flower clusters inoculated at bloom had the highest percentages (42–55%) of infected fruits, compared with those inoculated after fruit set at young fruits stage (20–30%), or with those of non-inoculated controls (20–42%), as determined by isolation tests on PDA during 1999. Flower clusters inoculated at petal fall stage resulted in intermediate percentages of infected fruit in this year (Table 1). Similar results were found in 2000; inoculations at early stage of bloom resulted in 56% of *A. alternata* recovery. Fruits of flower clusters inoculated at full bloom and petal fall were slightly, but not significantly, less infected, and those inoculated after fruit set caused the least number of infected fruits with *A. alternata*. However, the percentage of infected fruits of non-inoculated control was 46% and higher from those inoculated after fruit set (Table 1).

Efficacy of fungicides

Natural infection with *A. alternata* in fruits was relatively high, reaching 44% and 46% of the fruits on non-treated control trees in 1999 and 2000, (Tables 2 and 3). Four or eight foliar applications of the fungicides tested in 1999 provided similar levels of control (Table 2). Four applications of polyoxin B, difenoconazole or azoxystrobin reduced the number of infected fruits by 70%, 64% and 50.0%, respectively, compared to non-treated trees, as determined just prior to commercial harvest (13 September) (Table 2).

Four applications of these fungicides in 2000 provided similar results in both experiments (Table 3). Trifloxystrobin, added in 2000, significantly reduced incidence in the first, but not in the second experiment. There were no significant differences between two, three, four or six foliar applications of difenoconazole, in reducing disease incidence (14–20% infected fruits), neither between two or four applications of polyoxin B (20–22% infected fruits) (Experiment 1 – data not shown). Two foliar applications of azoxystrobin resulted in 30% of infected fruits, as compared to 46% infected fruits of the non-treated control with no significant difference between treatments. Adding of $CaCl_2$ (0.1%) as a tank mixture with difenoconazole (0.02%), did not improve efficacy, compared to difenoconazole alone (Experiment 2 – data not shown).

Results from the large-scale demonstration trial in a commercial orchard during 2000 supported those obtained in the experimental plots. Three foliar applications of difenoconazole reduced incidence of *Alternaria* and resulted in 7.7% of infected fruits, as compared to 31% infected fruits for the non-treated control.

In both experiments in 2000, *Alternaria* mycelia was recovered from the inner part of the core region in 88% and 71% of the fruits of the non-treated control, but was less frequently (50% and 17%) recovered from outside the core region (Table 4). A similar trend was found when fruits from difenoconazole and polyoxin B treated trees were examined. Both fungicides reduced the incidence of *A. alternata* at both the inner and outside regions of the core, as copmpared with control (Table 4).

Discussion

The ultimate value of any chemical compound as an agent for disease control, depends on the mode of action of the molecule on one or more components of the life cycle of the pathogen, at the physiological level. Among the stages in the life cycle of *A. alternata*, germination of conidia, hyphal growth and, subsequently, decay formation offer the highest potential for host infection and disease development (Rotem, 1994; van der Plank, 1963). Therefore, any chemical that significantly inhibits germination of conidia, mycelial growth and decay formation should reduce the ability of *A. alternata* to cause moldy-core disease. At appropriate concentrations, azoxystrobin, difenoconazole, polyoxin B and trifloxystrobin significantly inhibited germination of conidia, and mycelial growth *in vitro*, or decay development on inoculated post-harvested fruits (M. Reuveni, unpublished data), compared with non-treated controls, thus severly restricting the potential of *A. alternata* to infect plant tissue and cause disease.

No effective fungicide alternatives for control of *Alternaria* moldy-core in apple fruit are currently available. Attempts to control *Alternaria* and moldy-core by using foliar sprays of several fungicides e.g. benomyl, captan, dodine, iprodione, mancozeb or some of their combinations, have been unsuccessful (Biggs et al., 1993; Combrink et al., 1985; Ellis and Barrat, 1983). Fungicides used in this study appear to have a beneficial effect, with at least two fungicides, difenoconazole and the polyoxin B significantly reducing disease incidence relative to control. Artificial inoculations in the orchard indicate that a control programme based on spray applications for *Alternaria* on Red Delicious could be initiated at the beginning of flowering and continued until petal fall, presuming that an effective fungicide could be registered for this use in apple orchards. In fact, two foliar applications of difenoconazole or polyoxin B during the bloom period, were as effective as six or four applications, respectively.

The results of this study suggest that *Alternaria* is extremly common in the apple orchard and is closely associated with moldy-core. The fungus apparently colonizes flower parts and eventually grows (presumably through the open calyx tube) to the core or carpel regions. Results in apple orchards indicate that fruits can be infected with *A. alternata.* This can be attributed to the moderate temperatures (10–25 °C) and favourable RH conditions for moldy-core development during the spring in this region in Israel (Spotts, 1990). Although a large percentage of fruits were colonized by *Alternaria*, not all developed moldy-core symptoms. The reason for this is not clearly understood.

Each compound has a different mode of action (Koller and Scheinpflug, 1987; Reuveni, 2000; Reuveni et al., 2000a); therefore, these modifications could be incorporated into a disease management programme that would minimize the risk of development of resistance by *Alternaria* spp. and, at the same time, maximize disease control. The activities of azoxystrobin, difenoconazole, polyoxin B and trifloxystrobin on one or more stages of the life cycle of *A. alternata*, and on decay formation in post-harvested apple fruits (M. Reuveni, unpublished) and their efficacy in the field, indicate that these compounds could potentially provide control of moldy-core disease. It has been shown that strobilurins (Reuveni, 2000) and polyoxin B (Reuveni et al., 2000a) can be used to control foliar diseases in orchards.

Acknowledgements

The authors wish to thank Y. Mashal and A. Venezian for their assitance. This work was supportd by the Israeli Fruit Board, Makhteshim Ltd., Israel, C.T.S. Ltd, Israel, and Tivon-Chem Ltd., Israel.

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