

Occurrence and etiology of *Alternaria* leaf blotch and fruit spot of apple caused by *Alternaria alternata* f. sp. *mali* on cv. Pink lady in Israel

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Abstract Severe outbreaks of *Alternaria* leaf blotch and fruit spot were recently observed in cv. Pink Lady apples in northern Israel, especially on fruit. Such severe outbreaks have not been reported from other countries. Symptoms involved cracks and rot around the calyx and external rot of the fruit body. Up to 80 % of the fruit in some orchards were affected by the disease. Microscopic examinations, fulfillment of Koch's postulates and molecular (genetic) analyses confirmed the causal agent as *Alternaria alternata* f. sp. *mali*. The incidence of *Alternaria* increased as the degree of calyx cracking increased, or if fruit were both cracked and rotted. Injecting spore suspensions into the fruit produced typical rot symptoms. Injection assays of detached fruit of eight apple cultivars showed that cvs. Pink Lady and Golden Delicious were susceptible whereas cv. Jonathan was resistant. Pink Lady and Golden Delicious produced more fruit rot as the inoculum concentration increased. Rot in all three cultivars was moderate close to the skin but more severe close to the seed locule. Aqueous extracts taken from Jonathan fruit peel inhibited germ tube elongation of *A. alternata* f. sp. *mali* in vitro. This is the first report on heavy infection of Pink Lady fruit in Israel caused by *A. alternata* f. sp. *mali*.

Keywords *Alternaria mali* · *Alternaria alternata* Apple pathotype · *Alternaria* blotch · Cripps pink · Calyx cracks · Susceptibility

Introduction

Alternaria leaf blotch and fruit spot of apple (*Malus domestica* Borkh), caused by the fungus *Alternaria alternata* f. sp. *mali* (syn. *Alternaria mali* Roberts, or *Alternaria alternata* apple pathotype) (Grove et al. 2003; Hartevelde et al. 2013; Woudenberg et al. 2015) was first identified in 1924 in the USA (Roberts 1924). *Alternaria* leaf blotch and fruit spot is one of the most important diseases of apple in Southeast Asia, including Japan, South Korea and China (Filajdic and Sutton 1992), southeastern USA (Filajdic and Sutton 1991; Filajdic and Sutton 1992) and Australia (Hartevelde et al. 2013). It was observed also in Zimbabwe (Sawamura 1990). The first report from Europe was in 1996 from Yugoslavia (Bulajic et al. 1996). In 1999 it was observed in Italy (Marschall et al. 2006), in 2006 in Turkey (Ozgonen and Karaca 2006), and in 2007 in Iran (Soleimani and Esmailzadeh 2007). *Alternaria* leaf blotch and fruit spot affects cultivars such as Golden Delicious and offspring of cv. Golden Delicious, including cvs. Indo and Gala (Filajdic and Sutton 1991; Ferree and Warrington 2003; Rotondo et al. 2012). Initial symptoms appear on the leaves (ca. 1 month after petal fall) as small circular brown spots (Filajdic and Sutton 1991; Ferree and Warrington 2003) which enlarge in zonate circular or crescent-shaped rings. Leaf infection

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can result in 60–85 % defoliation in susceptible cultivars (Filajdic and Sutton 1992; Jung 2007). Fruit symptoms are usually limited to small, corky, dark lesions, often associated with the lenticels (Ferree and Warrington 2003). It may cause soft rot, particularly when the skin has already been damaged by other means (Jung 2007) such as mechanical wounds, red mites (Filajdic et al. 1995a), apple aphids (Filajdic et al. 1995b), or cracks around the apple calyx (Stern et al. 2013; Ginzberg et al. 2014). Developing new resistant cultivars is a high priority in many apple breeding programs throughout the world (Laurens et al. 2011). Resistance against *Alternaria* blotch in apple cultivars have been reported previously (Filajdic and Sutton 1991; Abe et al. 2010). However, assays to screen for resistance were not performed with fruit, but only with leaves in the orchard, or detached leaves in the laboratory. Inconsistent results on the level of resistance of several apples cultivars were reported (Abe et al. 2010). Therefore, information on the level of resistance of apple cultivars to *Alternaria* leaf blotch and fruit spot is needed; such information is essential for developing integrated control programs of the disease (Abe et al. 2010).

Cv. Pink Lady (also known as Cripps Pink) is a cross between cvs. Lady Williams and Golden Delicious (Cripps et al. 1993). Severe outbreaks of *Alternaria* leaf blotch and fruit spot were recently observed in Pink Lady in northern Israel, especially on fruit (Gur et al. 2013). Such a phenomenon of heavy infection on fruit is rare in other countries (Jung 2007; Li et al. 2013). *Alternaria* leaf blotch and fruit spot symptoms were observed in the late 1990's in Israel on cv. Golden Delicious, mainly on leaves but also on a few fruit late in the season (Reuveni and Oppenheimer, unpublished data). Cv. Pink Lady is a high-quality fruit in many countries, including in Israel, due to its distinct pink blush color on a pale green background, crisp texture, and a high sugar:acid ratio (Mackay et al. 1994). While leaf infection and defoliation caused by *A. alternata* f. sp. *mali* is the main problem caused by this fungus in most regions of the world (Jung 2007; Li et al. 2013), it seems that fruit rot can become a major problem in Israel. Fruit infections downgrading fruit value were reported in a few apple cultivars in Japan, Italy and Australia, in heavily defoliated orchards (Rotondo et al. 2012; Hartevelde et al. 2013; Li et al. 2013).

The objectives of this study were to: (i) identify the casual agent of *Alternaria* leaf blotch and fruit spot on cv. Pink Lady apple fruit in Israel; (ii) examine the

resistance to *Alternaria* fruit spot of apple cultivars grown in Israel; and (iii) determine the relationship between fruit susceptibility to *Alternaria* fruit spot and fruit maturity.

Materials and methods

Occurrence and isolation of *Alternaria* leaf blotch and fruit spot disease in Israel

The occurrence of *A. alternata* f. sp. *mali* in leaf and fruit lesions was demonstrated by fulfilling Koch's postulates. Leaves and fruit of cv. Pink Lady infected with typical *Alternaria* leaf blotch and fruit spot symptoms were collected from three orchards in Northern Golan, Israel, and used to isolate the pathogen. Both leaves and fruit were first sprayed with 70 % ethanol. Four mm diameter leaf disks or small pieces (1–2 mm) of the fruit pericarp tissue, adjacent to lesions exhibiting *Alternaria* leaf blotch and fruit spot symptoms, were removed and placed in a 0.3 % hypochlorite solution for 30 s, washed twice with sterilized water and placed on potato dextrose agar (PDA, 39 g of Difco potato dextrose agar in 1 l of distilled water) in Petri dishes and incubated at 25 °C for 10–12 days until conidia were produced (Reuveni and Sheglov 2002a, b). Thirty single spore cultures were produced, maintained on PDA and used for identification. Color, shape and dimensions of conidia and mycelium were examined under the microscope and compared to previously described literature (Roberts 1924; Sawamura 1990). Pathogenicity of six isolates was verified by inoculating 15 healthy leaves and 15 fruit of cv. Pink Lady with a conidial suspension as described below. Leaves and fruit inoculated with sterilized water served as negative control treatments. Finally, the pathogen was re-isolated from the deliberately infected apple tissues on PDA dishes with the similar method of isolation as described above and identified.

Molecular identification of the pathogen

For molecular identification of the pathogen, DNA of four single spore isolates was extracted from artificially-inoculated fruit or leaves, using Masterpure Yeast DNA purification Kit (Epicentre, Madison, USA). The isolates were marked: PL-EZ-B-2011-1 (body rot of cv. Pink Lady fruit from the Ein-Zivan orchard), PL-SH-B-3011 (body rot of cv. Pink Lady fruit from the Sha'al orchard), PL-OR-

C (calyx end rot of cv. Pink Lady fruit from the Ortal orchard), PL-EZ-1 (infected cv. Pink Lady leaf from the Ein-Zivan orchard). Two genes, endopolygalacturonase (endoPG) and *Alternaria* major allergen (Alt a1) were selected. PCR reactions were performed as described by Harteveld et al. (2013) using PG3 forward primer 5'-TACCATGGTTCTTTCCGA-3' and PG2b reverse primer 5'-GAGAATTCRCARTCRCTCYTGRTT-3' for endoPG, and Alt-for forward primer 5'-ATGCAGTTCACCACCATCGC-3' and Alt-rev reverse primer 5'-ACGAGGGTGAYGTAGGCGTC-3' for Alt a1. PCR products of the four isolates were purified (HiYield™ Gel/PCR DNA Fragments Extraction Kit (RBC bioscience, Taipei, Taiwan)), and sequenced (Hy Laboratories Ltd., Rehovot, Israel). All sequences were checked and aligned using BioEdit v7.0.1 package (<http://www.mbio.ncsu.edu:BioEdit/bioedit.html>). One representative sequence for each gene was submitted to GenBank (Accession No. KT582776 and KT582777). The concatenated sequences of the Alt a1 and endoPG genes of the four Israeli isolates were compared according to Harteveld et al. (2013) to 51 Australian isolates, and five *Alternaria* species reference isolates (EGS 34–016, EGS 39–128, EGS 34–015, EGS 38–029 and EGS 30–033) derived from the National Centre for Biotechnology Information (NCBI, National Library of Medicine, USA. <http://www.ncbi.nlm.nih.gov>). Multiple nucleotide sequence alignments were performed using ClustalW (BioEdit v7.0.1), and a phylogenetic tree was elaborated using MEGA V. 6 software (Tamura et al. 2013). A maximum likelihood phylogeny was obtained using the best-fit procedure: Kimura-2 parameter with a discrete gamma distribution (K2 + G). Tree stability was tested by using 1000 bootstrap replications.

Additionally, the presence of the gene AMT3, that is involved in AM-toxin biosynthesis (a host-specific toxin) by *A. alternata* f. sp. *mali* (Harimoto et al. 2007) was examined in the four Israeli isolates and compared to an isolate of *A. alternata* causing core rot in cv. Red Delicious fruit (isolate Sh42, Reuveni et al. 2007). A PCR reaction was performed according to Harimoto et al. (2007), using primer pair AMT3-F (5'-GCG GAT TAG TCT TTC TGC TCT TGC-3') and AMT3-R (5'-CCC ATG TGC TAA CGT AAA CTC GC-3').

Inoculation procedure

A single-spore culture of *A. alternata* f. sp. *mali* grown on PDA as described above was maintained on PDA in

Petri plates for 10–12 days until conidia were produced (Reuveni and Sheglov 2002a, b). Isolate PL-SH-B-3011 was used for all experiments. For inoculation, the previously described assay by Reuveni and Sheglov (Reuveni and Sheglov 2002a, b) was used. Briefly, cv. Pink Lady fruit were brought from the orchard, washed with soap and water, blotted dry with a paper tissue, and allowed to dry at room temperature. Each fruit was wounded at six sites of the surface with a 2 mm diameter sterilized plastic tip to a depth of ~3 mm and inoculated by pipetting 10 µl of conidial suspension containing 5×10^5 spores ml⁻¹ into each wound site. Following inoculation, the fruit were placed on trays containing wet filter paper, covered with plastic bags to maintain high humidity and kept in a growth chamber at 25 °C, in the dark. Lesion diameter was measured after 13–14 days at each inoculation site.

Population of additional microorganisms from fruit calyx cracks and rot

Fruit of cv. Pink Lady were collected from untreated trees from the Ein-Zivan orchard in the Golan region. The fruit were divided into four categories: fruit with no visible calyx cracks or rot, fruit with minor calyx cracks and no visible rot, fruit with developed open calyx cracks and no visible rot, and fruit with calyx cracks and visible rot. Ten fruit were included in each category. Fruit were washed with soap and water, blotted dry with a paper tissue, and allowed to dry at room temperature. Two small pieces (1–2 mm) of calyx tissue around the cracks/rot region were removed from each fruit and each category (20 pieces per category), and cultured on PDA in petri dishes as described above. Six days later, the developed pathogens were examined under the microscope. For imitation of calyx rot development, ten apples with minor calyx cracks and no visible rot were washed with soap and water, sprayed with 70 % ethanol and blotted dry. Each fruit was inoculated by pipetting 10 µl of conidial suspension containing 5×10^5 spores ml⁻¹ into the calyx cracks. Following inoculation, the fruit were placed on trays containing wet filter paper, covered with a plastic bag to maintain high humidity and kept in a growth chamber at 25 °C, in the dark for 14 days. Fruit with no visible calyx cracks or rot inoculated with conidial suspensions and cracked fruit inoculated with sterilized water served as controls. Fourteen days after inoculation fruit calyxes were examined for rot development, and re-isolation was made to confirm

the pathogen identity. Both experiments were conducted twice (in 2010 and 2011).

Inoculation of fruit lenticels

In order to examine the ability of the fungus to penetrate through the fruit lenticels into the pericarp and create rot, three inoculation procedures were undertaken. Healthy and similar sized fruit of cv. Pink Lady were collected three and one month before harvest from untreated trees. The fruit were washed with soap and water, blotted dry with a paper tissue, and allowed to dry at room temperature. Five mm diameter filter paper disks immersed in conidial suspension or 5 µl conidial suspension droplets (both at 5×10^5 spores ml⁻¹) were placed on the fruit lenticels. Disks immersed in sterilized water, or sterilized water droplets served as controls. Ten disks, or droplets were placed on each of eight fruit used for each treatment. In addition, eight fruit were sprayed to runoff with the above conidial suspension. Fruit sprayed with sterilized water served as control treatment. Fruit were placed on trays containing wet filter paper, covered with plastic bags to maintain high humidity and kept in a growth chamber at 25 °C, in the dark for 13 days. Rot development was examined under the the paper disks, and around the fruit lenticels.

Differential susceptibility of fruit of apple cultivars to *A. alternata* f. sp. *mali*

The susceptibility of eight apple cultivars commonly grown in Israel to *A. alternata* f. sp. *mali* was examined in 2010. Fruit of cvs. Pink Lady, Top Red (Red Delicious), Golden Delicious, Gala, Granny Smith, Akane, Johnny and Jonathan were collected from orchards not treated with fungicides at three to five dates (22 July, 12 August, 7 September, 12 October and 8 November). On September 7th fruits of cvs. Gala, Akane and Jonathan were collected from the trees 14 days after commercial harvest. Four fruit of each cultivar were inoculated in the laboratory at each date with a conidial suspension of the fungus as previously described. Each of the four fruit was inoculated at six sites of the surface (24 inoculation sites per cultivar in total). The experiment was conducted twice (fruit were collected from two different orchards (Ein-Zivan and Ortal)). Fruit were kept in moist conditions as described above (25 °C, in the dark) for rot development. The diameter of the lesion was masured 13 days after inoculation. A cultivar was considered as

resistant if lesions were 0–5 mm in diameter. Moderately resistant and susceptible cultivars developed lesions of 5–10 mm and >10 mm, respectively. In 2012 the susceptibility of apple cvs. Pink Lady, Golden Delicious and Jonathan was examined as described above. Fruit were collected and inoculated during the growing season, at eight dates, starting at 30 days after petal fall (DAPF) (April 26, 2012) until harvest (every 20–30 days). Eight fruit for each cultivar were used for inoculation at each collection date as described above. In all experiments fruit inoculated with sterilized water served as control treatments.

Relationship between fruit susceptibility to *Alternaria* fruit spot and maturity indices

The relationship between fruit susceptibility to *Alternaria* fruit spot and several fruit maturity indices was examined. This was made in parallell to fruit inoculation described in the above section in which fruit of cvs. Pink Lady, Golden Delicious and Jonathan were inoculated in 2012. At each of the eight collection dates, 10 additional fruit of each cultivar were used for each maturity indices examination. The pH of the fruit pericarp was measured by a pH-meter (ORION 720 A (Orion Research, Boston, USA) with an FC200B electrode), in juice produced by grinding the fruit pericarp (without the peel). The total soluble solids (TSS expressed as °Brix) was measured by a digital refractometer ATAGO PAL-1 (Atago Co. Ltd., Tokyo, Japan). Fruit firmness was measured by a penetrometer (Digital firmness tester, Agro Technologie, Wolfcraft, Germany). Starch level in the fruit was measured by a standard Iodine test in which the fruit were cut in half and the cut surface of each half was dipped in an IKI solution. The cut fruit were placed for two minutes on a tray (cut surface up), the fruit were photographed, and the photo was analyzed by Amylan magic 4.2 software (FruitSens, Paris, France) to detarmin the starch index (scale of 1–10, whereas 1 = least starch hydrolysis). Visual validation was scored using a 10-point CTIFL scale (Pomme code regression de l'amidon Starch conversion chart for apples, France).

Effect of inoculum concentration on rot development

Healthy fruit of cvs. Pink Lady, Golden Delicious and Jonathan from trees not treated with fungicides were brought from the orchard one month before harvest

and washed as described above. Four conidial suspensions containing 10^3 , 10^4 , 10^5 and 10^6 spores of *A. alternata* f. sp. *mali* ml^{-1} were prepared. Each of three fruit of each cultivar was inoculated with each conidial concentration. Each fruit was wounded at six sites as described above, and 10 μl of the appropriate conidial suspension were pipetted into each wound site. Fruit inoculated with sterilized water served as untreated controls. The fruit were kept in a growth chamber as described above. Lesion diameters were measured 7, 11 and 14 days after inoculation.

Susceptibility of different parts of the fruit to rot development

In order to examine if fruit susceptibility differed among different parts of the fruit tissue, healthy and similar sized fruit of cvs. Pink Lady, Golden Delicious and Jonathan from trees not treated with fungicides were brought from the orchard one month before harvest and washed as described above. Each of four fruit per cultivar was dipped in 70 % ethanol and work was conducted under aseptic conditions in a laminar flow table. A 22 mm diameter core was taken from each fruit from the peel through the seed locule, using a sterilized cork borer. Each core was sliced to six discs (3–4 mm thick), the peel was sliced with 1 mm of the mesoderm. Each of the six discs were placed onto sterilized moist filter paper in one Petri dish, with the side facing the peel up (except from the peel disc which was placed inverse). A 2 μl conidial suspension of *A. alternata* f. sp. *mali* (5×10^5 spores ml^{-1}) was placed at the center of each disc. Discs with 2 μl of sterilized water served as controls. The petri dishes were closed, sealed with plastic wrap and incubated at 25 °C in the dark. Six days after inoculation, the percentage of each disc surface with symptoms of the disease was recorded. The experiment was conducted twice.

Effect of extracts from different parts of the fruit on conidia germination

The effect of aqueous extracts from the mesoderm of fruit of cvs. Pink Lady, Golden Delicious and Jonathan on germination of *A. alternata* f. sp. *mali* conidia was examined. Healthy fruit of cvs. Pink Lady, Golden Delicious and Jonathan from trees not treated with fungicides were brought from the orchard and washed as described above. Cvs. Jonathan and Golden Delicious were of the

same maturity stage (about 2 weeks before harvest) and of cv. Pink Lady at about 7 weeks before harvest. Fruit of cv. Jonathan were collected from three different orchards in the Golan region: Alonei HaBashan (ALB), Sha'al (SH) and Merom Golan (MG). The peel of three fruit from each cultivar was removed, and 10 g of mesoderm tissue from each fruit (30 g tissue in total) were cut and



Fig. 1 Rot caused by *Alternaria* and cracks around the calyx of cv. Pink Lady apple fruit (a), A bin with fruit of cv. Pink Lady not treated with fungicides with symptoms of *Alternaria* fruit spot during the 2010 harvest at the Ein- Zivan orchard in Israel (b), *Alternaria* fruit spot on the surface of fruit of cv. Pink Lady (c)

Fig. 2 Maximum likelihood phylogenetic tree using the Kimura-2 parameter model with discrete gamma distribution of the concatenated sequences of the Alt a1 and endoPG genes of four Israeli *Alternaria* isolates (in bold) and 51 Australian isolates and *Alternaria* species reference isolates (EGS). Bootstrap values (only above 50 are shown) at the nodes are based on 1000 replicates. Clade 1 consists of *A. arborescens* and *A. arborescens*-like isolates, clade 2 *A. tenuissima*/*A. mali* isolates, clade 3 *A. alternata*/*A. tenuissima* intermediate isolates and clade 4 *A. longipes* and *A. longipes*-like isolates



mashed in a plastic bag. Two ml of the juice were removed from the plastic bag and placed in a small tube. The tubes were centrifuged at 7500 rpm for 3 min. Sixty three microliter of the supernatant, mixed with 7 μ l of a conidial suspension of *A. alternata* f. sp. *mali* (5×10^4 spores ml^{-1}), were pipetted into depressions on microscope slides. A conidial suspension mixed with sterilized water served as control. The slides were placed on wet filter paper in Petri dishes and incubated at 25 °C for 20 h in the dark. In another experiment, the effect of aqueous extracts from (i) the peel, (ii) the peel plus 5 mm of adjacent mesoderm, and (iii) the mesoderm (5 mm adjacent to the peel) of cv. Jonathan was examined. Extraction from the three different regions of the fruit was done as described above. In both experiments, the numbers of germinated conidia were counted under a microscope, and the germination inhibition percentages were calculated. The length of the ten longest germ tubes was measured in each depression, and the mean of the longest germ tube was calculated for each treatment. Three depressions were used for each treatment, and each experiment was conducted twice.

Data analysis

Each laboratory experiment was conducted at least twice. Data from repeated experiments were combined for analysis when the variance between experiments was homogeneous. All data were processed with the SAS version 9.2 (SAS Institute Inc., Cary, North Carolina, USA) GLM statistical software package or with the JMP statistics package (SAS Institute). For the experiments with inoculation of detached fruit, the Fisher's LSD K-ratio t-test was applied to determine whether differences between treatments were significant at $\alpha < 0.05$. Standard error of the mean was reported in other cases. Correlation between maturity indices and

rot development in different phenological stages for the three cultivars was examined according to the Pearson correlation coefficient (r).

Results

Occurrence and isolation of *Alternaria* leaf blotch and fruit spot disease in Israel

During 2009 dark brown lesions on leaves were observed in orchards of cv. Pink Lady in northern Israel, causing leaf defoliation. Small lesions on the surface of fruit, and adjacent to cracks around the calyx, developed during the season to large dark rots (up to 50 mm in diameter), causing major losses, reaching up to 80 % infected fruit in some orchards (Fig. 1a-c). The isolated pathogen from infected leaves and fruit from several orchards in northern Israel was identified as a fungus from the genus *Alternaria*. Morphological characteristics of the mycelia and conidia indicated that it was *A. alternata* f. sp. *mali* (formally *Alternaria mali* Roberts). The hyphae were dark olive and conidia were brown to black, obclavate, pyriform, 25–30 μ m long and 7.5–12 μ m wide, with one to five transverse septae, one to three longitudinal or oblique septae and a short conical beak. Healthy leaves and fruit artificially inoculated with conidial suspensions developed similar symptoms and rot formation as observed in the field, whereas control leaves and fruit remained healthy. The same pathogen was consistently re-isolated from infected leaves and fruit, and Koch's postulates were thus fulfilled.

Molecular identification of the pathogen

Phylogenetic analysis based on the genes *endoPG* and *Alt a1* of four Israeli isolates, PL-EZ-B-2011-1, PL-SH-

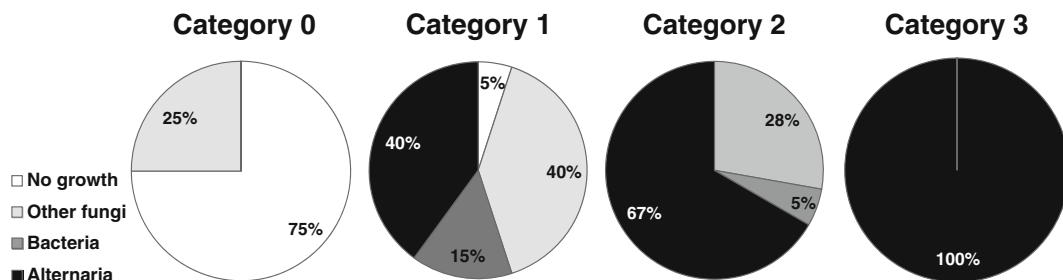


Fig. 3 Percentage of bacteria and fungi isolated from calyxes of fruit with or without cracks and rot. Category 0 = Calyx with no cracks, Category 1 = Calyx with minor cracks, Category 2 = Calyx with open cracks and no rot, Category 3 = Calyx with cracks and rot

Table 1 Rot development in fruit of eight apple cultivars following artificial inoculation with *A. alternata* f. sp. *mali* conidia

Apple cultivar	Lesion diameter (mm) ^a (Mean $\pm$ S.E.)				
	22 July	12 August	7 September	12 October	8 November
Pink Lady	5.0 $\pm$ 0.32	8.3 $\pm$ 0.26	6.2 $\pm$ 0.77	8.3 $\pm$ 0.39	13.9 $\pm$ 1.34
Golden Delicious	5.1 $\pm$ 0.51	11.4 $\pm$ 0.68	13.3 $\pm$ 0.35	30.6 $\pm$ 1.65	30.0 $\pm$ 0.00
Akane	5.6 $\pm$ 0.64	12.9 $\pm$ 1.28	16.4 $\pm$ 1.16 ^b	n.t. ^c	n.t
Gala	6.7 $\pm$ 0.73	6.6 $\pm$ 1.09	10.8 $\pm$ 1.40 ^b	n.t	n.t
Granny Smith	3.5 $\pm$ 0.29	5.7 $\pm$ 0.61	8.1 $\pm$ 0.33	8.3 $\pm$ 0.41	n.t
Red Delicious	6.3 $\pm$ 0.69	5.4 $\pm$ 0.52	6.8 $\pm$ 0.56	n.t	n.t
Johnny	3.3 $\pm$ 0.34	4.9 $\pm$ 0.40	3.7 $\pm$ 0.46	n.t	n.t
Jonathan	3.2 $\pm$ 0.38	5.6 $\pm$ 0.54	10.7 $\pm$ 1.97 ^b	5.1 $\pm$ 1.11	3.3 $\pm$ 0.22

^a Lesion diameter on apple skin was measured 13 days after inoculation

^b Fruit left on the trees 14 days after the commercial harvest

^c n.t = not tested due to early harvest

B-3011, PL-OR-C and PL-EZ-I, and 51 Australian isolates associated with *Alternaria* leaf blotch and fruit spot of apple (Harteveld et al. 2013) and five *Alternaria* species reference isolates showed that the Israeli isolates were located in clade 2 identified as *A. mali* / *A. tenuissima* group (Fig. 2). Isolate PL-SH-B-3011 (GenBank Accession No. KT582776) is representing the 480 bp fragment of the *Alt* *al* gene of the Israeli isolates, and isolate PL-OR-C (GenBank Accession No. KT582777) is representing the 423 bp fragments of the *endoPG* gene of the Israeli isolates. All four Israeli isolates contained the *AMT3* gene, opposed to the *A. alternata* isolate (isolate Sh42, Reuveni

et al. 2007) causing core rot in cv. Red Delicious fruit which did not exhibit this gene.

Population of additional microorganisms from fruit calyx cracks and rot

Incubation of tissue samples obtained from calyxes of fruit at various categories showed that the incidence (as percentage) of *Alternaria* spp. increased as the degree of cracked or rotted fruit increased (Fig. 3.). *Alternaria* spp. was not isolated from healthy, non-cracked fruit (category 0). The incidence increased to 67 and 100 % in cracked fruit and fruit with cracks and rot, respectively. On the other hand, the incidence of bacteria, or other

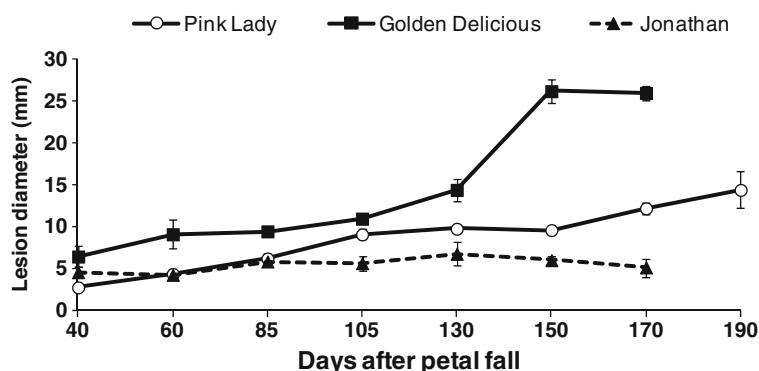


Fig. 4 Rot development in detached fruit of three apple cultivars following inoculation with *A. alternata* f. sp. *mali* conidia at 7–8 consecutive dates during the season. Lesion diameter measured 13 days after inoculation. Commercial harvest of Jonathan,

Golden Delicious and Pink Lady took place 130, 150 and 190 days after petal fall (DAPF), respectively. After commercial harvest, fruit were collected from cold storage (4–6 °C) prior to inoculation. Vertical bars indicate standard error of the mean

fungi decreased as number of cracked and rotted fruit increased (Fig. 3).

Inoculation of fruit lenticels

Inoculation of fruit lenticels with paper disks immersed in conidial suspension, or with conidial suspension droplets, yielded less than 3 % infected lenticels (data not shown). In those cases, rot development on 13 days after inoculation was limited and reached only 2–3 mm in diameter. Fruit inoculation by spraying the fruit with conidial suspension did not cause any rot.

Differential susceptibility of apple varieties to *A. alternata* f. sp. *mali*

In addition to cv. Pink Lady, seven other apple cultivars were examined in 2010 for their susceptibility to *A. alternata* f. sp. *mali* following inoculation of detached fruit. Cvs. Jonathan and Johnny were the most resistant, with a mean lesion diameter of less than 5 mm. Cvs. Top Red (Red Delicious), Gala and Granny Smith were moderately resistant (5–10 mm lesion diameter), and the most susceptible were cvs. Akane, Golden Delicious and Pink Lady (15–30 mm lesion diameter) (Table 1). Regardless of the tested cultivar, fruit that had been left on the trees after the commercial harvest were more susceptible to *Alternaria* fruit spot following artificial inoculation than fruit picked at normal harvest time (Table 1). A further study in which the most susceptible cultivar (Golden Delicious) and the most resistant cultivar (Jonathan) were compared to cv. Pink Lady fruit during the 2012 season, confirmed that Jonathan was resistant throughout the entire season and even after being kept in cold storage (4–6 °C) after commercial harvest (Placed in cold store from 130 days after petal fall (DAPF)). The susceptibility of fruit of cv. Pink Lady increased during the season starting from 105 DAPF and towards ripening, and susceptibility of cv. Golden Delicious increased mainly close to harvest (150 DAPF) (Fig. 4).

Relationship between fruit susceptibility to *Alternaria* fruit spot and maturity indices

Examination of fruit maturity indices of cvs. Pink Lady, Golden Delicious and Jonathan showed that pH values of Golden Delicious were higher during the entire season than those of cvs. Pink Lady and Jonathan (Fig. 5a).

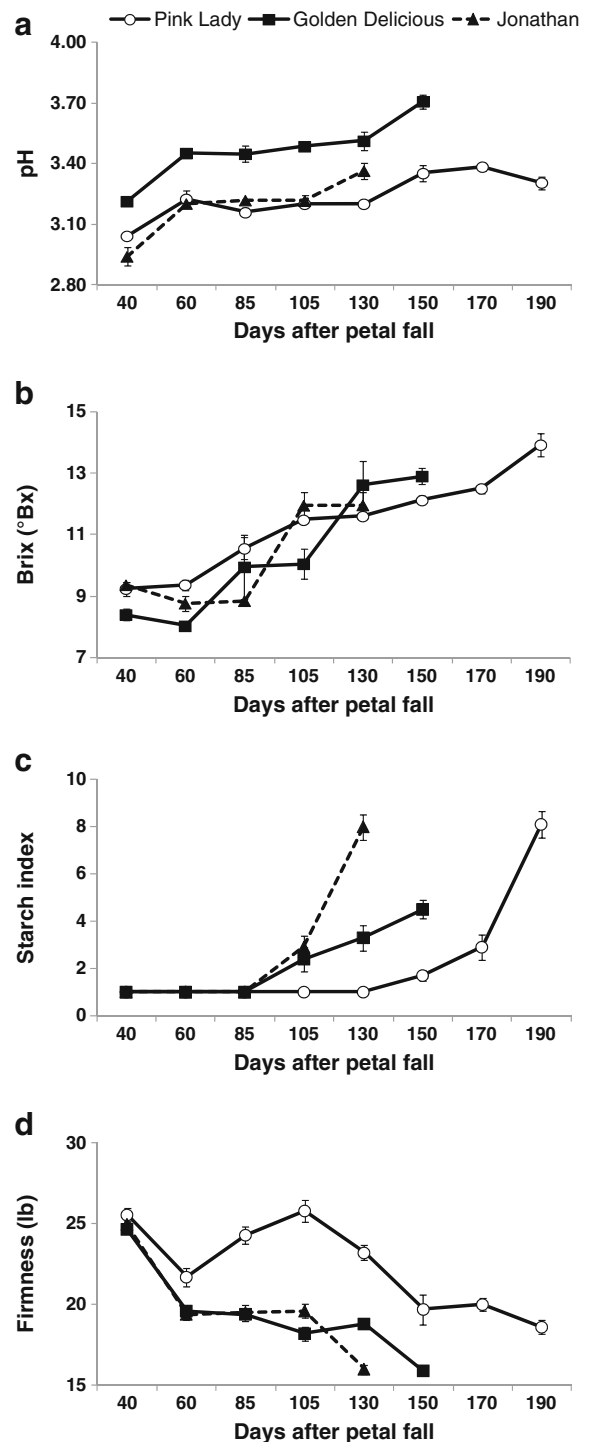


Fig. 5 Fruit maturity indices **a** pH, **b** Brix, **c** starch and **d** firmness of fruit of cvs. Pink Lady, Golden Delicious and Jonathan. Measurements were conducted during the growing season in the Ein-Zivan orchard in parallel to fruit inoculation in the laboratory. Vertical bars indicate standard error of the mean

Similar levels of brix were observed during the season in fruit of the three cultivars (Fig. 5b). Starch levels remained stable for all three cultivars in the beginning of the season (until 85 DAPF), and starch hydrolysis (high starch index) increased towards commercial harvest time of each cultivar (Fig. 5c). Decrease in fruit firmness along fruit maturation was observed in all three cultivars, while the firmness of cv. Pink Lady fruit was higher during the entire season, followed by that of cvs. Golden Delicious and Jonathan (Fig. 5d). Pearson correlation coefficients (r) showed a significant positive correlation between pH, brix, starch and rot development for cvs. Pink Lady and Golden Delicious, but not for cv. Jonathan. A negative correlation was observed for all three cultivars for fruit firmness (Table 2).

Effect of inoculum concentration on rot development

Increasing the inoculum concentration enhanced rot development (Fig. 6). Lesion diameter in fruit of cv. Golden Delicious increased over time (7, 11 and 14 days post inoculation) for all four concentrations. Results were similar for fruit of cv. Pink Lady, with the exception of a concentration of 10^3 conidia ml^{-1} in which lesion diameter remained relatively low (below 5 mm). Regardless of inoculum concentration, cv. Jonathan showed almost no rot development over time (7, 11 and 14 days after inoculation) (data not shown). Fourteen days after inoculation lesion diameter was larger in the cv. Golden Delicious at all four concentrations compared to cvs. Pink Lady and Jonathan (Fig. 6). Fruit inoculated with 10^6 conidia ml^{-1} , reached 32 and 37 mm in diameter for cvs. Pink Lady and Golden Delicious respectively, significantly larger than at lower inoculum concentrations ($P < 0.05$, data not shown). For cv. Pink Lady, rot development was moderate when 10^4 or 10^5 conidia ml^{-1} were used for inoculation, and very little rot developed when fruit were inoculated with 10^3 conidia ml^{-1} (Fig. 6).

Susceptibility of different parts of the fruit to rot development

Inoculation of fruit mesoderm discs showed that cvs. Jonathan and Pink Lady fruit were less sensitive than Golden Delicious (Fig. 7). For all three cultivars the resistance was highest close to the skin and decreased towards the seed locule (Fig. 7).

Effect of extracts from different parts of the fruit on conidia germination

Germination was not affected (above 95 %) in any of the aqueous extractions from the mesoderm of cvs. Pink Lady, Golden Delicious and Jonathan (data not shown). However, conidia in the water-treated control developed only 1–2 germ tubes, while those in fruit extracts developed few and shorter germ tubes (data not shown). Thus the length of the longest germ tube was chosen to describe the differences among treatments. The length of the longest germ tube in mesoderm extracts of cv. Jonathan was significantly shorter than those of cvs. Pink Lady and Golden Delicious, regardless of the orchard from which the cv. Jonathan fruit were collected (Fig. 8). Peel extract inhibited germ tube elongation significantly, more than a mixture of peel and mesoderm extract, or mesoderm extract alone (Fig. 9).

Discussion

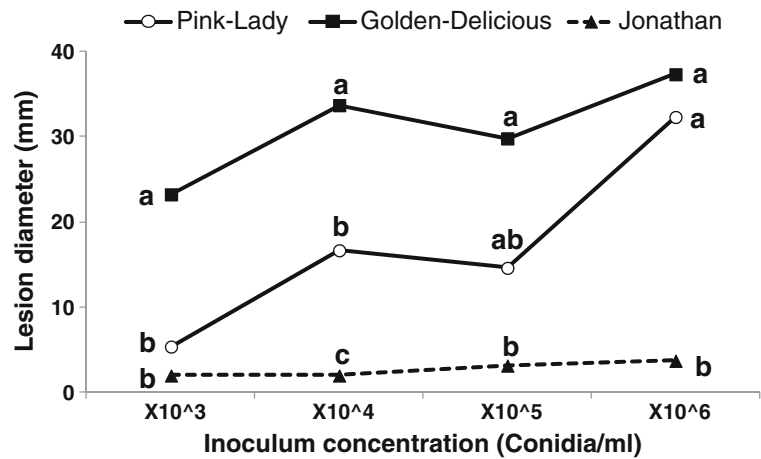
In recent years, severe outbreaks of *Alternaria* fruit spot were observed on fruit of apple cv. Pink Lady in the orchards of Golan and Galilee regions in Israel. Such severe attacks and fruit rots, particularly on cv. Pink Lady, were not reported from other regions of the world. Furthermore, the severe rot of cv. Pink Lady fruit associated with calyx cracking is a new phenomenon. Microscopic examinations, fulfillment of Koch's postulates

Table 2 Pearson correlation coefficients between pH, Brix, starch, firmness and rot development at different phenological stages for cvs. Pink Lady, Golden Delicious and Jonathan

Apple Cultivar	pH	<i>P</i> -value	Brix	<i>P</i> -value	Starch	<i>P</i> -value	Firmness	<i>P</i> -value
Pink Lady	0.776 ^a	0.023	0.986	>0.001	0.728	0.040	-0.671	0.068
Golden Delicious	0.892	0.016	0.834	0.039	0.926	0.008	-0.780	0.067
Jonathan	0.724	0.166	0.687	0.200	0.802	0.102	-0.706	0.182

^a Each number represents correlation between values of lesion development and maturity index at 5–8 inoculation dates

Fig. 6 Effect of inoculum concentration on rot development in detached fruit of cvs. Pink Lady, Golden Delicious and Jonathan. Inoculum concentrations are in number of conidia ml^{-1} . Different letters indicate significant ($P < 0.05$) differences between means among cultivars for each concentration 14 days after inoculation, according to the Fisher's LSD t-test



and molecular-based studies showed that the pathogen causing the rots on Pink Lady apple leaves and fruit in Israel is *A. alternata* f. sp. *mali*. This is the first report about the causal agent of Alternaria leaf blotch and fruit spot of apple in Israel.

The present investigation showed that *A. alternata* f. sp. *mali* is common in Israeli apple orchards, in association with Alternaria leaf blotch and fruit spot of cv. Pink Lady. The fungus apparently colonizes cracks around the fruit calyx or the skin of the fruit body and eventually penetrates (presumably through open cracks) into the parenchyma of the fruit of cv. Pink Lady. The fungus was more frequently isolated from fruit with elevated levels of fruit cracking or rot, than from fruit with few minor cracks.

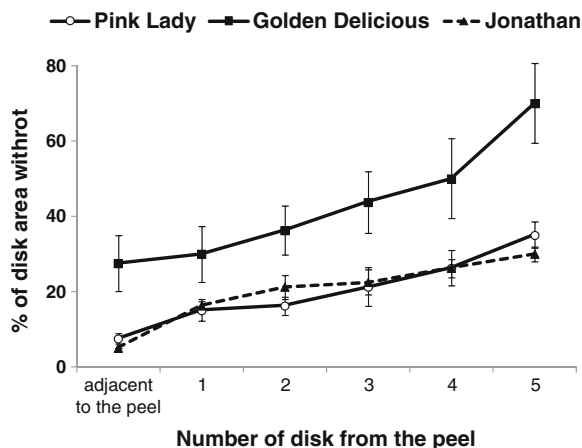


Fig. 7 Rot development on fruit mesoderm disks of cvs. Pink Lady, Golden Delicious and Jonathan following artificial inoculation. Each disk represents different regions in the fruit mesoderm from adjacent to the peel through the seed locule (5). Vertical bars indicate standard error of the mean

Alternaria spp. associated with Alternaria leaf blotch and fruit spot have been isolated from apple fruit in previous studies (Rotondo et al. 2012; Hartevelde et al. 2013), but successful infection following inoculation of detached fruit was limited (Rotondo et al. 2012). In some cases, no symptoms were observed on the detached inoculated fruit (Hartevelde et al. 2014). When symptoms have appeared, they were limited to small lesions (a few mm), similar to those observed in the field in Italy and Australia (Rotondo et al. 2012; Hartevelde et al. 2014). Disease incidence reported was also very low (less than 2 %) (Hartevelde et al. 2014), unlike in Israel where the percentage of rotted fruit with lesion diameters ≥ 30 mm reached 80 %. Although not tested, it may seem that the Israeli isolates are more virulent compared to the Italian or Australian isolates, and/or the climatic conditions in Israel are more conducive for pathogen development. For the best of our knowledge no reports on inoculation of cv. Pink Lady fruit were documented before. The ability of the fungus to infect apple fruit depends on the presence of a wound on the apple pericarp (Rotondo et al. 2012). We found this crucial, as unwounded fruit produced no infection even when inoculated with a high dose of conidial suspension. We concluded that the fungus can penetrate into the fruit pericarp barrier only through a wound.

All attempts to artificially inoculate fruit via the lenticels failed. It is possible that the high radiation and temperatures that prevail in the orchard during the Israeli summer prevent fusion of micro-cracks around the fruit lenticels, and also causes melting of the wax at the base of the lenticels. As a result, the unprotected cells below the cuticle become exposed, and are subject to pathogen attack (Curry 2003). This may serve as a penetration site for *A. alternata* f. sp. *mali* causing fruit rot.

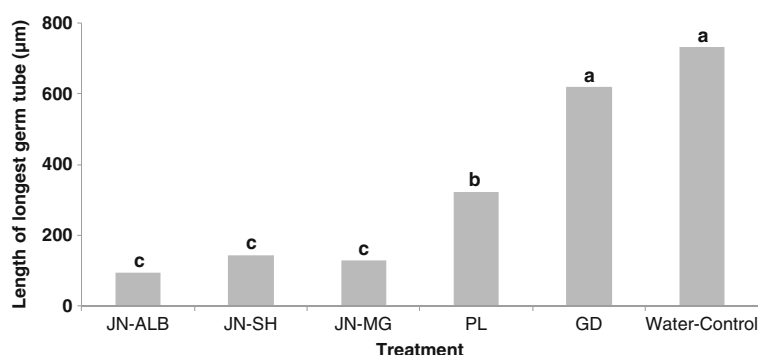


Fig. 8 Effect on length of the germ tube of *A. alternata* f. sp. *mali* conidia by aqueous extracts from the fruit mesoderm of cvs. Pink Lady (PL), Golden Delicious (GD) and Jonathan (JN). Fruit of cv. Jonathan were collected from three orchards: Alonei HaBashan

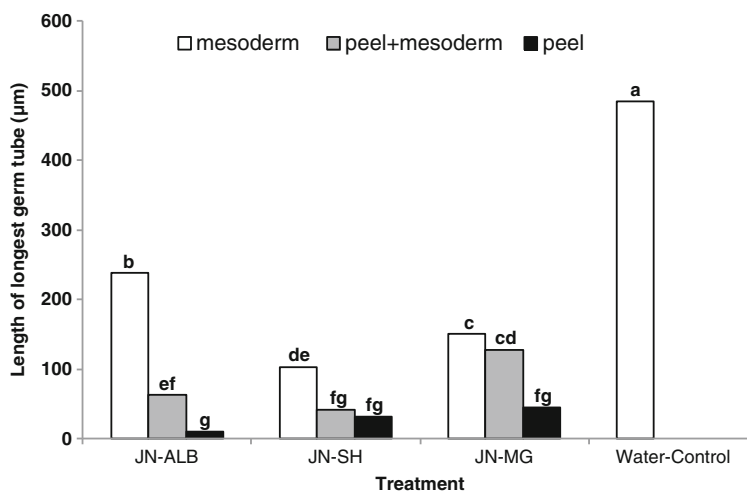
(ALB), Sha'al (SH) and Merom Golan (MG). Data represent the length of the longest germ tube. Different letters indicate significant ($P < 0.05$) differences between treatments according to the Fisher's LSD t-test

Here we report on the resistance to *Alternaria* fruit spot among apple cultivars commonly grown in Israel. Previous studies reported on cultivar resistance of apple leaves (Filajdic and Sutton 1991; Saito et al. 2001; Abe et al. 2010), because leaf infection (and defoliation) was the main damage in most countries. In most cultivars, resistance against *A. alternata* f. sp. *mali* in leaves was not correlated with resistance of the fruit (Saito et al. 1978). Foliage of cvs. Golden Delicious and Gala were evaluated as resistant to the pathogen in Japan (Abe et al. 2010) while regarded as moderately resistant in North Carolina (Filajdic and Sutton 1991), and susceptible in Italy and Australia (Horlock 2006; Rotondo et al. 2012). Adequate inoculation procedures can provide better assessment of resistance to *Alternaria* fruit spot. Our results showed that under laboratory conditions, fruit of all major Israeli cultivars (including Pink Lady) became infected to a certain degree. Regardless of the

tested cultivar, fruit that were left on the trees after the commercial harvest were more susceptible to *Alternaria* fruit spot following artificial inoculation, probably due to an increase in sugar or carbohydrate contents in the fruit mesoderm. The most susceptible cultivars were Pink Lady, Golden Delicious and Akane, but the latter was found to be resistant in Japan (Abe et al. 2010). Similar to our findings, cv. Jonathan was considered one of the most resistant cultivars in Japan (Abe et al. 2010), however, the Japanese work was done with apple leaves and we inoculated fruit. Another important aspect of the present work is the possibility to create a base-line susceptibility level for all important apple cultivars growing in Israel. That may be used to evaluate any change that might occur in the future in the etiology of the disease.

Germ tube lengths of *A. alternata* f. sp. *mali* conidia incubated in mesoderm extract of cv. Jonathan were

Fig. 9 Effect of aqueous extracts from different parts of Jonathan (JN) fruit tissue, on germ tube elongation of *A. alternata* f. sp. *mali* conidia. Fruit of cv. Jonathan were collected from three orchards: Alonei HaBashan (ALB), Sha'al (SH) and Merom Golan (MG). Different letters indicate significant ($P < 0.05$) differences between treatments according to the Fisher's LSD t-test



significantly shorter compared to those emerging from conidia incubated in extracts of cvs. Pink Lady or Golden Delicious. This indicates on the presence of inhibitory substances in the fruit tissue of cv. Jonathan. Examination of the susceptibility of the cvs. Pink Lady, Golden Delicious and Jonathan following artificial inoculation of fruit at different phenological stages during the growing season, revealed that cv. Jonathan was relatively resistant throughout the season, while cvs. Pink Lady and Golden Delicious had low susceptibility at the beginning of the season, and increased towards fruit maturation. The pH values of cv. Golden Delicious were higher during the entire season than those of cvs. Pink Lady and Jonathan. The expression of genes encoding enzymes such as β -1, 4 endoglucanase and exoglucanase indicate that their secretion is enhanced at high pH, and this has been suggested to be a regulatory mechanism of pathogenicity under alkaline environments in apple and persimmon (Eshel et al. 2002; Niem et al. 2007). It is possible that the low acidity of the susceptible cv. Golden Delicious tissue encourages development of *Alternaria* after penetration and rot formation. Brix and starch hydrolysis values were similar for all three cultivars. The firmness of fruit of cv. Pink Lady was higher than that of cvs. Golden Delicious and Jonathan. Spotts et al. (1988) ascribed apple fruit resistance to pathogenic fungi that penetrate through injuries, to the firmness of the fruit tissue. In our case, that cannot be the reason for the difference in resistance between cvs. Golden Delicious and Jonathan. No significant correlation was found between maturity indices and resistance in fruit of cv. Jonathan. Fruit of cvs. Pink Lady and Golden Delicious exhibited susceptibility to rot development following artificial inoculation even at low inoculum concentration (10^3 conidia ml^{-1}), while cv. Jonathan fruit were highly resistant (no signs of external rot) even at elevated inoculum concentration (10^6 conidia ml^{-1}). Rot lesions in the fruit mesoderm of cv. Jonathan were significantly smaller than in the susceptible cultivars. This finding led to examination of the susceptibility of different parts of the fruit. Inoculation of fruit mesoderm disks confirmed high resistance close to the fruit surface and decreasing resistance towards the seed locule. This was true for all three cultivars. Peel extract from cv. Jonathan inhibited germ tube elongation more than the mesoderm extract, suggesting that inhibitory substances in the resistant cultivar may be concentrated in the fruit peel layer, the zone first interacting with the pathogen. Secondary metabolites like phenolics, known for their antifungal properties (Wilson

and Wisniewski 1989; Lattanzio et al. 2006), may be responsible for the resistance of cv. Jonathan. More research to confirm this hypothesis is required.

In conclusion, we have shown here that the fungal pathogen *Alternaria alternata* f. sp. *mali* is the causal agent of the heavy fruit rot in cv. Pink Lady in Israel; fruit infection require wound and therefore cracks around the calyx enhance the disease; cv. Jonathan is the most resistant, and resistance in cv. Jonathan is not related to fruit firmness or maturity but may be related to inhibitory compounds present in the peel. These finding may be used to develop disease control strategies against *Alternaria* leaf blotch and fruit spot of apple in Israel and elsewhere.

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