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Occurrence, characterization and management of fruit rot of immature cucumbers under greenhouse conditions in Oman

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Summary. A study was undertaken to characterize and manage pathogens associated with fruit rot of immature cucumber fruits in greenhouses in Oman. A survey over five growing seasons from 2008 to 2010 in 99 different greenhouses in Oman showed that the disease was prevalent in 91 (92%) greenhouses and resulted in losses of 10 to 60% (avg. 33%) of immature fruits per plant. Incidence of the disease was not found to be affected by growing seasons, which could be attributed to the limited fluctuations in ambient temperatures in greenhouses. Isolations from diseased cucumber fruits yielded *Alternaria alternata* (isolation frequency = 52%), *Fusarium equiseti* (40%), *Cladosporium tenuissimum* (27%), *Botrytis cinerea* (6%), *F. solani* (6%), *Corynespora cassiicola* (3%), *Aspergillus* spp. (2%), *Curvularia* sp. (1%) and *Bipolaris* sp. (1%). With the exception of the *Curvularia* and *Bipolaris* species, all other fungi were pathogenic on cucumber fruits, with *F. equiseti* being the most aggressive, followed by *Co. cassiicola, B. cinerea* and *A. alternata*. *Cladosporium* and *Aspergillus* spp. were found to be weakly pathogenic. Comparing the efficacy of foliar and soil applications of carbendazim fungicide on fruit rot of cucumber showed that foliar applications significantly reduced fruit rot and increased cucumber yield when compared to soil application or to untreated experimental controls ($P<0.01$). This is the first report of the association of *Co. cassiicola* and *F. equiseti* with fruit rot of immature greenhouse cucumbers. This is also the first report in Oman for the association of *Cl. tenuissimum* with fruit rot of immature cucumbers. Factors affecting disease control in greenhouses using carbendazim are discussed in light of the experimental results from the study .

Key words: aggressiveness, gray mold, ITS rDNA.

Introduction

Oman is an arid region located in the eastern part of the Arabian Peninsula, where temperatures can exceed 50°C in the summer. The unfavorable environmental conditions to profitable production of commercial vegetable varieties in these areas have resulted in shifting most profitable vegetable production to protected agriculture «greenhouses». In greenhouses, temperatures can be brought down to 25 to 32°C in most times of the year to satisfy the growing conditions for the most sensitive vegetable crops. As a result, cucumber (*Cucumis sativus*) became the most important greenhouse crop in Oman, occupying over 95% of greenhouses throughout the country.

Despite increased demand for this crop in the country and in other parts of the world, cucumber cultivation and production has been limited by a number biotic and abiotic stresses. Among the most serious biotic stresses, soilborne pathogens represent the most important challenge, with losses reaching 50% in some greenhouses (Stanghellini and Phillips, 1975; Al-Sa'di *et al*., 2007, 2011b). Fruit rot is a serious disease in many greenhouses, being the second most important biotic challenge

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to greenhouse cucumber production in Oman after soilborne diseases. Typical symptoms on infected fruits are in the form of yellowing which usually starts from the flower end of each immature fruit, followed by browning and rotting. Gray mold may develop on the infected fruits, especially under conditions of high humidity (Blancard *et al*., 2005). In Oman, these symptoms are common only on immature cucumber fruits, which are not ready for harvest (3–8 cm in length) and which are considered highly susceptible to the disease. Despite some studies which have addressed this problem (e.g. Moghal *et al*., 1993), little information is available concerning prevalence, losses and effects of growing seasons on severity of cucumber fruit rot in greenhouses in arid regions.

Rotting symptoms on immature cucumber fruits have been related to infection by several pathogenic fungi including *Alternaria tenuis, Botrytis cinerea, Choanephora cucurbitarum, Didymella bryoniae*, *Geotrichum candidum*, *Penicillium oxalicum*, *Phytophthora capsici* and *Rhizopus nigricans* (van Steekelenburg, 1983, 1986; Moghal *et al*., 1993; Blancard *et al*., 2005; Gevens *et al*., 2006). In Oman, a limited survey attributed disease to infection by *B. cinerea*, *A. alternata* and *R. nigricans* (Moghal *et al*., 1993). Despite efforts to characterize the causal agents of this disease in Oman (Moghal *et al*., 1993) and elsewhere (van Steekelenburg, 1983, 1986; Jarvis *et al*., 1990), most of these studies were limited and only targeted one or few pathogenic fungi. This tells little about the relative occurrence of different fungi in relation to seasonality and level of aggressiveness. In addition, little is known about causal agents of this disease in greenhouses in arid areas of the world.

Management of fruit rot of immature cucumbers in different parts of the world has relied on the use of cultural practices, host resistance, plant nutrition and fungicides (Yunis *et al*., 1991; Elad *et al*., 1993a; Ando and Grumet, 2006). Carbendazim-containing fungicides are known to have broad spectrum activity against many fungi, including *B. cinerea* and *Fusarium* spp. (Yunis *et al*., 1991; Bao *et al*., 1992; Elad *et al*., 1992, 1993b; Thomidis *et al*., 2009). Despite some reports indicating effective management of fruit rot of cucumber using carbendazim (Yunis *et al*., 1991; Elad *et al*., 1993b), there is a lack of knowledge about the most efficacious method of fungicide application for management of the disease.

Due to the lack of knowledge of the occurrence and causal agents of fruit rot of immature cucumbers in greenhouses in arid regions, as well as the most efficacious method for application of carbendazim fungicide, this study was initiated to characterize and manage pathogens associated with this disease. Specific objectives included:

1. to determine the occurrence, incidence and severity of fruit rot of immature cucumber fruits.
2. to characterize pathogens associated with the disease and determine their pathogenicity.
3. to test the efficacy of two fungicide application methods for management of fruit rot and effects on cucmber yield.

Knowledge in these areas may help reduce losses due to this disease in greenhouse systems in arid regions.

Materials and methods

Incidence and severity of cucumber fruit rot

Surveys were conducted during 2008 to 2010 in order to characterize the pathogens associated with fruit rot of immature cucumbers in Oman. The surveys were carried out in six major cucumber growing districts; Barka, Muscat, Musana'a, Khaboura, Suwaiq and Samael. A total of 99 different greenhouses were visited over five different growing seasons. Except for one farm with four greenhouses using hydroponic systems, all the surveyed greenhouses use soil-based systems for growing cucumber crops. Cucumbers were sown directly on raised soil ridges or transplanted when plants were 7–14 days old. In order to determine incidence of the disease in different greenhouses, the number of cucumber plants with immature fruits showing symptoms of fruit rot was divided by the total number plants grown in the greenhouse (approx. 850–1000 plants) and then multiplied by 100. Disease severity was determined for ten randomly selected plants per greenhouse by estimating the percent fruits per plant developing fruit rot symptoms.

Pathogens associated with fruit rot of cucumber

Fruit samples developing rotting symptoms were collected from each greenhouse (ten-20 fruits per greenhouse) during the course of the surveys.

Fruit tissues were surface sterilized in 1% sodium hypochlorite (1 min), washed in sterile distilled water and then dried on sterile filter paper. Fruit tissues (4–6 mm) were plated on 2.5% potato dextrose agar (PDA, Oxoid) for 2–5 days. Emerging fungal growth was excised, transferred to new PDA plates, purified using mycelium tip culture and then preserved at 22°C in PDA slants for further use.

The isolated fungi were first identified to the species level using morphological characteristics (Barnette and Hunter, 1998; Leslie and Summerell, 2006). Identity of selected isolates (1 to 5) from each fungal species was confirmed using sequences of the internal transcribed spacer region of the ribosomal DNA (ITS rDNA). To do this, mycelium from 4–7-day-old cultures was harvested in 1.5 mL sterile Eppendorf tubes followed by freeze drying. DNA was extracted from the freeze dried mycelium following a modified protocol of Lee and Taylor (1990) as described by Al-Sa'di *et al*. (2007). About 40 mg of freeze dried mycelium was ground into fine powder followed by addition of lysis buffer, purification using phenol: chloroform: isoamyl alcohol (25:24:1), precipitation using 3M NaAc and isopropanol and then washing of the DNA pellet using 70% ethanol. The DNA pellet was resuspended in 100 µL of TE buffer and stored at -20˚C until required.

The PCR reaction mixture and conditions were as described by Al-Sadi and Deadman (2010) using primers ITS1 and ITS4 (White *et al*., 1990). The PCR reaction mixture consisted of PuReTaq™ Ready-To-Go™ PCR beads, 0.4 µM ITS1 primer, 0.4 µM ITS4 primer, 2 µL of diluted DNA (12 ng μL^{-1}) and Milli-Q water up to a final volume of 25 µL. The thermocycling was as follows: 95°C for 10 min followed by 35 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 90 s. The final extension was performed at 72°C for 10 min. The PCR products were purified from primers and dNTPs using the ultraclean PCR clean-up kit (Mo-Bio Laboratory, Carlsbad, CA, USA) according to the manufactures' protocol. Samples were then sent for sequencing to Macrogen, Korea using the primers ITS1 and ITS4.

The forward and backward ITS sequences for each isolate were first aligned and edited using ChromasPro. The most closely related sequences were downloaded from the National Center for Biotechnology Information (NCBI) using BLAST search. Alignment of sequences was done using Clustal W (Thompson *et al.*, 1994), which was followed by construction of phylogenetic trees using Clustal X 2.09 (Larkin *et al.*, 2007) for the newly reported fungal species in Oman. Bootstrap 50% majority-rule consensus trees were generated using 1000 replications.

Pathogenicity test

Representative fungi isolated from diseased cucumber fruits were tested for pathogenicity using the following protocol. Immature cucumber fruits (4–5 cm in length) obtained from 5–7 week old cucumber plants were used. The fruits were detached from plants and the flowers were excised. The fruits were then surface sterilized using 70% ethanol, and were placed into Petri dishes (three fruits per dish) onto 90 mm diam. Whatman filter papers moistened with sterile distilled water. Each fruit was inoculated from the flower end with a 3 mm diam. mycelial disc obtained from a 3–6 day old fungal culture grown on 2.5% PDA. Two randomly selected isolates were used for inoculation for each fungal species. Control fruits were inoculated with 3 mm diam. PDA plugs in the same way. At least three replicate Petri dishes were used for each fungal isolate and the control, and the experiment was repeated once. Severity of fruit rot was determined 4 days after inoculation by measuring the area of the fruit showing fruit rot symptoms.

Influence of different carbendazim application methods on severity of fruit rot

A commercial greenhouse was used to determine whether foliar applications of carbendazim (Bavistin®DF, BASF, New Zeland Limited, Auckland, NZ) are superior to soil applications in reducing losses due to fruit rot. The greenhouse contained 840 plants of the cucumber variety Miracle, transplanted in the winter growing season, in six double rows.. The greenhouse was divided lengthwise into four blocks. The three treatments, starting 28 days after transplanting, were replicated in each block using three central rows with 140 plants per row. The first treatment consisted of weekly foliar applications of carbendazim at 150 g ai ha^{-1}. The second treatment consisted of weekly soil applications of carbendazim at 50 mL 100 L^{-1}

water (5.5 L solution m^{-2}, 150 g ai ha^{-1}). The application rates were based on the recommended rates of application of Bavistin®DF. The third treatment served as an experimental control, with no fungicide applications. Disease intensity was measured by counting the numbers of diseased fruits on ten randomly selected plants in each block and expressing this as a percent of the total number of fruits per plant. The experiment was repeated using the same method of planting, treatments, number of replicates and disease evaluation.

Data analysis

Data generated from surveys and fungicide tests were analyzed using Statistical Analysis System Version 8 (SAS Institute, Inc., Cary, NC, USA). Means were separated using Tukey's Studentized Range test.

Results

Incidence and severity of cucumber fruit rot

Fruit rot of cucumbers was observed in 91 out of 99 (92%) greenhouses surveyed in 2008, 2009 and 2010 (Table 1). The disease was observed in all districts. Symptoms were characterized by yellowing of eac affected fruit from one end followed by browning and rotting. Symptoms were observed in fruits which were 3–8 cm in length. The percentage of cucumber plants affected with the disease varied from 10 to 100 (avg. 55%) per greenhouse. Severity of fruit rot represented as percent symptomatic fruits of the total fruit number varied from 10 to 60%, with a mean fruit loss of 33% (Table 1). No significant differences were observed in the severity of fruit rot among different growing seasons (Table 1; $P>0.05$).

Pathogens associated with fruit rot of cucumber

Isolations from diseased cucumber fruits yielded nine different fungi: *Alternaria alternata* (isolation frequency = 52%), *Fusarium equiseti* (40%), *Cladosporium tenuissimum* (27%), *Botrytis cinerea* (6%), *F. solani* (6%), *Corynespora cassiicola* (3%), *Aspergillus* spp. (2%), *Curvularia* sp. (1%) and *Bipolaris* sp. (1%). *Fusarium equiseti* and *A. alternata* were recovered from all the surveyed districts, while the next most frequently recovered fungus was *Cl. tenuissimum* (four districts) (Table 2).

Amplification and sequencing of the ITS rDNA

Table 1. Mean severity of cucumber fruit rot in different greenhouses and seasons in Oman districts.

Season	District	Sample size (No. of greenhouses)	Mean severity of fruit rot (%) [a]	Mean severity per season [b]
Summer 2008	Khaboura	6	46	31a
	Barka	22	20	
	Musana'a	7	53	
	Suwaiq	2	38	
Fall 2008	Samael	2	50	18a
	Barka	8	10	
Winter 2009	Muscat	5	30	43a
	Suwaiq	6	53	
Spring 2010	Barka	10	35	37a
	Samael	1	60	
Fall 2010	Samael	8	33	43a
	Barka	16	49	
	Suwaiq	5	38	

[a] Indicates percent symptomatic fruits out of the total fruits (10 plants per greenhouse).
[b] Means with the same letter in the same column are not significantly different ($P<0.05$; Tukey's Studentized Range test).

Table 2. Frequency of isolation and aggressiveness level of different fungi on immature cucumber fruits.

Fungus	Isolation frequency (%)	Geographic distribution [a]	Season [b]	Aggressiveness test [c]
Fusarium equiseti	40	K, B, M, Q, S, C	S, F, W, P	+++
Fusarium solani	6	B, S	F, P	?
Alternaria alternata	52	K, B, M, Q, S, C	S, F, W, P	++
Cladosporium tenuissimum	27	B, S, C, Q	S, F, W, P	+
Botrytis cinerea	6	B, Q	W, P	++
Corynespora cassiicola	3	S	P	++
Curvularia spp.	1	B	P	-
Bipolaris spp.	1	B	P	-
Aspergillus spp.	2	B	P	+

[a] Geographical distribution (K, Khaboura; B, Barka; M, Musana'a; Q, Suwaiq; S, Samael; C, Muscat).
[b] Seasons (S, summer; F, Fall; W, winter; P, spring).
[c] Aggressiveness test: –,no chlorosis/rotting; +, size of chlorosis/rotting <3 mm; ++, chlorosis/rotting from 3 to 10 mm; +++, indicates chlorosis/rotting >10 mm; ?, not tested.

region helped to confirm identify of the isolated pathogens. Since most of the fungi have been reported in Oman and sequenced in previous studies (Moghal *et al*., 1993; Al-Sadi and Deadman, 2010; Al-Sadi *et al*., 2011a, 2011b), a phylogenetic tree was only constructed for the newly reported pathogen in Oman, *Co. cassiicola*. The Omani isolate of *Co. cassiicola* (Q8) clustered with sequences of two isolates of *Co. cassiicola* from GenBank (EU822319 and FJ649317). This cluster was separated from the closely related species *Co. smithii* with a very high bootstrap support (100%) (Figure 1). The ITS rDNA sequences for isolate Q8 of *Co. cassiicola* was deposited in GenBank under the accession number HQ453184.

Pathogenicity test

Differences were observed among the isolated fungi in their aggressiveness on cucumber fruits. *Fusarium equiseti* was highly pathogenic, followed by *B. cinerea, Co. cassiicola* and *A. alternata* (Table 2). *Cladosporium* and *Aspergillus* species were weakly pathogenic and *Curvularia* and *Bipolaris* species were non-pathogenic. No symptoms developed on the control fruits. Symptoms in most of the inoculated fruits were characterized by yellowing of the tissue, followed by browning and fruit rot. The pathogenic fungi which induced symptoms in the inoculated fruits were re-isolated from symptomatic tissue.

Influence of different carbendazim application methods on severity of fruit rot

In both trials, the percent diseased fruits was significantly reduced, both by soil and foliar applications of carbendazim ($P<0.01$, Figure 2). In the experimental control, an average of 29–37% of immature fruits per plant was lost due to the disease. This number was reduced to 22–24% with soil application of carbendazim, and to 14–16% following foliar application of the fungicide.

Fruit weight was significantly increased by the application of carbendazim in the first trial ($P<0.01$, Figure 2B). When the fungicide was applied through foliar application, mean fruit weight increased from 4.30 kg to 5.36 kg per plant, an increase of 25% relative to the control. The increase in fruit weight following soil application of carbendazim was statistically significant but less than for foliar application, equivalent to 17.2%, or 0.75 kg per plant per season. When the experiment was repeated, the increase in fruit weight was only significant for the foliar application (Figure 2B). Isolations from randomly selected diseased cucumber fruits yielded *F. equiseti* and *A. alternata*.

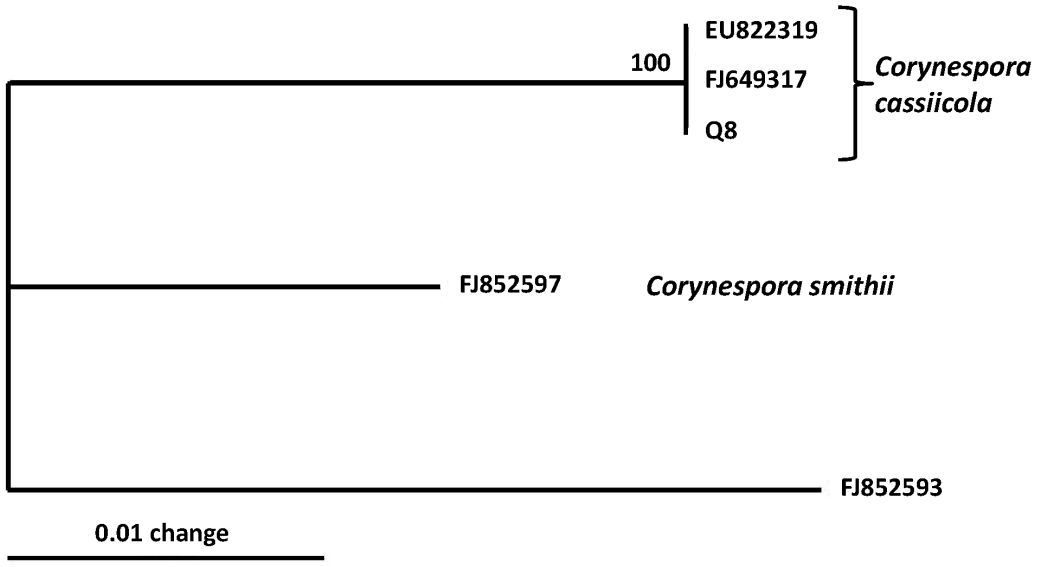

Figure 1. Phylogram representing the relationship of *Corynespora cassiicola* isolate from Oman (Q8) to sequences of *Co. cassiicola* and two other *Corynespora* species based on the ITS rDNA sequences. Numbers within the tree represent the bootstrap values (values above 50% are indicated; 1000 replications). The tree was rooted to *Co. citricola* (FJ852593).

Discussion

Survey results and pathogenicity tests provided evidence for fruit rot symptoms of immature cucumber fruits in Oman to be caused primarily by *F. equiseti* and *A. alternata*. Other fungi were found to be either less pathogenic (e.g. *Cl. tenuissimum*) or with a low level of recovery (e.g. *B. cinerea*, *Co. cassiicola*). These findings may appear to disagree with the reports emphasizing the major role of *B. cinerea* as a causal agent of fruit rot of immature cucumbers (Yunis *et al*., 1991; Blancard *et al*., 2005). One possible explanation is that symptoms of fruit rot in Oman were characterized by yellowing of fruits followed by browning and rotting; typical *B. cinerea*-induced gray mold was rarely observed (Yunis *et al*., 1991; Blancard *et al*., 2005).

Fruit rot of cucumber was found to be prevalent in most (92%) of the greenhouses in Oman, resulting in an average loss of 33% of immature fruits per plant. Due to lack of data about losses due to this disease in greenhouse cucumbers in other parts of the world, the apparently high levels of fruit losses in greenhouses in Oman might be due to one of several reasons. Firstly, environmental conditions, particularly high humidity and low temperatures (<25°C), have been considered as important factors in increasing severity of fruit rot (Yunis *et al*., 1990). These conditions are prevalent in greenhouses in Oman, especially during the period from October to March. Secondly, findings from the present study provide evidence for several pathogenic fungi being associated with fruit rot of cucumber in Oman, suggesting possible synergy between pathogens. Co-infection of the same fruits with more than one pathogen, especially *Alternaria* and *Fusarium* spp., was observed in this study.

Different studies have emphasized the effect of growing seasons on different soilborne and foliar diseases of cucumber (Yunis *et al*., 1990; Al-Sa'di *et al*., 2007; Al-Sadi *et al*., 2011b). However, no significant differences were observed in the incidence of the fruit rot between different growing seasons. This may be related to the fact that the range of temperature inside greenhouses (18–30°C) is narrow compared to the wide variations in the ambient temperature in Oman (18–50°C). This could have resulted in a constant level of disease throughout the year.

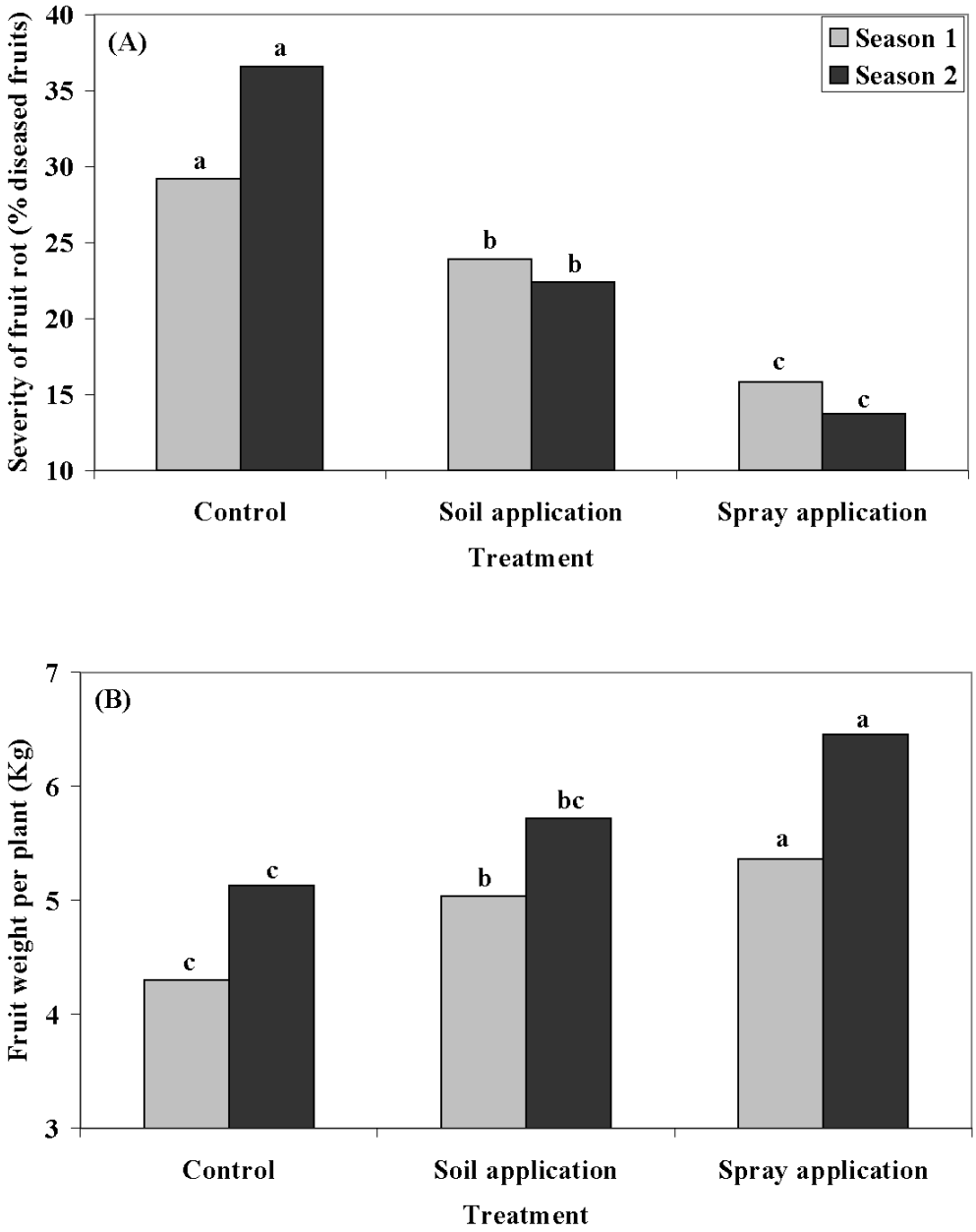

Figure 2 Mean percent diseased fruit (A) and mean fruit yields (B) of winter-season greenhouse cucumber crops that were either untreated or treated with foliar or soil applications of carbendazim. Means within the same category (i.e. season 1 or 2), and which have the same letter are not significantly different $P<0.05$).

Application of carbendazim was found to significantly reduce fruit rot symptoms and increase yield of cucumber plants. However, foliar application of the fungicide (avg. 55% disease reduction) was found to be superior to soil drenching (29%) for management of fruit rot of immature cucumbers. Direct foliar application may result in more direct effects whilst soil applications may be lost due to leaching, adsorption or biodegradation (Sharma and Awasthi, 1997; Al-Sa'di *et al*., 2008). Although carbendazim is a commonly used fungicide for the management of different diseases including cu-

cumber fruit rot (Bao *et al*., 1992; Moyano *et al*., 2004; Sun *et al*., 2010), this is the first reported study to compare efficacy of soil drenching and foliar application of carbendazim in the management of cucumber fruit rot.

The fact that carbendazim only offered 55% disease control might imply that the fungicide is not effective against all causal agents of fruit rot of cucumber. Previous studies have provided evidence that carbendazim is effective for diseases caused by *Fusarium* spp. and *Botrytis* spp. (Yunis *et al*., 1991; Thomidis *et al*., 2009), which were found associated with fruit rot of cucumber in Oman. However, carbendazim is not efficacious in the management of diseases caused by *A. alternata* (Nallathambi *et al*., 2009; Thomidis *et al*., 2009), one of the most common fungi associated with fruit rot symptoms in Oman. Another explanation might be the development of fungicide resistance among *Fusarium* (Chen *et al*., 2007; Chung *et al*., 2009; Luo *et al*., 2009) or *Botrytis* species (Elad *et al*., 1992; Kim *et al*., 2001; Moyano *et al*., 2004; Sun *et al*., 2010; Al-Sadi *et al*., 2011a), or that other factors such as nutritional deficiencies also contribute to fruit rot symptoms of greenhouse cucumber in Oman (Elad *et al*., 1993a). Future studies may therefore be required to evaluate resistance of *Fusarium* and *Botrytis* spp. to carbendazim in Oman, and to evaluate the possible role of nutrient deficiencies in increasing fruit rot of cucumber in the country.

The present study is the first report of the association of *Co. cassiicola* and *F. equiseti* with fruit rot of immature greenhouse cucumbers. We also report the association of three new pathogenic fungi with fruit rot of immature cucumbers in Oman. Future studies should consider evaluating the effect of abiotic factors on severity of the fruit rot and the efficacy of other fungicides in management of the disease to avoid potential future resistance to carbendazim, which is an important active ingredient.

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