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Iprodione and a mixture of Carbendazim and Diethofencarb fungicides resistance in Botrytis cinerea population collected from Palestinian vegetable greenhouses

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Abstract: The acquired resistance of Botrytis cinerea isolates, the causal agent of grey mould disease to synthetic fungicides Iprodione (Rovral®) and a mixture of Carbendazim and Diethofencarb (Resec®) was evaluated on 84 isolates collected from vegetable greenhouses throughout the West Bank. The fungicide-resistant isolates were evaluated in vitro in terms of mycelial growth rate and conidial germination, in vivo as grey mould lesion growth rate on detached bean leaves, and on grey mould disease severity on bean whole plants. The effective concentrations (EC_{50}) of the fungicides varied in vitro and in vivo. The Iprodione EC_{50} (µg ml^{-1}) on the isolates in vitro (mycelial growth rate and conidial germination) were 0.8 and 4.31 respectively, and in vivo (grey mould lesion growth rate and disease severity) were 16.7 and 336.4, respectively. However, the Carbendazim and Diethofencarb EC_{50} (µg ml^{-1}) on the isolates in vitro were 1.19 and 10.43 respectively, and in vivo (grey mould lesion growth rate and disease severity) were 16.4 and 447.2, respectively. The Iprodione resistance was found in 49.4% of the isolates; Carbendazim and Diethofencarb resistance on the other hand was evident in 44.4% of the isolates, and resistance for both fungicides occurred in 35.7% of the isolates. The highest percentages of resistance were found in the Northern agricultural areas (Jenin, Nablus, Tulkarem and Qalqelia greenhouses) cultivated traditionally with cucumbers and tomatoes.
Keywords: fungicide resistance, Iprodione, Carbendazim and Diethofencarb, Botrytis cinerea.

المناعة المكتسبة عند المسبب لمرض العفن الرمادي (فطر Botrytis cinerea) للمبيدي Iprodione و Carbendazim and Diethofencarb داخل البيوت البلاستيكية المزروعة بالخضروات في الضفة الغربية

ملخص: تم دراسة وتقييم المناعة المكتسبة عند فطر Botrytis cinerea للمبيدين الأكثر استخداماً Iprodione و Carbendazim & Diethofencarb داخل أربع وثمانون من البيوت البلاستيكية المزروعة بالخضروات (البندورة، الخيار، الفاصوليا، الكوسا، الباذنجان، والفراولة) والمنتشرة في الضفة الغربية. تم جمع عزلات الفطر من البيوت البلاستيكية يستخدم فيهن المبيدين خلال فترة وبائية المرض. وتم تقييم اثر المبيدين على مستويين؛ المستوي الأول: اثر المبيدات على معدل نمو الفطر على الوسط الغذائي الممزوج بتركيزات مختلفة ونسبة أنبات الابواغ، والمستوي الثاني: اثر المبيدات على معدل تطور المرض على أوراق الفاصوليا وشدة الإصابة على نباتات الفاصوليا. وبعد

تقييم التركيز الفاعل (EC_{50}) لكلا المبيدين على المستويين لجميع عزلات الفطر؛ تبين أ ن التركيز الفاعل ($\mu g\ ml^{-1}$) لمبيد Iprodione على معدل نمو الفطر ونسبة أنبات الابواغ هو 0.8 و 4.31 على التوالي ، وعلى معدل تطور المرض على الأوراق وشدة الإصابة على النباتات هو 16.7 و336.4 على التوالي. بينما التركيز الفاعل ($\mu g\ ml^{-1}$) للمبيدCarbendazim & Diethofencarb على معدل نمو الفطر ونسبة أنبات الابواغ هو 1.19 و 10.43 على التوالي ، وعلى معدل تطور المرض على الأوراق وشدة الإصابة على النباتات هو 16.4 و 447.2 على التوالي. وبعد التحليل الإحصائي والتوزيع التكراري وتطبيق المعايير على جميع العزلات تبين أ ن نسبة العزلات التي تطورت فيها مناعة للمبيد Iprodione هي 49.4% وللمبيد Carbendazim and Diethofencarb كانت 44.4% ، بينما نسبة العزلات التي تطورت فيها المناعة لكلا المبيدين 35.7%. ووجد أ ن أعلى نسبة لتطور المناعة في العزلات التي جمعت من البيوت البلاستيكية من شمال الضفة الغربية (جنين، نابلس، طولكرم، قلقيلية) والتي كانت مزروعة بالبندورة والخيار.

Keywords: fungicide resistance, Iprodione, Carbendazim and Diethofencarb, *Botrytis cinerea*.

Introduction

Botryis cinerea Pers., the causal agent of grey mould, is a major pathogenic fungus of a number of crops worldwide (Prins et al., 2000). Chemical control of the disease often depends on frequent fungicide applications, utilizing compounds representing ca. 10 chemical classes, and targeting specific cellular processes in fungi (e.g. respiration, sterol biosynthesis etc.). Unfortunately, B. cinerea is a classical "high-risk" pathogen (Brent & Hollomon, 1998). The occurrence of resistance to different fungicides with specific mode of action, as well as residues of fungicides in food have been frequently reported (Yourman and Jeffers, 1999 ; Yourman et al., 2000; Kim et al., 2001; Dianez et al., 2002; Baroffio et al., 2003; Moyano et al., 2004; Zhang et al., 2007; Keith and Hollomon, 2007; Yoon et al., 2008; Kim et al., 2009; Bardas et al., 2010 ; Leroch et al., 2011 ; Yin et al., 2011.; Billard et al., 2012; Bernard et al., 2013).

In Palestine, grey mould is one of the most serious diseases of greenhouse vegetable crops such as cucumber, tomato, eggplant, squash, pepper and

beans which cover a cultivated area of approximately 2506 hectare. Chemical control of the disease was traditionally achieved by a number of fungicides applied every two weeks, from November to April. Various fungicides such as Iprodione (Rovral®) and a mixture of Carbendazim and Diethofencarb (Resec®) have been intensively used during growing seasons for many years. The unsatisfactory grey mould control and usefulness of these fungicides have been lately notified by vegetable growers from different areas in the West Bank. The development of resistance by B. cinerea to a mixture of Carbendazim and Diethofencarb in the field was reported earlier by Leroux et al., 1999; Yourman and Jeffers, 1999 and Kim et al., 2009. The resistance of B. cinerea to a mixture of Carbendazim and Diethofencarb, and Iprodione was documented in cucumber greenhouses (Elad, 1992), vegetable greenhouse crops (Moyano et al., 2004), and strawberry in Spain (Vallejo et al., 2003). The objective of this study was to evaluate the level of resistance of Botrytis cinerea isolates collected from vegetable greenhouses in various agricultural areas of the West Bank to Iprodione (Rovral®) and a mixture of Carbendazim and Diethofencarb (Resec®) fungicides.

Materials and Methods

Collection of B. cinerea isolates

Grey mould infected plants were collected in January and March 2011, from eighty four greenhouses cultivated with various vegetable crops (cucumber, tomato, eggplant, squash, bean, and gourd) throughout the West Bank; plants in these greenhouses were continuously sprayed with Rovral® and Resec® according to a grower questionnaire distributed previously. Plant parts including fruits, stems, flowers, and leaves exhibiting symptoms of grey mould were detached from plants and placed in clean plastic bags and

stored for day in the refrigerator before sampling. A small piece of infected plant part was surface sterilized by 1% sodium hypochlorite for 3 minutes, washed three times with sterilized distilled water and placed in the center of a 90mm diameter Petri plate containing potato dextrose agar (PDA) amended with 500 mg l^{-1} chloramphenicol (10 ml PDA medium per plate). Petri plates were then incubated at 22 °C for 4 days. Mycelium from the edge of growing fungal colonies was subcultured on three Petri plates containing PDA for each fungal isolate and incubated again at 22 °C. After 10 days, conidia were harvested from plates by pipetting 5 ml sterilized distilled water in plates, and gently scraping colonies with sterile glass rods. Conidial suspensions obtained were filtered through a double layer of sterilized muslin membrane to remove all traces of mycelium and placed into sterile glass tubes. Conidial suspensions (200µl) were seeded on 90-mm PDA Petri plates and incubated at 22ºC. After 2 days, singly germinated conidia were removed using a fine sterile needle and transferred to a new PDA plates. Inoculated plates were incubated and maintained as mentioned above. Single conidial colonies obtained were used later in the fungicides experiments.

Effect of fungicides on Botrytis cinerea isolates

Fungicides most commonly used in local commercial greenhouses were Iprodione (Rovral®, SC 500 gl^{-1} active ingredient (a.i), Rhone Poulenc, France), Dicarboximide class and a mixture of Carbendazim and Diethofencarb (Resec® , SC 250 g l^{-1} a.i, for each, Sumitomo Co. Ltd.).

Fungicides efficiency against B. cinerea isolates was evaluated in vitro as mycelial growth rate and conidial germination, and in vivo as grey mould lesion growth rate on detached bean leaves, and on grey mould disease severity on bean whole plants.

Iprodione and a mixture of Carbendazim and Diethofencarb

In vitro studies

Mycelial growth rate

The effect of fungicides on B. cinerea isolates was carried out in vitro using defined minimal medium, which was modified from the Difco Czapek-Dox recipe, to avoid possible interaction of fungicides with complex growth media components (Yourman and Jeffers, 1999). The medium (1 liter) contained 12g agar, 10g glucose, 3g $NaNO_3$, 1g KH_2PO_4, 1g K_2HPO_4, 0.5g $MgSO_4.7H_2O$, 0.5g KCl, and 0.01g $FeSO_4.7H_2O$ and 0.5g chloramphencol; the final pH was 6.6. Erlenmeyer flasks (1L), each containing a standard volume of the mentioned medium were placed on hot plate with magnetic stirrer to dissolve and homogenize the components; flasks were then autoclaved and allowed to cool down to 55-60^{o}C. Appropriate volumes of both fungicides stock solutions (1000 µg ml^{-1}, a.i of each) were prepared in distilled water and added to the growth media to give final concentrations of 0.2, 0.5, 1, 2 and 3 µg ml^{-1} a.i of each fungicide. Growth media (14 ml) were dispensed into each Petri plates (90 mm diameter). The experimental design was completely randomized (CRD) with five Petri plates for each fungicide concentration and fungal isolate. Petri plates were then inoculated with 5 mm mycelium disks from 5 day-old cultures of the B. cinerea isolates. Plates were then incubated in growth chamber at 22 ^{o}C. Colony diameters were measured after 24 and 72 h and the mycelium growth rate (MGR, cm^2 day^{-1}) was calculated using the following equation (Barakat and Al-Masri, 2005):

$$R = \{(D/2)^2 * л - (d/2)^2 * л \}/T$$

where R- mycelium growth rate, D - average diameter of colony (cm) after 72 h, d - average diameter of colony (cm) after 24 h, л - 3.14, and T- time of incubation (day).

The experiment was repeated as described above and the means of MGR of all isolates of both experiments were correlated with fungicides concentrations. This was used to calculate linear regression to identify the fungicides effective concentration which reduced the growth of B. cinerea isolates by 50% (EC_{50}).

Germination assays

In vitro germination of B. cinerea, conidia were evaluated in 24 wells microtiter sterilized plates (Greiner bio-one, Germany). Conidia were harvested from 10 days old B. cinerea cultures growing on PDA medium and incubated at 22^{o}C, by scraping the mycelium with a glass rod using 10 ml sterile DW. Harvested conidia were filtered using double layers of sterilized muslin membrane to remove all traces of mycelia and placed into sterile tubes. Conidial suspensions were then centrifuged at 2308 xg (RCF) for 3 minutes and precipitated pellets were washed three times by 10 ml DW. Conidial concentrations of all B. cinerea isolates were then adjusted using the haemocytometer to 2×10^4 conidia ml^{-1}. The germination assays used the fungicides Iprodione at the concentrations 0, 0.5, 1, 1.5, 2, 3, 4, 5, 7, 9, and 11 µg ml^{-1} (a.i) and Carbendazim and Diethofencarb at 0, 0.5, 1, 1.5, 2, 3, 4, 5, 7, 9, 11, 13, 16, and 19 µg ml^{-1} (a.i). The experimental design was completely randomized using three wells (Replicates) of a 24 wells microtiter sterilized plates for each fungicide concentration and fungal isolate. In each well, 480 µl of each fungicide concentration and 20µl of conidial suspension of each B. cinerea isolate were added. Plates were then incubated for 24 hours in growth chamber at 22°C. Germination in wells was assayed by inverted microscope at 20X (Olympus CKX41, Japan). Percentage of germinated conidia for each B. cinerea isolate and fungicide concentration was determined of the reading five microscope fields and

three wells per treatment. The experiment was repeated as described above and the means of germination percentages of both experiments were correlated with fungicides concentrations. This was used to calculate linear regressions to identify the EC_{50} of both fungicides for all B. cinerea isolates.

Effect of fungicides on Botrytis cinerea isolates in vivo

Disease severity on detached bean leaves

Two week-old bean seedlings (cv. Hilda) were transplanted in 15 x 30 cm pots in a glasshouse. The planting medium consisted of a mixture of peat moss and perlite (2:1, v:v). Plants were irrigated and a 20:20:20 NPK fertilizer was added twice a week. At flowering (40 days after planting), healthy young leaves were collected and placed in the bottom of plastic boxes (40 x 25 x 15cm), on a double layer of a plastic mesh platform. The plastic mesh was placed on a sterilized wet paper towel to maintain high humidity in the box. Each box contained six leaves; leaves were sprayed with Iprodione or Carbendazim and Diethofencarb using a micro sprayer, and the fungicide concentrations used were 0, 2, 4, 8, 12, 16, 20, 40, 100, 200, and 400 µg ml^{-1} water (a.i). After the treatment solutions were absorbed by leaves, the detached leaves were inoculated by placing on top a 5-mm diameter agar disc taken from 6-day-old PDA cultures of B. cinerea isolates. The boxes were covered by a transparent plastic film and incubated at 22ºC under a 12 h photoperiod. Evaluation of disease development on detached leaves was carried out by measuring the rotting leaf area (cm^2 day^{-1}) around the inoculum disks after 96 hours of incubation. The experimental design was a completely randomized design, where each six leaflets of bean plants were considered as six replicates for each concentration and isolate. The experiment was repeated as described above and the means of lesion area of both experiments were correlated with fungicides concentrations.

This was used to calculate linear regressions to identify the EC_{50} of both fungicides for all B. cinerea isolates.

Disease severity on bean plants

Five 40-day old bean plants were sprayed until runoff with 0, 100, 200, 300, 400, 500 and 600 μg ml^{-1} (a. i) of Iprodione and 0, 100, 200, 300, 400, 500, 600 and 700 μg ml^{-1} (a. i) of Carbendazim and Diethofencarb against each of the B. cinerea isolates (Bc1, Bc29 and Bc43). After 3 hours, the solutions were absorbed by plants, and inoculated with 20ml of conidial suspension ($2x10^5$ colony-forming unit (CFU)) in deionized sterile water containing 2g l^{-1} glucose and 1g l^{-1} potassium dihydrogen phosphate (KH_2PO_4). Plants were then covered with transparent plastic bags, and incubated in a growth chamber at 22ºC, under a photoperiod of 12h. The severity of grey mould was evaluated by estimation of the percentage of leaf mould coverage after 10 days of incubation. The experimental design was completely randomized, where five bean plants were considered as five replicates for each fungicide concentration and B. cinerea isolate. The experiment was repeated and from the means of both trials, linear regression was calculated between the disease severity and the concentration of both fungicides. EC_{50} of both fungicides was calculated and used later to identify the isolates for resistance or tolerance. The experiments were repeated twice at concentrations of EC_{50} of both fungicides by using five seedlings (replicate) for 84 isolates. The bean seedling were treated and inoculated in the same manner described previously.

Statistical analysis

The EC_{50} values (effective fungicide concentration causing 50% reduction in mycelial growth rate, conidial germination, grey mould lesion growth rate, and disease severity) were estimated statistically for both fungicides

and each B. cinerea isolates using linear regression of the means for the two experiments. The frequency distribution of EC_{50} values of both fungicides and fungal isolates in all experiments was used to identify the resistance /sensitivity of fungal isolates. In addition, the data of mycelial growth rate, conidial germination, lesion growth rate, and grey mould disease severity were statistically analyzed by using one way ANOVA repeated measurement and Fisher LSD test. Probit analysis (statistical software Sigma Stat® 2.0 program, SPSS Inc., USA) was used.

Results

Botrytis cinerea isolates

During the epidemics of grey mould disease in January and March 2011, about 84 isolates of B. cinerea were collected from eighty four greenhouses in seven geographical areas (Hebron, Jericho, Jordan valley, Jeninn, Nablus, Tulkarem, and Qalqelia) cultivated with various vegetable crops (cucumber, tomato, eggplant, squash, bean, and gourd) (Table 1). B. cinerea isolates were collected from greenhouses exposed to severe grey mould attack in spite of regular applications of Iprodione and/or a mixture of Carbendazim and Diethofencarb. Sixty seven isolates were recovered from greenhouses cultivated with cucumber, tomato, gourd and squash and located in northern West Bank.

Effect of fungicides on Botrytis cinerea isolates In vitro

The fungicide Iprodione was able to reduce the mycelium growth (MGR) of B. cinerea isolates grown on amended medium at the concentrations (0.2- 2 µg ml^{-1}) compared to the control (Fig. 1A). MGR values ranged from 0.67 $cm^{-2}d^{-1}$ for B.c1 (sensitive isolate) to 2.99 $cm^{-2}d^{-1}$ for B.c 98 (resistant isolate), and MGR reduction was positively correlated with increasing fungicide concentrations (r^2=0.60). The values of effective concentration

(EC_{50}) of Iprodione against mycelium growth of B. cinerea isolates ranged from 0.4- >2.8 µg ml^{-1} (Fig. 3A), and the mean was 0.8 µg ml^{-1} (Table 2). According to EC_{50} values obtained, 64 % of B. cinerea isolates were resistant to Iprodione as far as mycelium growth rate was in vitro.

Iprodione significantly reduced as well conidial germination of B. cinerea isolates at concentrations (3-11µg ml^{-1}), and reduction was positively correlated with increasing fungicide concentrations (r^2=0.83) (Fig.1 B). Germination was completely inhibited at concentrations ≥ 9 µg ml^{-1} for most B.cinerea isolates. The values of effective concentrations (EC_{50}) of Iprodione against conidial germination ranged from 2- >5 µg ml^{-1}, and the mean was 4.31 µg ml^{-1} (Fig. 3B & Table 2). According to the EC_{50} values obtained, 51 % of B.cinerea isolates were resistant to Iprodione as far as coinidia germination percentages were in vitro.

The fungicide mixture of Carbendazim and Diethofencarb (Carb & Dieth) was able to reduce the mycelium growth (MGR) of B. cinerea isolates grown on amended medium at the concentrations (0.2- 3µg ml^{-1}) compared to the controls (Fig. 2A). MGR values ranged from 0.2 $cm^{-2}d^{-1}$ for Bc 17 (sensitive isolate) to 13.2 $cm^{-2}d^{-1}$ for Bc98 (resistant isolate); MGR reduction was positively correlated with increasing fungicide concentrations (r^2=0.62). The values of effective concentration (EC_{50}) of Carb & Dieth against mycelium growth of B. cinerea isolates ranged from 1.8- 6 µg ml^{-1} (Fig. 4A), and the mean was 1.19 µg ml^{-1} (Table 2). According to EC_{50} values obtained, 63 % of B. cinerea isolates were resistant to Carb & Dieth as far as mycelium growth rate was in vitro.

Carb & Dieth significantly reduced as well conidial germination of B. cinerea isolates at concentrations (4-18 µg ml^{-1}), and reduction was positively correlated with increasing fungicide concentrations (r^2=0.98)

Iprodione and a mixture of Carbendazim and Diethofencarb

(Fig.2 B). Germination was completely inhibited at concentrations ≥ 18 µg ml^{-1} for most B.cinerea isolates. The values of effective concentrations (EC_{50}) of Carb & Dieth against conidial germination ranged from 8-12 µg ml^{-1}, and the mean was 10.34 µg ml^{-1} (Fig. 4B). According to the EC_{50} values obtained, 47 % of B.cinerea isolates were resistant to Carb & Dieth as far as coinidia germination percentages were in vitro.

In vivo

The fungicide Iprodione significantly reduced the grey mould lesion growth (LGR) on detached bean leaves at the concentrations (2-400 µg ml^{-1}) compared to the controls (Fig.1C). LGR values ranged from 0.22 $cm^{-2}d^{-1}$ for B.c 101 to 2.96 $cm^{-2}d^{-1}$ for B.c 27, and reduction was positively correlated with increasing fungicide concentrations (r^2=0.84). The values of effective concentrations (EC_{50}) of Iprodione against grey mould lesions growth ranged from 5.5- 30.2 µg ml^{-1}(Fig.3C), and the mean was 16.7 µg ml^{-1}. According to the EC_{50} values obtained, 51 % of B.cinerea isolates were resistant to Iprodione as far as grey mould lesion growth was in vivo. In addition, Iprodione significantly reduced the disease severity (%) on bean plants at the concentrations (200- 600 µg ml^{-1}) compared to the controls. Disease severity reduction was positively correlated with increasing fungicide concentrations (r^2=0.97). The mean value of effective concentration (EC_{50}) of Iprodione against grey mould disease severity on bean plants was 336.4 µg ml^{-1}; values of EC_{50} ranged, however, from 182 to ≤683 µg ml^{-1}. According to the EC_{50} values obtained, 52% of B. cinerea isolates were resistant to Iprodione as far as grey mould disease severity was on bean plants.

The fungicide Carb & Dieth significantly reduced the grey mould lesion growth (LGR) on detached bean leaves at the concentrations (2-400 µg ml^{-1})

compared to the controls (Fig. 1 C). LGR values ranged from 0.11 $cm^{-2}d^{-1}$ for B.c 3 to 3.43 $cm^{-2}d^{-1}$ for B.c 52, and reduction was positively correlated with increasing fungicide concentrations (r^2=0.72). The values of effective concentrations (EC_{50}) of Carb & Dieth against grey mould lesions growth was ranged from 6.9- 39.1 µg ml^{-1}(Fig.2C), and the mean was 16.4 µg ml^{-1}. According to the EC_{50} values obtained, 47% of B.cinerea isolates were resistant to Carb & Dieth as far as grey mould lesion growth was in vivo. In addition, Carb & Dieth significantly reduced disease severity (%) on bean plants at the concentrations (400- 700 µg ml^{-1}) compared to the controls. Disease severity reduction was positively correlated with increasing fungicide concentrations (r^2=0.91). The value of effective concentration (EC_{50}) of Carb & Dieth against grey mould disease severity on bean plants was 447.2 µg ml^{-1}; the values of EC_{50} ranged however, from 182 to ≤849 µg ml^{-1}. According to the EC_{50} values obtained, 44% of B.cinerea isolates were resistant to Carb & Dieth as far as grey mould disease severity was on bean plants.

Fungicides resistance levels in B.cinerea isolates

The resistant isolate to fungicides was identified if its data seemed to be resistance in both in vitro and in vivo as mentioned previously. Iprodione resistance was found in 49.4% of the isolates, Carb & Dieth in 44.4% of the isolates, and resistance to both fungicides was found in 35.7% of the isolates (Table 3 and 4). The highest resistance (%) was found in the Northern area of the West Bank (Jenin and Nablus greenhouses) and the lowest was found in the Southern (Hebron greenhouses). The mean resistance (%) to Iprodione, Carb & Dieth, and to both was 23.3, 21.4, and 19% respectively, in Jenin and Nablus greenhouses. Resistance (%) in the Tulkarem and Qalqelia isolates to Iprodione, Carb & Dieth, and to both was 13%, 14.6%,

and 10.7%, respectively. In terms of cultivation, the highest resistance were recovered from greenhouses cultivated with cucumber, tomato and squash; Iprodione resistance was 20.4, 15.6, and 6, and Carb & Dieth resistance was 24, 8.4, and 7.2, respectively, mainly in the Northern area.

Discussion

B. cinerea is a classical "high-risk" pathogen for development of resistance to selective fungicides due to its short generation time and abundance of sporulation (Jarvis, 1992). The phenomenon of failure to fully control grey mould disease with Iprodione and a mixture of Carbendazim and Diethofencarb in protected vegetables in the West Bank has been lately observed by farmers and extension agents especially in areas with intensive production system. Resistance in the population of B. cinerea isolates to Iprodione and a mixture of Carb & Dieth was evaluated. Iprodione resistance was found in 49.4% of the inspected isolates, Carb & Dieth resistance in 44.4% of the isolates, and resistance for both fungicides was found in 35.7% of the isolates. Similar results were found in some populations of B. cinerea to Iprodione and Dicarboximide in protected crops in the Mediterranean (Katan, 1982 and Petsikos- Panayotarou et al., 2003). Furthermore, the resistance of B. cinerea isolates to Iprodione and a mixture of Carbendazim Diethofencarb was found in cucumber greenhouses (Elad, 1992), in Spanish greenhouses (Raposo et al., 1996), in B. cinerea population collected from infected plants of strawberry, tomato and cucumber (Kim et al., 2001), from strawberry fields in Spain (Vallejo et al., 2003) and from vegetable greenhouses crops (Moyano et al., 2004).

Resistance to Iprodione (36%) and to Carb & Dieth isolates (32.4%) was found mainly in greenhouses cultivated with cucumber and tomato. In addition, high resistance to both fungicides was observed in the isolates

collected from Jenin, Nablus, Tulkarem and Qalqelia in the North. This may be due to the frequent application of both fungicides during the growing season. The total number of foliar applications during growing season of cucumber and tomato was estimated to be 8 and 12 times, respectively. Similar results were observed in greenhouses of South Europe and Israel in which the total number of foliar applications was 8-10 applications during the period of grey mold outbreaks (Elad, 1992; Petsikos- Panayotarou et al., 2003).

The EC_{50} values of Iprodione and Carb & Dieth in vitro as MGR of B. cinerea inspected isolates was 0.8 and 1.19 µg ml^{-1} and in vivo on bean leaves was 16.7 and 16.4 µg ml^{-1}, respectively. Similar results observed by Petsikos- Panayotarou et al. (2003) which found that the EC_{50} values in vitro were 1.35 and 1.96 µg ml^{-1} and in vivo were 4.37 and 9.86µg ml^{-1}, respectively prior treatment on tomato leaves and increased after repeated applications of fungicides. The EC_{50} of local population was higher than other populations because of high applications of fungicides and other ecological factors. In addition, the variation between EC_{50} values in vitro as mycelium growth and conidial germination were observed in inspected isolates (Yourman et al., 2000).

The fungicides at lower concentrations highly affected the mycelial growth of the isolates more than spore germination, lesion development on bean leaves and disease severity on bean plants did. The Iprodione EC_{50} (µg ml^{-1}) values were 0.8, 4.3, 16.7, 336.4, and Carb & Dieth EC_{50} were 1.2, 10.4, 16.4, 447.2 on MGR, germination, grey mould lesion and disease severity, respectively. This variation may be due to the sensitivity of mycelium and conidia of the fungus, and the plant tissues to the fungicides. Furthermore, the mean of EC_{50} values of both fungicides on local isolates varied on the

other isolates in some related investigations; Petsikos- Panayotarou et al. (2003) reported that Iprodione EC_{50} value was 1.35 µg ml^{-1}, and Carb & Dieth EC_{50} value 1.96 µg ml^{-1} in vitro.

Several investigators attempted to explain the mechanisms of acquired resistance in B. cinerea isolates (Leroux et al. 2002; Kim et al., 2009). A mixture of Carb & Dieth resistance was correlated with a single mutation in ß–tubulin gene of B. cinerea amplified with primers causing a change of glutamate to alanine at amino acid position 198 and the substitution of valine for glutamate led the resistance (Kim et al., 2009).

In conclusion, high resistance was observed in Botrytis cinerea isolates to the Iprodione and Carbendazim and Diethofencarb fungicides. The highest percentages of resistance were found in the Northern agricultural areas (Jenin, Nablus, Tulkarem and Qalqelia greenhouses) cultivated traditionally with cucumbers and tomatoes.

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Table 1 : *Botrytis cinerea* isolates collected from eighty four greenhouses during January and March 2011.

Area	No. of greenhouses	Isolates	Cultivation
Hebron	7	Bc1	Bean
		Bc2, Bc79, Bc 87	Cucumber
		Bc3, Bc4, Bc17	Tomato
Jericho and Jordan valley	10	Bc5, Bc6, Bc10, Bc11, Bc14	Egg plant
		Bc7, Bc12	Strawberry
		Bc8, Bc9, Bc13	Tomato
Jeninn and Nablus	45	Bc20, Bc25, Bc26, Bc31, Bc 65, Bc82, Bc83, Bc91, Bc92, Bc93	Gourd and Squash
		Bc27, Bc28, Bc64, Bc66, Bc72, Bc73, Bc86, Bc88	Tomato
		Bc29, Bc30, Bc32, Bc 33, Bc 34, Bc 35, Bc 36, Bc 37, Bc 38, Bc 39, Bc 40, Bc42, Bc 43, Bc 44, Bc60, Bc 61, Bc62, Bc67, Bc 68, Bc 69, Bc74, Bc80, Bc89,	Cucumber
		Bc19, Bc63, Bc78, Bc90,	Bean
Tulkarem and Qalqelia	22	Bc 45, Bc 46, Bc 47, Bc 48, Bc 49, Bc 50, Bc 52, Bc 53, Bc 54, Bc 56, Bc 57, Bc 59, Bc94, Bc95, Bc98, Bc99, Bc100	Cucumber
		Bc96, Bc101,	Bean
		Bc55, Bc102, Bc103	Tomato
Total	**84**		

Table 2. Effective fungicides concentration (EC_{50}) against mycelial growth rate (cm^2 day^{-1}), conidial germination (%), grey mould lesion growth rate (cm^2 d^{-1}), and grey mold disease severity (%), induced by *B. cinerea* isolates.

Treatments	Effective concentration EC_{50} (µg ml^{-1})	
	Iprodione	Carbendazim and Diethofencarb
Mycelial growth rate	0.8	1.19
Conidial germination	4.31	10.43
Lesion growth rate	16.7	16.4
Disease severity	336.4	447.2

Table 3. Percentages of *B. cinerea* isolates resistant to the fungicides Iprodione and the mixture of Carbendazim and Diethofencarb in the West Bank greenhouses.

Area	Iprodione	Carbendazim &Diethofencarb	Both fungicides
Hebron	4.8	2.4	1.2
Jericho and Jordan valley	8.3	6	4.8
Jenin and Nablus	23.3	21.4	19
Tulkarem and Qalqelia	13	14.6	10.7
Total	49.4	44.4	35.7

Table 4. Percentages of *B. cinerea* isolates resistant to the fungicides Iprodione and the mixture of Carbendazim and Diethofencarb in West Bank greenhouses according to cultivated crops.

Area	Iprodione					
	Cucumber	Tomato	Bean	Squash	Eggplant	Straw berry
Hebron	1.2	2.4	1.2	0	0	0
Jericho and Jordan valley	1.2	2.4	0	0	3.6	2.4
Jenin and Nablus	9.7	7.2	2.4	6	0	0
Tulkarem and Qalqelia	8.3	3.6	1.2	0	0	0
Total (%)	**20.4**	**15.6**	**4.8**	**6**	**3.6**	**2.4**
Carbendazim and Diethofencarb						
Hebron	0	0	1.2	0	0	0
Jericho and Jordan valley	2.4	2.4	0	0	2.4	0
Jenin and Nablus	12	2.4	1.2	7.2	0	0
Tulkarem and Qalqelia	9.6	3.6	0	0	0	0
Total (%)	**24**	**8.4**	**2.4**	**7.2**	**2.4**	**0**

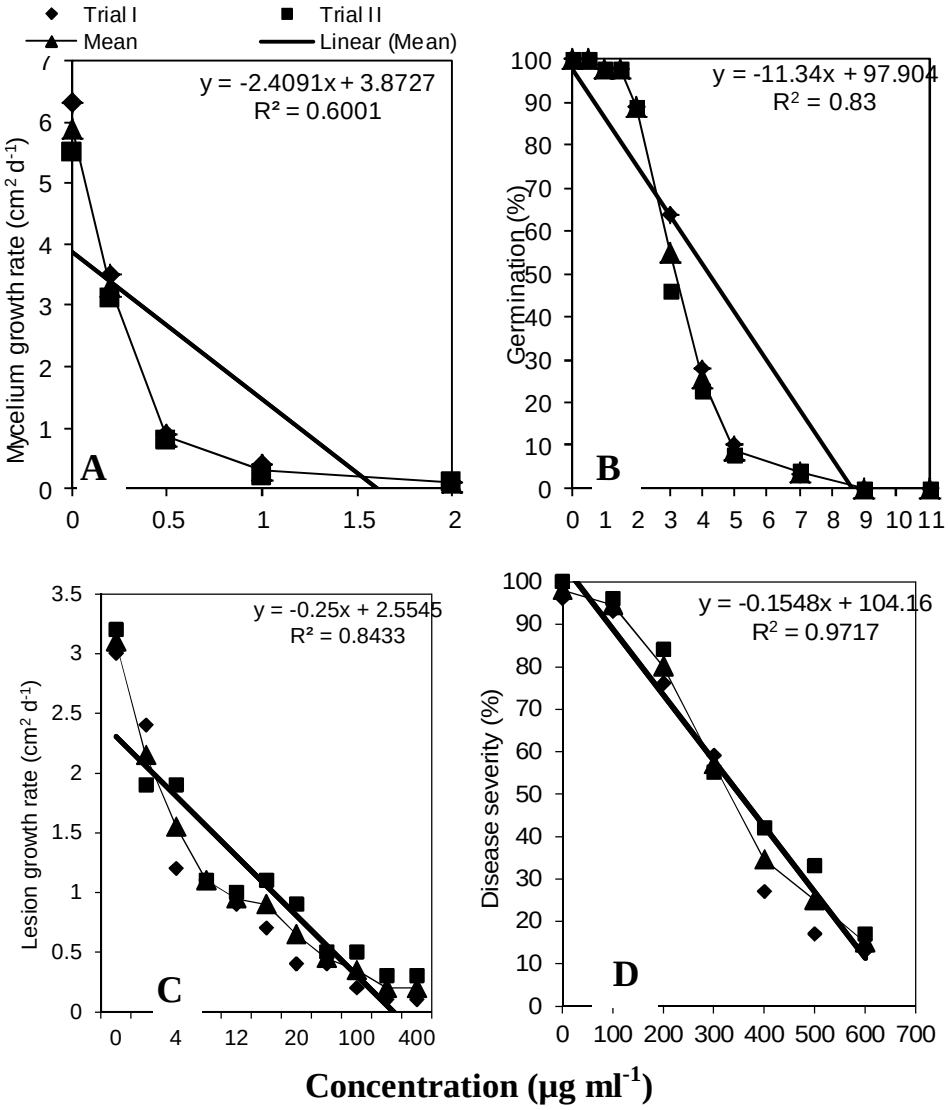

Fig.1. Effect of the fungicide Iprodione (Rovral®) on eighty four isolates of *B. cinerea*: A- mycelium growth rate (cm^2 day^{-1}), B- conidial germination (%), C- disease lesion growth rate (cm^2 d^{-1}) on detached bean leaves, and D- grey mold disease severity (%) on bean plants.

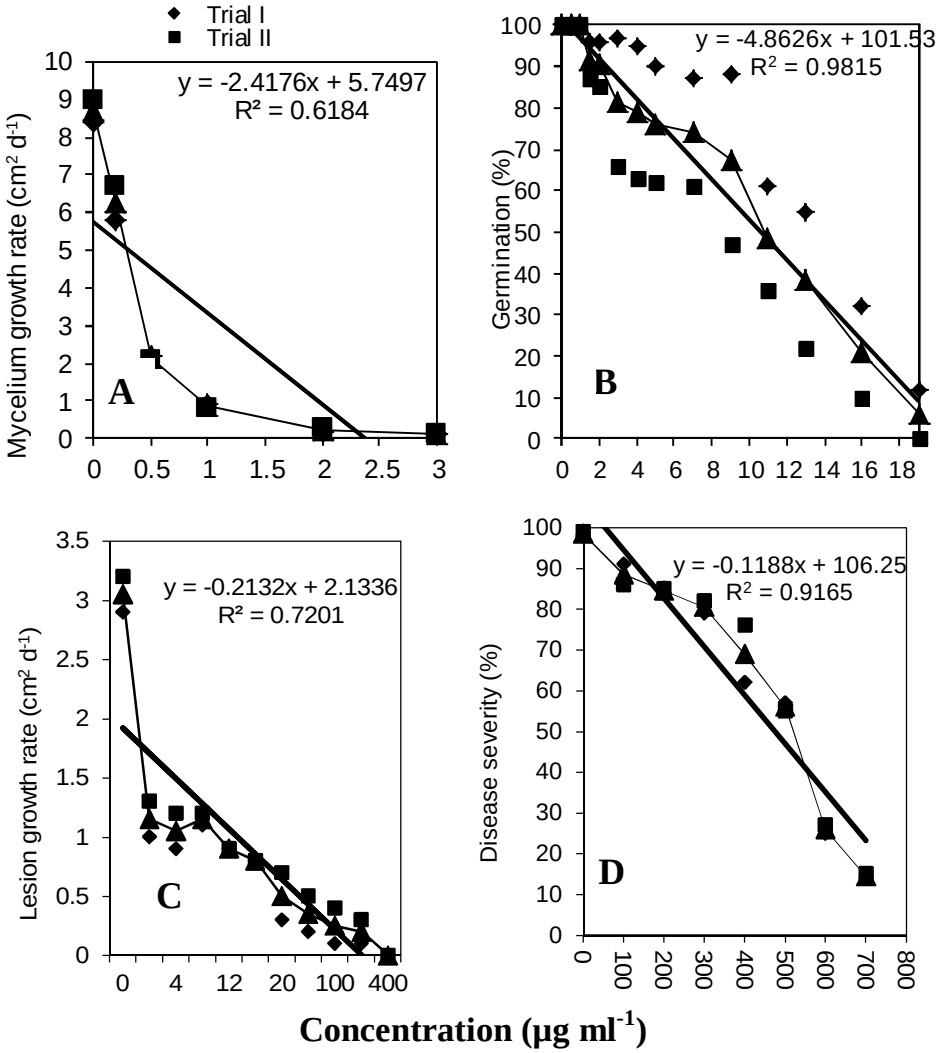

Fig.2. Effect of the fungicide mixture of Carbendazim and Diethofencarb (Resec®) on *B. cinerea*: A- mycelium growth rate (cm^2 day^{-1}), B- conidial germination (%), C- disease lesion growth rate (cm^2 d^{-1}) on detached bean leaves, and D- grey mould disease severity (%) on bean plants.

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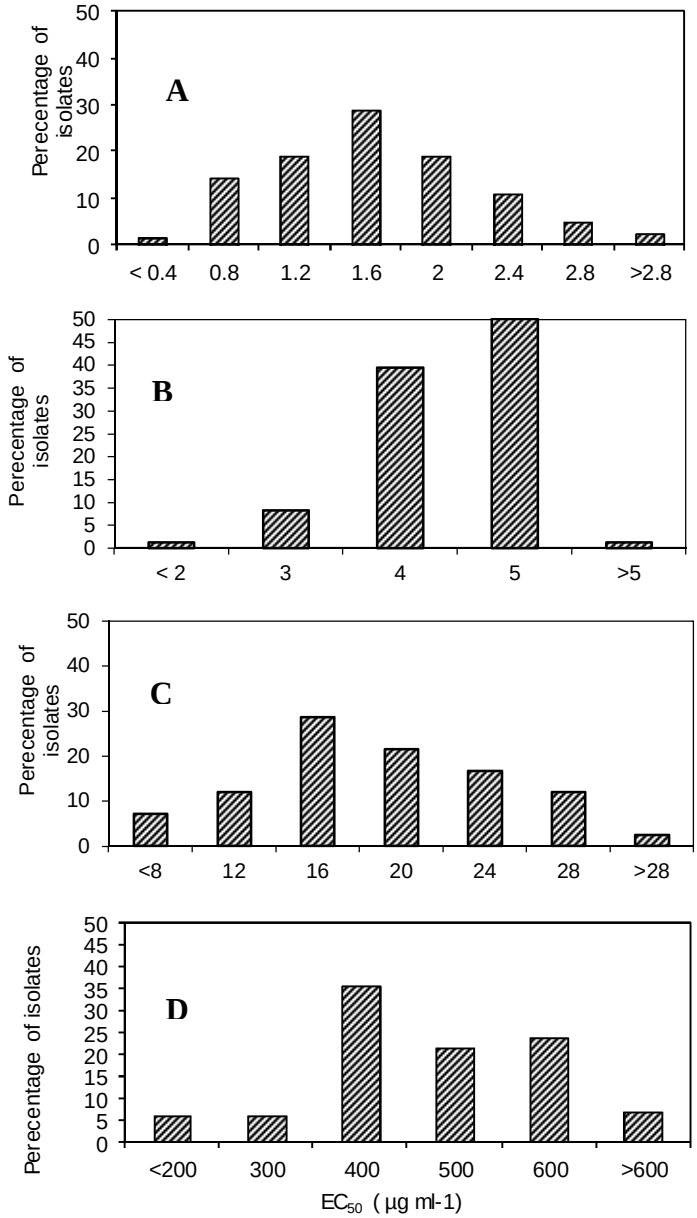

Fig.3. Frequency distribution of EC_{50} values for Iprodione determined for isolates of *B. cinerea* (%): A- mycelium growth rate (cm^2 day^{-1}), B- conidial germination (%), C- disease lesion growth rate (cm^2 d^{-1}) on detached bean leaves, and D- grey mould disease severity (%) on bean plants.

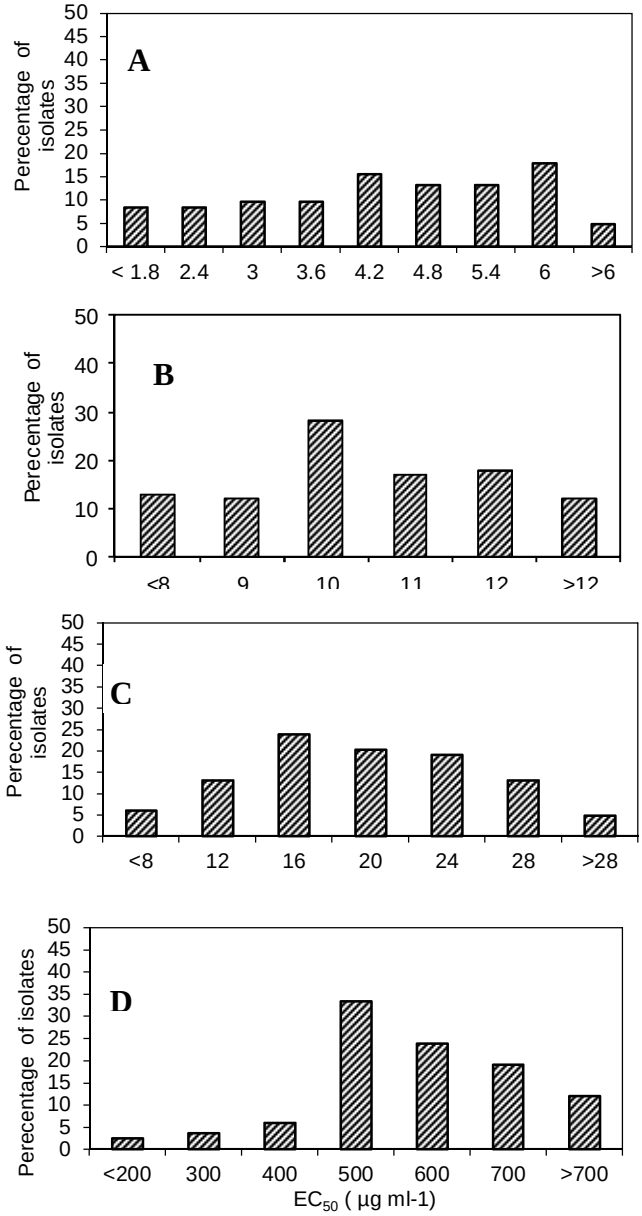

Fig.4. Frequency distribution of EC_{50} values to the mixture of Carbendazim and Diethofencarb determined for isolates of *B. cinerea* (%): A- mycelium growth rate (cm^2 day^{-1}), B- conidial germination (%), C- disease lesiongrowth rate (cm^2 d^{-1}) on detached bean leaves, and D- grey mould disease severity (%) on bean plants.