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Development of a reliable screening technique for determining tolerance to *Macrophomina phaseolina* in strawberry

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Abstract *Macrophomina phaseolina* is a typical soilborne fungal pathogen causing crown and root rot in strawberry (*Fragaria* × *ananassa* Duch.) worldwide. *M. phaseolina* has become a major problem for strawberry growers parallel to the phase-out of methylbromide since 2004 and is considered the most destructive soilborne pathogen of strawberry since then. Global warming is characterized by extreme weather conditions, in the Mediterranean area, as reflected by long, hot, dry summers without rain and relatively short, cold rainy winters. This together with regulatory restrictions on toxic fumigants creates favorable conditions for *M. phaseolina* to thrive. Screening for resistant germplasm is currently the most effective and sustainable approach for managing the disease. In order to screen for susceptible/tolerant strawberry cultivars, various inoculation techniques were assessed on five strawberry cultivars. Artificial inoculation of growth medium with naturally infected plant material and the use of the 'toothpick' method resulted in no significant differences. However, the use of artificially produced sclerotia in a soil mix at concentrations of 2.5×10^3 sclerotia/g soil exhibited differential cultivar mortality rates. High variation was found among 32 tested strawberry varieties (Israeli and US) grown under outdoor conditions in a screenhouse. Cultivars 'Pelican' (US), 'Orly', 'Tamir' and 'Rotmy' were considered tolerant compared to cultivars 'Florida 90' (US) and 'Peles' that were the most susceptible. The overall results indicate that the choice of certain Israeli and US cultivars may provide future germplasm for resistance breeding against *M. phaseolina*.

Keywords Charcoal rot · Inoculation techniques · *Macrophomina phaseolina* · Soilborne pathogens · Strawberry wilt

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Introduction

In the Mediterranean region, strawberry is an economically important winter producing crop, cultivated for local consumption and export to European markets. In Israel, transplants for production fields are obtained from disease-free nurseries, where they are propagated from mother plants during the spring months (Freeman and Gnayem 2005). Spring and summer propagated field nurseries are routinely monitored for soilborne fungal pathogens (e.g., *Phytophthora* spp., *Rhizoctonia* spp., *Colletotrichum* spp., *Verticillium* spp., and *Macrophomina phaseolina*) throughout the season until late summer transplanting to the fruiting fields (Freeman

and Gnayem 2005; Freeman and Katan 1997; Sharon et al. 2007; Zveibil et al. 2012). In Israel, *M. phaseolina* has become the main threat to cultivation of strawberry causing up to 20% in losses of yield and revenue (Freeman et al. unpubl.), and has become a major pathogen of importance in other countries growing strawberry in the Mediterranean region. As an annual crop system, strawberry is grown year after year without rotation (Freeman and Gnayem 2005). At the end of the growing season, farmers dry the fields by terminating irrigation and plants are destroyed by applying herbicides, mowing off the foliage and ploughing into the soil. The development and proliferation of *M. phaseolina* in the remaining plant material, generates inoculum for the following season's crop (Chamorro et al. 2015; Lisker and Meiri 1992; Reuveni et al. 1982; Strand 1994; Zveibil and Freeman 2009). This, together with elevated soil temperatures, creates optimal conditions for the development and infection of strawberry by *M. phaseolina* (Chamorro et al. 2015; Strand 1994; Zveibil et al. 2012).

Traditionally, strawberry production relied on soil fumigation with methyl bromide as the main control method against weeds, nematodes and soilborne pathogens including *M. phaseolina* (Chamorro et al. 2015; Hummer and Hancock 2009). However, due to the negative impact of methyl bromide on the ozone layer, it was banned from use in many countries and alternative fumigation measures have not been as effective, making it more difficult to manage soilborne diseases (Albajes et al. 1999; Carter et al. 2005). Several approaches for managing *M. phaseolina* in strawberries have been examined in order to provide an alternative solution to chemical pesticides that have a negative impact on human health, cause unprecedented changes to the environment, and detrimentally affect soil fertility and/or water quality (Avilés et al. 2009; Duniway 2002).

A combination of different approved fumigants to manage the disease has provided only a partial solution, and thus has proven to be ineffective as well as detrimental to the ecosystem. Hutton et al. (2013) found that serious losses due to *M. phaseolina* have all occurred in strawberry crops planted in soils treated with alternatives to methyl bromide in comparison to methyl bromide treated soils. Chamorro et al. (2016) assessed the efficiency of different fumigant treatments on *M. phaseolina* in strawberries. Alternatives to methyl-bromide included, chloropicrin (PIC), 1,3-dichloropropene (1,3D), dimethyl disulfide (DMDS), potassium *N*-methyldithiocarbamate (Kpam) and sodium methyldithiocarbamate (Vapam). Furthermore, Chamorro et al. (2016) found most of the alternatives to be efficient against *M. phaseolina* although in developed countries, the majority of useful chemical alternatives have a limited future, due to regulatory restrictions. Soil solarization provided adequate control against several soilborne pathogens but did not achieve favorable results against *M. phaseolina*, especially at a 10–20 cm soil depth due to the ability of microsclerotia to survive elevated temperatures (Yildiz et al. 2010). In a study conducted by Chamorro et al. (2015), the focus on biosolarization treatments has been considered due to the increased restrictions on the use of other toxic fumigants i.e., treatments reduced or stabilized *M. phaseolina* sclerotia populations in soil compared to the untreated control only when applying combined biosolarization with chicken manure or sugar beet.

M. phaseolina has a wide host range and affects more than 500 botanical species worldwide (Gupta et al. 2012; Kaur et al. 2012; Ndiaye et al. 2007; Singh et al. 1990). As a typical soilborne pathogen that lacks a sexual stage, *M. phaseolina* forms microsclerotia, a condensed mass of hardened cells (Dhingra and Sinclair 1975). The structure can survive in organic matter and in the soil for several years, serving as the primary source of inoculum from season to season (Meyer et al. 1974; Ndiaye et al. 2007). In strawberry, symptoms in the field are characterized by wilting of foliage and necrosis of older leaves while the younger leaves usually remain green. When dissecting the crowns of infected plants, the internal vascular and cortical tissues appear dark to orange-brown in color, while the external tissues become necrotic, resulting in symptoms termed 'charcoal rot' (Koike et al. 2016). The fungus penetrates the plant via an appressorium formed from the germ tubes and penetrates through the host epidermis of roots and crowns by secreting CWDEs (cell-wall degrading enzymes) or via indirect penetration through natural openings or wounds (Bowers and Russin 1999; Mayek-Pérez et al. 2001). Mycelia colonize the vascular tissue by growing through the cortex, then entering the xylem vessels (Abawi and Pastor-Corrales 1990). Once inside vascular tissues, the fungus spreads through the tap root system and plugs the vessels resulting in wilting and eventual mortality of the plant (Kaur et al. 2012).

M. phaseolina first appeared in strawberry in Israel in 2004 and has since become the major soilborne

pathogen affecting both nursery and field cultivation (Zveibil and Freeman 2005). Unlike other soilborne pathogens of strawberry that favor a change in moisture conditions, *M. phaseolina* thrives in areas where climate change results in longer drought periods and higher temperatures (Mihail et al. 1992; Saleh et al. 2010). Mediterranean countries are characterized by long, hot, dry summers without rain and relatively short, cold rainy winters (Goldreich 2003). These climatic conditions favor *M. phaseolina* and as a result, many crops are attacked, such as melon, cotton and strawberry, causing considerable economic losses (Lisker and Meiri 1992; Reuveni et al. 1982; Zveibil and Freeman 2005). Considering the failure in developing a viable controlling strategy, and given the need to reduce and eventually eliminate chemical fumigation, screening for resistant/tolerant strawberry germplasm to *M. phaseolina* is of paramount importance. This strategy has proven successful in other botanical species such as sorghum and soybean (Mayek-Pérez et al. 2001; Pecina-Quintero et al. 1999).

To date, no *M. phaseolina*-disease-resistant strawberry varieties have been found, although several studies have reported a differential response of various cultivars to the disease (Avilés et al. 2009; Baggio et al. 2019; Fang et al. 2012; Koike et al. 2016; Sánchez et al. 2016). Avilés et al. (2009) found variation among three commercial strawberry cultivars in Spain, where 'Camarosa' was more tolerant than other tested cultivars. Furthermore, Fang et al. (2012) screened three commercial strawberry cultivars in Western Australia under controlled conditions, and found that 'Albion' was more resistant compared to 'Camarosa', the most susceptible cultivar. Fang et al. (2012) also assessed the disease severity between fumigated and non-fumigated fields on eight commercial cultivars, and found variation in fruit yield and disease susceptibility with 'Camino-Real' showing the most resistance. However, a recent study by Gomez et al. (2020) indicated that 'Camarosa is more resistant than 'Albion' in contrast to Fang et al. (2012). Sánchez et al. (2016) found significant differences in susceptibility among twelve strawberry germplasms with cultivar 'Florida Fortuna' being the most susceptible. In addition, two QTLs conferring resistance to *M. phaseolina* were found recently by researchers at the University of Florida in two consecutive growing seasons and validated in a set of cultivars and advanced selections (Nelson et al. 2019).

One of the challenges in assessing the reaction of cultivars to a pathogen is to determine the most reliable inoculation technique to differentiate, and at the same time generate disease symptoms as similar as possible to those caused by the pathogen under field conditions. Therefore, the main goals of this study were to: (i) evaluate the most reliable inoculation technique for screening a large collection of representative strawberry germplasm against *M. phaseolina* under controlled conditions, and (ii) screen for resistant/tolerant strawberry germplasm by evaluating an international collection of selected cultivars and variants for disease in a screenhouse under natural conditions.

Materials and methods

Fungal cultures and growth conditions

A representative isolate M1 of *M. phaseolina* from strawberry, originating from necrotic, wilted crowns of cultivar 'Yuval' from the Sharon region of central Israel, was chosen from a total of 190 isolates in the authors collection, and used for screening for resistant/tolerant strawberry germplasm (Zveibil and Freeman 2005). Three additional isolates [M5 isolated from a diseased cultivar in Ein-Sarid (unknown cultivar), M71 isolated from cultivar 'Hadas' in Kalanswa, and M101 isolated from cultivar '330' in Tira, all from the Sharon region in central Israel], were used in selected experiments, using all the tested methods of inoculation, to verify the results obtained for isolate M1 (data not shown). Isolation of the fungus from infected plants was performed by removing roots and foliage with a scalpel and exposing the inner part of the crown or stem of the plant. The plant material was surface sterilized in 70% ethanol for 20 s and thereafter, 1% sodium hypochlorite (NaClO) for 3.5 min and washed in sterile distilled H_2O. The plant tissue was then dried on sterile paper towels and placed aseptically on Petri plates containing potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA) amended with 0.25 g/l chloramphenicol (PDAC) or semi-selective medium, if required (Sharma et al. 2017; Zveibil and Freeman 2009). Isolation of the fungi was performed by the hyphal-tip method; from PDAC plates containing the plant material, squares of agar with *M. phaseolina* were transferred aseptically to 1% PDA plates for 24 h. After 24 h an individual hypha was transferred to a 45 mm PDA plate for storage. All

isolates collected over the years since 2005 were stored in 15% (*v*/v) glycerol/sterile H_2O at −80 °C.

Detection of a reliable inoculation technique under controlled environmental conditions

Experiments were conducted under controlled conditions in growth chambers with constant temperatures of 30 °C, the optimal growth temperature for *M. phaseolina* under natural light conditions (Zveibil et al. 2012). Experiments assessing three different artificial inoculation techniques (see below) were performed at least twice on five commercial strawberry cultivars (including US and Israeli cultivars) representing the most tolerant (1) to the most susceptible (5): (1) 'Dandi'; (2) 'Gili'; (3) 'Hadar'; (4) 'Rocky' and (5) 'Festival', based on preliminary surveys conducted by extension specialists in the field. For inoculation, plants were transplanted from 5 to 10 cm pots (300 ml volume) adding the infested soil mix at the desired concentration or adding growth medium (when the inoculation was conducted by the 'toothpick' method, see below) while two plants from each cultivar served as untreated controls. Disease severity was evaluated weekly following inoculation using a five-degree ranking scale based on Koike et al. 2016 and was converted to percentage to express plant mortality: 1 = no symptoms (equaling 0% plant mortality); 2 = wilting, chlorosis and/or necrosis of lower older leaves (equaling 25% plant mortality); 3 = wilting, chlorosis and/or necrosis of less than 50% of the foliage including symptomatic younger leaves (equaling 50% plant mortality); 4 = wilting, chlorosis and/or necrosis of more than 50% of the foliage (equaling 75% plant mortality); 5 = complete plant collapse and mortality (equaling 100% plant mortality).

Artificial inoculation using the 'toothpick' method

Wooden toothpicks were soaked in dH_2O overnight, dried on a paper towel and autoclaved. Five sterile toothpicks were placed on seven-day-old cultures of *M. phaseolina* in a 90-mm PDA Petri plates for five days in an incubator at 25 °C under dark conditions (Cohen et al. 2016). When the toothpick was completely covered with the fungal hyphae and microsclerotia were visible, it was inserted into the crown of the plant to a depth of 1.5 cm. Sterile toothpicks were used as controls.

Artificial inoculation of growth medium with naturally infected plant material

One-hundred strawberry plants, cultivars of 'Hadar' and 'Rocky' with visible wilt disease symptoms, were collected from naturally infected fields in the Sharon region. Twenty plants were inspected randomly by plating infected crowns on PDA before the experiments in order to verify that disease symptoms were caused by *M. phaseolina*. The crowns and roots of each infected plant were rinsed thoroughly under running tap water and cut into 0.2–0.3 mm pieces, then dried in the sun for 5 h. Naturally infested soil that surrounded the infected plants in the field was collected and strained through a 700-μm strainer, then mixed with the dried infected plant material to produce the inoculum. Tested strawberry plants (eight plants per cultivar) were transplanted from 5 to 10 cm pots (300 ml) adding the infested soil together with 6 g of infected plant material per pot (0.02 infected plants/ ml soil). Two control plants, from each cultivar, were transplanted from 5 to 10 cm pots, twice adding sterilized soil-less coconut and styrofoam (3:1 *v*/v) growth medium.

Inoculation of growth medium with artificially produced sclerotia in a soil mix

Ten pieces of 0.5 cm^2 disks of a seven-day-old *M. phaseolina* culture of isolate M1 growing on PDA medium were transferred to 250 ml Erlenmeyer flasks containing 100 ml PDB (Difco Laboratories, Detroit, MI, USA) amended with chloramphenicol at 250 mg/l. The Erlenmeyer flasks were then transferred to a rotary shaker incubator, and adjusted to 130 rpm at 25 °C under dark conditions for ten days. After ten days a dark liquid formed with noticeable fungal spheres. The Erlenmeyer flask contents were blended in a sterile blender for 30 s then filtered through Miracloth (Calbiochem, La Jolla, CA, USA). The Miracloth filter containing the sclerotia and mycelium was then dried in an 18 cm sterile glass Petri dish on three layers of sterile paper towels for seven days until the mycelium was completely dry. The dry mycelium was grinded thoroughly with a mortar and pestle and then strained through a 500-μm strainer followed by a 177-μm strainer until a powder of sclerotia and mycelium was produced (Zveibil et al. 2012). To test fungal viability, 0.01 g of inoculum was diluted tenfold in 0.85% NaCl (saline solution, with 100 μl/l Tween 80). From each

dilution, 100 μl was transferred to PDA medium, three replicas per dilution and seeded with glass beads for an even spread on the plate. After 24 h, colony forming units (CFU's) were enumerated by counting each sclerotium colony that developed per plate.

Calibration of the concentrations of artificially produced sclerotia (sc) in a soil mix for screening for susceptible/tolerant strawberry germplasm

In order to determine the most effective concentration of artificially produced sclerotia in the soil mix, four different concentrations were tested; 2.5×10^2, 1.2×10^3, 2.5×10^3 and 2.5×10^4 sclerotia/g soil. The inoculum was mixed well with sterile soilless coconut and styrofoam (3:1 *v*/v) growth medium that autoclaved twice during a 24-h period. The susceptible strawberry cultivar 'Gili' served as an indicator for inoculation assays.

Plant material

All plant material screened in this work originated from the strawberry germplasm collection of ARO, the Volcani Center that included: Israeli cultivars; 'Angel', 'Aya', ARO-line (196), 'Dandi', 'Daniel', 'Gili', 'Hadar', 'Hadas', 'Peles', 'Rocky', 'Rotmy','Shaked', 'Shani', 'Tamar', 'Tamir', 'Yael' and 'Yasmin'. US cultivars; 'Camarosa', 'Carmine', 'Douglas', 'Festival', 'Florida 90′, 'Gaviota', 'Parker', 'San Andreas' and 'Seascape'. European cultivars; 'Candonga' from Spain, 'S. pantagruella' from Germany; Japanese cultivar 'Top Otome'; and advanced US selections imported to Israel as anthracnose resistant cultivars; 'Pelican' and 'US70' (Smith et al. 1998).

Screening for tolerance of strawberry cultivars under screenhouse conditions

Experiments were conducted twice, during the summer season (May–October of 2016 and 2017) in a white-colored shaded outdoor net house under conditions similar to those in the field, with temperatures ranging from 40 to 45 °C during the day and 20–25 °C at night. Plants were watered three times daily for 3 min with drip irrigation (4 l/h) and fertilized at a ratio of 2:1:3 (N, P_2O_5, K_2O). Thirty-three commercial strawberry cultivars (Table 1) that were screened for tolerance to *M. phaseolina*, were received from certified disease-free nurseries in the Sharon region; Romano Nurseries Ltd., Tel Yitzhak and Yosef's Farm Ltd., Kfar Malal, and from the Israeli strawberry germplasm collection in the ARO. Tolerance to *M. phaseolina* was tested using artificial inoculation of growth medium with artificially produced sclerotia in the soil mix at 2.5×10^3 sclerotia/g soil. Inoculation was conducted using eight plants per cultivar, as described previously, with two plants per cultivar serving as the control. Disease severity was evaluated weekly following inoculation using a five-degree ranking scale, as described above.

Data analyses

Detection of a reliable inoculation technique under controlled environmental conditions

Disease progress curves were generated from the disease severity data expressed as percent plant mortality following inoculation. Trials were conducted and analyzed as a complete randomized design. Results obtained were subjected to an analysis of variance (ANOVA), and the means were separated by the Tukey- Kramer HSD test ($\alpha = 0.05$) (Ozkilinc et al. 2011). The experiment was conducted twice and analysis of variance was performed in order to determine significance between the trials. All data were analyzed using JMP®, Version 13. SAS Institute Inc., Cary, NC, 1989–2019.

Calibration of the concentrations of artificially produced sclerotia (sc) in a soil mix for screening for susceptible/tolerant strawberry germplasm

Trials were conducted and analyzed as a complete randomized design. The relative area under the mortality progress curve (RAUMPC in % × days) was calculated on the basis of plant mortality assessments over the duration of each experiment (Philosoph et al. 2018). To enable analysis of variance, the disease severity values were normalized by arcsine square-root multiple comparisons of the means, evaluated by the Tukey- Kramer HSD test ($\alpha = 0.05$). The experiment was conducted twice and analysis of variance was performed to determine significance between the trials. All data was analyzed using JMP®, Version 13. SAS Institute Inc., Cary, NC, 1989–2019.

Table 1 RAUMPC relative area under the mortality progress curve, according to mortality percentage of thirty-two (year 2016) and thirty-three (year 2017) tested strawberry cultivars inoculated with *Macrophomina phaseolina* under natural screenhouse conditions

Trial (year)			
Cultivar	2016 (%)[a]	2017 (%)	
Peles	78.5 A[b]	23.8 A-D	
Florida90	78.3 A	42.3 A	
Gaviota	77.2 AB	26.2 AB	
Parker	76.6 AB	24.4 A-D	
San Andreas	74.3 A-C[c]	26.2 AB	
Yael	74.2 A-C	24.7 A-C	
Seascape	74.1 A-C	24.7 A-D	
Carmine	74.1 A-C	25.3 A-C	
Shaked	72.3 A-D	23.8 A-E	
Toq-Otome	72.1 A-D	26.5 A	
Rocky	71.2 A-D	26.1 A	
Douglas	68.3 A-E	25.1 A-C	
Chandler	66.2 A-F	27.2 A	
Gili	64.4 B-G	22 A-E	
Hadar	61.9 D-G	7.4 H	
S.Pantagruella	61.6 C-H	22.3 A-E	
Hadas	60.9 C-I	18.9 B-F	
Tamar	60.7 C-J	20.4 A-E	
Aya	59.3 D-J	18.1 C-G	
Shani	57.4 E-K	26.9 AB	*[d]
Camarosa	56.6 E-L	26.9 A	*
Daniel	55.8 E-L	16.4 E-G	*
ARO-line 196	53.8 F-L	26 AB	*
Dandi	53.7 F-L	25.3 A-C	*
Candonga	53.2 F-L	24.9 A-C	*
Yasmin	51.4 G-L	25.1 A-C	*
Angel	49.8 H-L	10.9 G-H	*
US70	47.6 I-L	27.2 A	*
Orly	47 J-L	11.8 F-H	*
Rotmy	44.2 KL	17.2 D-G	
Tamir	43.5 L	18.3 C-G	
Pelican	1.9 M	10.8 GH	
Festival	ND[e]	26.2 AB	
P Value	<.0001	<.0001	

[a]Disease severity (%), was evaluated weekly following inoculation using a five-degree ranking scale based on Koike et al. (2016), and was converted to percentage to express plant mortality

[b]Numbers with a common letter within each column indicate no significant differences at $P = 0.05$

[c]Hyphenated letters with the symbol (−) indicate the range between the first to last letters after statistical analysis (example: A-C indicates ABC, C-G indicates CDEFG etc.…)

[d]Asterixs represent cultivars that were not significantly different in susceptibility and/or tolerance between the two trials

[e]ND – not determined

Screening for tolerance of strawberry cultivars under screenhouse conditions

Data from both trials was subjected to analysis of variance [Student's t test ($\alpha = 0.05$)], since there was a significant difference between the two trials (summers of 2016 and 2017). The relative area under the mortality progress curve (RAUMPC in % × days) was therefore calculated separately. Comparison of RAUMPC means from each trial were compared in a linear regression for each cultivar. All data were analyzed using JMP®, Version 13. SAS Institute Inc., Cary, NC, 1989–2019.

Results

Evaluation of different inoculation methods for screening for tolerance/susceptibility in strawberry germplasm under controlled conditions

Three different inoculation techniques were assessed in order to determine the most reliable for screening for tolerance/susceptibility of germplasm to *M. phaseolina*. The experiment was conducted on five commonly used cultivars creating a 'ranking scale' of susceptibility to the disease, based on preliminary surveys conducted by extension specialists in the field. In all experiments, infected plants inoculated with *M. phaseolina* isolate M1, exhibited crown discoloration and the pathogen was recovered when surface-sterilized infected tissue was excised and plated onto Petri plates. Disease symptoms were not observed in any of the control plants. The toothpick-inoculated plants displayed high mortality rates after four days (Fig. 1a). By day eight all inoculated plants had completely collapsed with no significant difference in mortality between the five tested cultivars except for cultivar 'Hadar', exhibiting 80% mortality ($P = 0.05$). Artificially inoculated plants with growth medium mixed with naturally infected plant material (0.02 g infected crowns/ml soil) began to display symptoms four weeks after inoculation on average (Fig. 1b). A significant difference was recorded between the cultivars as expressed by a more rapid disease progress for the cultivars 'Dandi', 'Festival', 'Rocky' and 'Gili' in comparison to 'Hadar' that exhibited disease symptoms only from week twelve (Fig. 1b). Twenty-one weeks after inoculation 100% mortality of all cultivars was observed apart from the control plants. Artificial inoculation of plants with growth medium containing artificially produced sclerotia in a soil mix (concentration 2.5×10^3 sclerotia/g soil; after calibration, see next section) began to display symptoms in cultivars 'Rocky' and 'Gili' after two weeks on average, followed by 'Festival' at week three (Fig. 1c). Symptoms were observed in cultivar 'Dandi' from week five, however, at week six the average disease severity reached 60%, with a rapid increase. Cultivar 'Hadar' began to display symptoms at week seven and together with 'Gili' exhibited a relatively moderate increase in disease severity throughout the experiment (70% dead plants at week fourteen). A significant difference was recorded in the artificial inoculation of growth medium with artificially produced sclerotia in a soil mix, with the cultivar 'Hadar' exhibiting significant tolerance compared to the other cultivars, although after 18 weeks complete mortality of all the plants was observed ($P = 0.0004$). The cultivars 'Festival' and 'Dandi' were the most susceptible to disease regardless of the tested inoculation method, while 'Gili' and 'Rocky' represent cultivars of average susceptibility.

Calibration of the concentrations of artificially produced sclerotia (sc) in a soil mix

Four different concentrations were assessed to determine the most reliable concentration for screening for tolerance/susceptibility germplasm to *M. phaseolina* on the representative susceptible cultivar 'Gili'. All plants regardless of inoculum concentration, exhibited symptoms of disease throughout the experiment. All control plants remained healthy throughout the experiment (Fig. 2). At a concentration of 2.5×10^4 sc/g soil, all plants wilted and died within five weeks on average (mean RAUMPC score = 80%). At concentrations of 1.2×10^3 sc/g soil and 2.5×10^2 sc/g soil, similar plant mortality values were recorded as indicated by Tukey's HSD test (mean RAUMPC score = 43% and 38%, respectively; $P = 0.0049$), apart from the concentration of 2.5×10^2 sc/g soil that caused 90% plant mortality in the second trial. Furthermore, the concentration of 2.5×10^3 sc/g soil resulted in significantly different plant mortality percentages among tested cultivar 'Gili' and at faster rates compared with the other concentrations, therefore was chosen for the following experiments (mean RAUMPC score = 0.55%).

Screenhouse experiments

A total of 32 strawberry cultivars were assessed for screening susceptibility/tolerance against *M. phaseolina* under controlled conditions (Table 1). Experiments were conducted twice; in the summer of 2016 (trial 1) and 2017 (trial 2). In trial 1, a significant difference was recorded among the most susceptible and tolerant of the thirty-two tested cultivars ($P = 0.05$). The most susceptible cultivars were 'Peles' and 'Florida 90' (RAUMPC =78.5% and 78.3%, respectively) while cultivars 'Pelican', 'Tamir and 'Rotmy' were the most

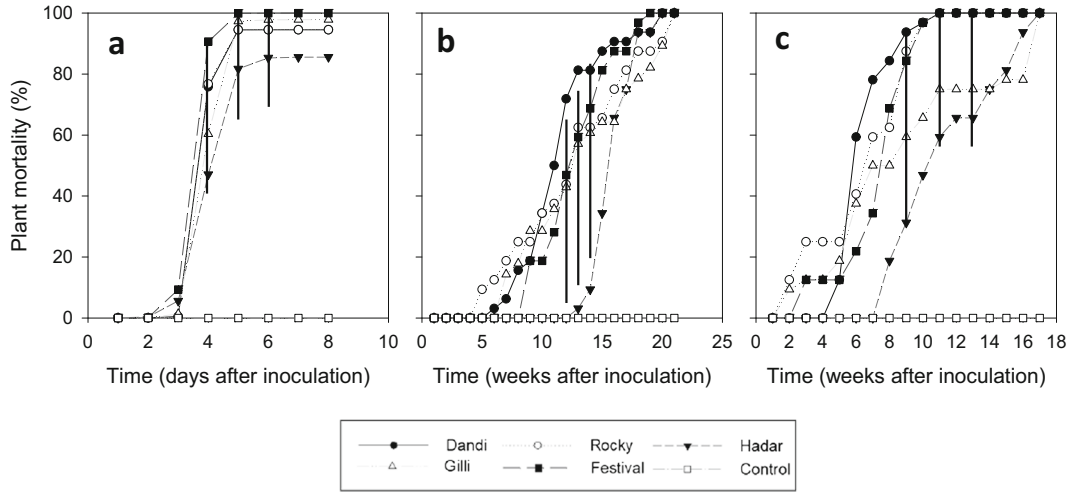

Fig. 1 Strawberry plant mortality (percent dead plants) over time (days/weeks after inoculation) using three different inoculation methods; (a) toothpick, (b) artificial inoculation of growth medium mixed with naturally infected plant material, and (c) artificial inoculation of growth medium with artificially produced sclerotia (concentration 2.5×103 sclerotia/g soil) in a soil mix, inoculated with isolate M1 of *Macrophomina phaseolina*. After inoculation, plants were maintained in growth chambers at constant temperatures of 30°C. Each data point represents the mean of 16 replicate plants. Vertical bars represent the least significant difference (α = 0.05) as determined by Tukey's HSD test.

tolerant (RAUMPC = 1.9%, 43.5% and 44.2% respectively) (Table 1). In trial 2, 33 cultivars were tested adding cultivar 'Festival' cultivar which was considered to be relatively susceptible according to preliminary and published evaluations, and as expected, was susceptible in this experiment (RAUMPC = 26.2%). Furthermore, a significant difference was found among the most susceptible and tolerant of the thirty-three tested cultivars ($P = 0.05$). The most susceptible cultivar was 'Florida 90′, similar to trial 1 (RAUMPC = 23.8%) whereas cultivars 'Hadar', 'Pelican' and 'Orly' were the most tolerant (RAUMPC = 7.4%,10.8% and 11.8% respectively).

In order to assess the effects of both trials on the tested cultivars, the mean RAUMPCs of each cultivar in each trial was combined in a linear regression (Fig. 3). Eleven cultivars were inconsistent in mean RAUMPC values, and were responsible for the significant difference between trials. Cultivars that were inconsistent in

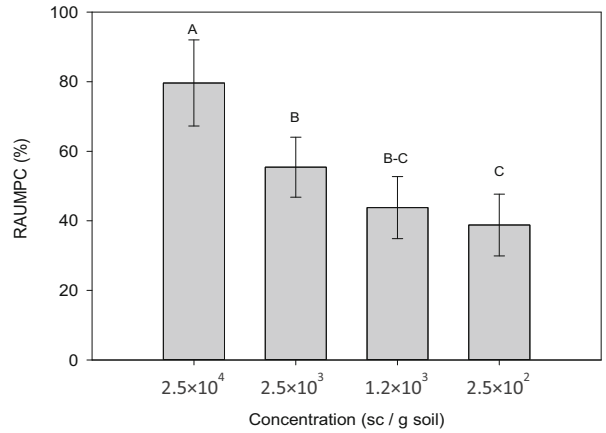

Fig. 2 RAUMPC, relative area under the mortality progress curve for four concentrations of sclerotia/g soil (2.5×10^4, 2.5×10^3,1.2×10^3 and 2.5×10^2 sc/g soil) of *Macrophomina phaseolina*, artificially inoculated in a soil mix. After inoculation, plants of the representative susceptible cultivar 'Gili' were maintained in growth chambers at a constant temperature of 30°C. Each bar represents the mean and standard error of 16 replicate plants per concentration. Values in each column followed by a common letter are not significantly different ($P < 0.05$) according to Tukey's HSD test

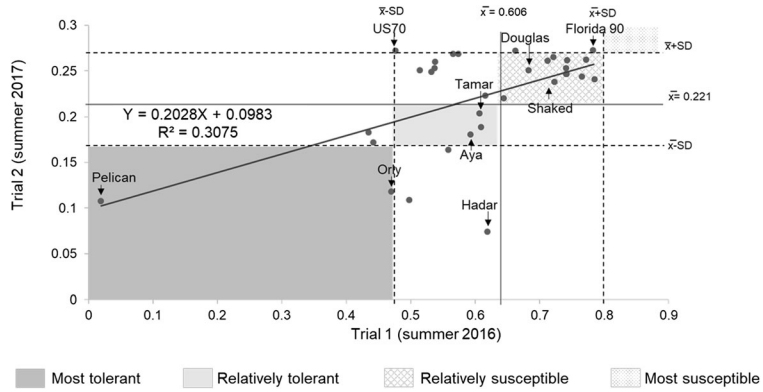

Fig. 3 Linear regression (black solid line) in a scatter plot according to the mean of each strawberry cultivar in both trials tested for tolerance/susceptibility to *Macrophomina phaseolina* under outdoor, screenhouse conditions. The solid grey line represents the mean ($\bar{x}$) of each trial. The dashed black line represents the variance of means for each trial. The plants were cultivated outdoors under similar conditions as in the field, with temperatures ranging from 35 to 40°C during the day and 20-25°C at night.

both trials appear far from the linear regression, such as 'US70' and 'Hadar'. Nevertheless, the majority of cultivars (twenty-one) were consistent throughout the experiments, thus demonstrating a correlation of $R = 0.554$ between trials, indicating a significant difference in the tolerance/susceptibility of the majority of tested strawberry cultivars to *M. phaseolina*.

Discussion

Macrophomina phaseolina was first encountered in strawberry in Israel and other Mediterranean countries parallel to the phase-out of methyl bromide, a pre-plant treatment used for soil disinfestation (Angelini and Nad Faedi 2010; Avilés et al. 2008; Zveibil and Freeman 2005). Therefore, the elimination of methyl bromide is considered the main reason for recent outbreaks of the disease in strawberry worldwide (Albajes et al. 1999; Koike 2008; Mertely et al. 2005; Zveibil et al. 2012). The elevated temperatures that prevail in the Mediterranean region are suitable for development of *M. phaseolina*, thus new approaches are required for disease management. Since the current soil disinfestation methods are not effective enough for eradication of *M. phaseolina* in infested soils, the most reasonable approach is the use of tolerant/resistant germplasm.

The present study identified a reliable inoculation technique that enabled screening of thirty-three strawberry cultivars for tolerance/susceptibility to *M. phaseolina* under natural outdoor conditions in the screenhouse. The commonly used 'toothpick' method was found to be invasive and unreliable for screening as all plants, regardless of cultivar, collapsed and died within one week (Fig. 1a). An additional method that was evaluated in our study was artificial inoculation of plants with growth medium mixed with naturally infected plant material. Similarly, Baggio et al. (2019) evaluated the ability of strawberry crowns to serve as a source of inoculum for the following year's crop by inserting infected crowns into the soil and found this inoculation method to be successful. However, in our study, this method required a longer period to initiate disease symptoms (overall 22 weeks, Fig. 1b) compared to other methods involving pure culture inoculum. Nevertheless, the five tested cultivars were not differentiated in susceptibility/tolerance by this method, and considering the lack of ability to create an even and equal distribution of inoculum for the tested plants, this inoculation method was discontinued. This further emphasizes the importance of choosing the most reliable inoculation method. The third method using artificial inoculation of growth medium with artificially produced sclerotia in a soil mix, proved to be reliable, resulting in significant variability among the five tested strawberry cultivars after 18 weeks (Fig. 1c). This method was also concentration-dependent and required calibration of the concentrations of sclerotia, thus becoming an even more reliable inoculation method. Of the four concentrations tested (2.5×10^2, 1.2×10^3, 2.5×10^3 and 2.5×10^4 sc/g soil), 2.5×10^3 sc/g soil was the most effective allowing reliable differentiation between cultivars. Higher concentrations of inoculum resulted in total collapse of the plants within five weeks, while lower concentrations did not cause significant mortality of plants within the tested time period of 20 weeks (Fig. 2).

Therefore, all further experiments conducted in this study, including the screening of susceptible/tolerant strawberry germplasm in the screenhouse under natural conditions, were conducted using an inoculum concentration of 2.5×10^3 sc/g soil. To the best of our knowledge, this study is the first to describe this inoculation method after evaluating its reliability compared to various methods used in other studies. This is also the first time that different inoculum concentrations were tested, thus, raising the question of screening of cultivars using less reliable methods or uncalibrated concentrations and accuracy of results. For example, Avilés et al. (2009) evaluated two inoculation techniques, the 'toothpick' and infested oat seeds methods on three common strawberry cultivars, and found that the most tolerant cultivar to *Macrophomina* was 'Camarosa'. Similar to our study, the authors found the 'toothpick method' to show no evidence of germplasm tolerance to the pathogen, while the second inoculation technique exhibited significant differences in strawberry germplasm susceptibility and/or tolerance over a prolonged period of approximately one month. However, Gomez et al. (2020) reported that 'Camarosa was similarly susceptible to 'Albion', based on an inoculation technique using a blended PDA microsclerotia mix of a single concentration (1.4×10^3 sclerotia/mL) of 50 mL drenched into a soil mix. This is in contrary to the results of Fang et al. (2012), which may be associated to varying inocula concentrations and volumes, inoculation techniques and environmental conditions. Sánchez et al. (2016) evaluated two artificial inoculation methods: (i) infested oat seeds, and (ii) a suspension of sclerotia although not concentration dependent, and found 'Camarosa' to be tolerant, opposed to results found in our study (Table 1). On the other hand, Koike et al. (2016) reported that 'Camarosa' was susceptible using the 'toothpick method' after inoculating five different strawberry cultivars even though, after eight weeks, no evidence of tolerant or susceptible cultivars was found.

Germplasm screening under screenhouse conditions indicated a high variation of plant susceptibility to the pathogen with the tested cultivars. Cultivars 'Pelican' (used in a US breeding program of the Southern Eastern USA; Smith et al. 1998), and 'Orly', 'Tamir' and 'Rotmy' (commonly used commercial cultivars in Israel), were the most tolerant to *M. phaseolina*. In contrast, 'Florida 90' (a US cultivar) and 'Peles' (an Israeli cultivar) were the most susceptible. This indicates that the chosen inoculation method of artificial inoculation with sclerotia in the soil mix could be comparable to natural infections taking place under natural outdoor conditions, as demonstrated in the screenhouse trials. Similar to our work, Baggio et al. (2019) found that 'Festival' was highly susceptible, 'Florida Beauty' moderately susceptible and 'Winterstar' moderately resistant. Interestingly, cultivar 'Peles', which was considered to be susceptible in trial 1, was located in the middle of the scale in trial 2. Cultivar 'Camarosa' which was the most tolerant cultivar in the study conducted by Avilés et al. (2009) and among the susceptible cultivars in the study conducted by Fang et al. (2012) and Gomez et al. (2020), was also relatively susceptible in our study.

Screening for tolerance/susceptibility in the screenhouse resulted in significant differences between the two trials. Even though the trials were inconsistent for eleven cultivars, the majority of the tested cultivars, twenty-one of thirty-two, resulted in a significant correlation between the two trials ($R = 0.554$), thus strengthening the assumption of the existence of a large variation between different strawberry cultivars (Fig. 3). The mean RAUMPC for the first trial was 60.7% whereas the second trial was 22.3%. We assume that the inconsistency is a result of differentiation in climatic conditions and heat stress under natural conditions that prevailed in the screenhouse, which may have resulted in different vulnerability levels after infection. At present, no *Macrophomina*-resistant Israeli strawberry germplasm has been found although there have been reports of QTL discovery for resistance to the pathogen in the US (Nelson et al. 2019). However, this comprehensive study strongly indicates that variation exists in strawberry and demonstrates that two relatively tolerant cultivars, 'Pelican' and 'Orly', may be used in future breeding programs in Israel and elsewhere, in order to develop resistant/tolerant strawberry germplasm.

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