agronomy

MDPI

Article

Investigation of Seed transmission in *Peronospora belbahrii* the Causal Agent of Basil Downy Mildew

Lidan Falach-Block, Yariv Ben-Naim and Yigal Cohen *

Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan 5290002, Israel; lidanf1@walla.co.il (L.F.-B.); yar2710@gmail.com (Y.B.-N.)

* Correspondence: ycohen@biu.ac.il; Tel.: +972-35318251

Received: 9 April 2019; Accepted: 20 April 2019; Published: 23 April 2019

Abstract: Downy mildew in sweet basil (*Ocimum basilicum* L.) caused by the oomycete pathogen *Peronospora belbahrii* Thines was first recorded in Israel in 2011. Within one year, the pathogen has spread all over the country, causing devastating economic damage to basil crops. Similar outbreaks were reported in Europe, the USA, and Asia. Seed transmission and seedling trade were suggested as possible explanations for this rapid spread. Here, we show that *P. belbahrii* can develop systemically in artificially inoculated basil plants in growth chambers. It may reach remote un-inoculated parts of the plant including the axillary buds but not the roots or seeds. To verify whether transmission of the disease occurs via seeds, we harvested seeds from severely infected, field-grown basil plants. Harvests were done in four seasons, from several basil cultivars growing in three locations in Israel. Microscopic examinations revealed external contamination with sporangia of *P. belbahrii* of untreated seeds, but not of surface-sterilized seeds. Pathogen-specific PCR assays confirmed the occurrence of the pathogen in untreated seeds, but not in surface-sterilized seeds. Contaminated seeds were grown (without disinfection) in pasteurized soil in growth chambers until the four–six leaf stage. None of several thousand plants showed any symptom or sporulation of downy mildew. PCR assays conducted with several hundred plants grown from contaminated seeds proved no latent infection in plants developed from such seeds. The results confirmed that (i) *P. belbahrii* can spread systemically in basil plants, but does not reach their roots or seeds; (ii) sporangia of *P. belbahrii* may contaminate the surface, but not the internal parts, of seeds produced by infected basil plants in the field: and (iii) contaminated seeds produce healthy plants, which carry no latent infection. The data suggest that *P. belbahrii* in Israel is seed-borne, but not seed-transmitted.

Keywords: basil downy mildew; epidemiology; oomycetes; over-seasoning; systemic infection

1. Introduction

Sweet basil (*Ocimum basilicum*) is an economically important herb due to its pleasant aroma and taste. Basil downy mildew (BDM) caused by the oomycete *Peronospora belbahrii* is currently the most destructive disease of sweet basil worldwide [1]. It was first reported in Uganda in 1932 [2], then emerging in Switzerland in 2001 [3], USA in 2009 [4], and in Israel in 2011 [5]. Typically, the disease appears in the field as chlorotic lesions on leaves, which gradually turn necrotic and abscise. Under favorable humid conditions, a characteristic purplish, furry growth of sporangiophores, and sporangia is evident on the lower epidermis of infected leaves. Oospores were erratically seen inside the mesophyll of infected leaves [5].

The reasons for the sudden appearance and quick global spread of the pathogen are not known. They may be related to infected seeds and/or trade of basil cuttings [1,3,6]. Another reason may be related to latent infection of *P. belbahrii* in basil plants [7]. Latent infection is known to occur in other downy mildews such as *Peronospora halstedii* in sunflowers [8] and *Peronospora effusa* in spinach [9].

Garibaldi et al. [6] and Farahani-Kofoet et al. [7] reported on systemic infection of this pathogen and its transmission by seeds. Garibaldi et al. [6] tested basil seeds samples from various sources in two locations in Italy and observed downy mildew in 4 out of 16 samples at 0.33%–0.66% incidence of infected plants. Farahani-Kofoet et al. [7] detected *P. belbahrii* by PCR in 80%–90% of randomly investigated commercial seed stock samples. Wick (see Wyenandt et al. [10]) recovered sporangiophores and sporangia in wash water of basil seeds (previously tested positive for BDM by PCR). Gilardi et al. [11] and Pintore et al. [12] used isolates of *P. belbahrii* that were originated from infected basil seeds. Wyenandt et al. [13] demonstrated that basil seeds, regardless of basil species and whether symptoms are visible on foliage or the plant is immune or resistant to downy mildew, can test positive for the presence of *P. belbahrii* using a real-time PCR assay following exposure of plants to the pathogen during the natural development of downy mildew under field conditions. Wyenandt et al. [13] did not germinate the seeds to follow whether or not downy mildew develops on the seedlings. None of the above studies, however, reported on the occurrence of *P. belbahrii* inside the seed/embryo of basil.

The downy mildews constitute a distinct fungal-like group due to their molecular, morphological and epidemiological characteristics. Some cause local lesions only (e.g., *Pseusoperonospora cubensis* in cucurbits, *Bremia lactuca* in lettuce, *Peronospora manshurica* in soybean), while others cause systemic infection (e.g., *Scleropora sorghi* in maize, *Pseudoperonospora humuli* in hop, *P. halsdedii* in sunflower) [14]. Seed transmission is known to occur in systemic downy mildews such as sunflower downy mildew caused by *P. halstedii* [15] as well as in non-systemic downy mildews such as cucurbit downy mildew caused by *P. cubensis* [16].

The aim of the present study was to examine the ability of *P. belbahrii* to spread systemically in basil plants, with the premise that systemic spread may allow the pathogen to reach the seeds and thus enables seed infection and transmission of the disease. We also examined the ability of *P. belbahrii* to cause latent infection in basil plants. Latent infection may explain the rapid global spread of the disease. We used symptoms, sporulation, microscopy and PCR to follow the presence of the pathogen in seeds and plants.

2. Materials and Methods

2.1. Pathogen

The systemic spread of the pathogen in basil plants was studied with isolate Knafo-3 of *P. belbahrii*. This isolate was collected in 2013 from infected basil plants on the farm of A. Knafo in Ein-Tamar, Southern Jordan Valley, Israel. It is resistant to mefenoxam, but sensitive to mandipropamid, dimethomorph, and oxathiapiprolin. This isolate is very aggressive to all sweet basil varieties grown in the country but avirulent to varieties carrying the *Pb1* resistance gene [17]. We maintained it by repeated inoculations of Sweet Basil plants in growth chambers at 25 °C.

2.2. Plants

The systemic spread of the pathogen was studied with the susceptible *Ocimum basilicum* 'Sweet Basil' (Genovese type, Genesis Seeds Ltd., Ashalim, Israel). Plants were grown in 250 mL pots filled with peat/perlite, 1:1, *v*/*v*, one plant per pot. Sowing was done at weekly intervals in order to obtain plants of different ages. Plants were grown in the greenhouse and used when reached the desired age. Seeds were always stored in aluminum bags at 4 °C.

2.3. Inoculation

Sporangia of *P. belbahrii* were washed from freshly sporulating leaves into cold distilled water and the suspension was adjusted to 1×10^4 sporangia/mL. One 10 µL droplet of spore suspension was inoculated onto the upper surface of each cotyledon or leaf of plants of different ages as demonstrated in Figure 1. Control plants were similarly treated with tap water. The control and inoculated plants were placed overnight in a dew chamber at 18 °C in the dark to ensure infection. Care was taken to

avoid any contact between the plants. The plants were then placed in a growth chamber at 25 °C (60%–70% RH) under continuous illumination (60 µmol m^2 s^{-1}) to allow for symptom production. At 7–14 days post inoculation (dpi), symptoms were recorded and the plants were placed overnight at 100%RH at 20 °C in darkness to allow for sporulation of the pathogen.

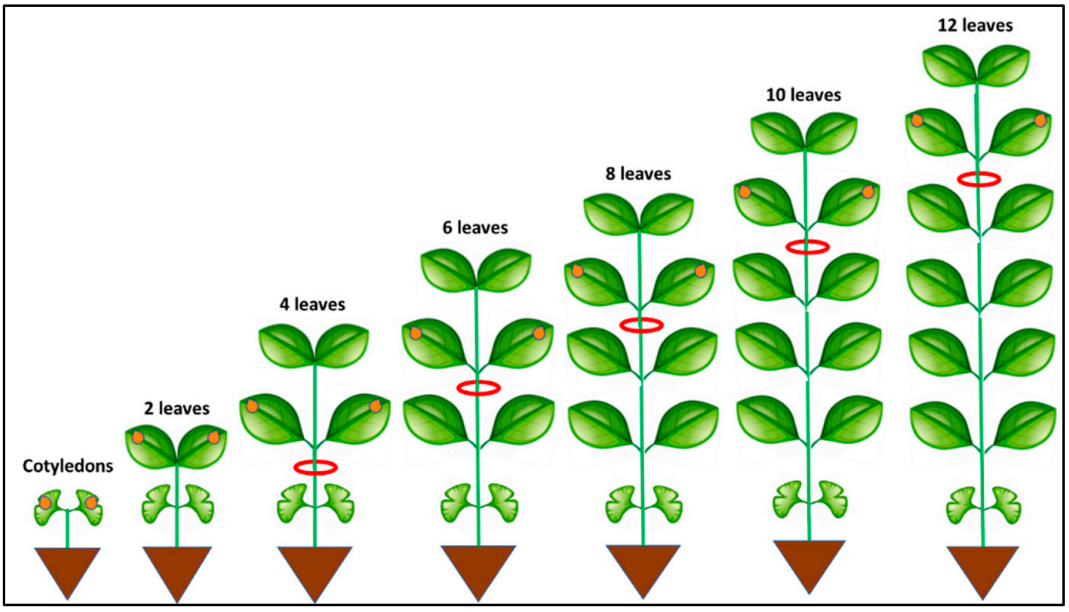

Figure 1. Experimental design for testing the systemic spread of *Peronospora belbahrii* in basil plants. Plants of various ages, from the cotyledons stage to the 12-leaf stage, were inoculated on two leaves per plant with two 10 µL droplets (orange dots) each containing 100 sporangia of the pathogen. The inoculated pair of leaves were located above the red ring on the stem. The inoculated plants were incubated in a dew chamber to allow infection and thereafter at 25 °C to develop systemic infection. Water-inoculated plants served as controls.

2.4. Disease Assessment

Disease symptoms and sporulation were visually estimated at 7, 10, or 14 dpi, as described before [18], depending on plant age at time of inoculation.

2.5. Microscopy

Stem internodes were excised from the inoculated plants and processed as described before [19]. Briefly, stem internodes (1 cm long) were sectioned longitudinally with a razor blade into thin (about 0.5 mm) slices. They were clarified in boiling ethanol, placed in aniline blue solution (0.05% aniline blue in 0.05 M K_2HPO_4, pH 8.9) at 4 °C for 24 h, stained with 0.01% calcofluor (Sigma) and examined with an Olympus A70 epi-fluorescent microscope. Fungal structures inside the stem showed green-yellow fluorescence. To observe the pathogen on seeds, untreated seeds were placed (5 seeds per group, 10 groups per seed batch) on a glass slide, 20 µL sterile water were added per group, and seeds were to covered with a glass cover slip. Seeds were examined at 30 min after preparation with a dissecting microscope at ×400 magnification.

2.6. DNA Extraction

DNA was extracted using a modified CTAB-protocol [20]. Stem internodes, hypocotyls, roots, axillary buds, apices, whole seedlings or seeds were placed in aluminum foil and frozen in liquid nitrogen. Special precautions (changing gloves and disinfesting cutting tools) were taken to prevent cross-contamination of samples. The samples were groundto powder with a mortar and pestle and collected into 1.5 mL Eppendorf tubes. CTAB extraction buffer (600 µL, 100 mM Tris pH 8, 1.4 M

sodium chloride, 20 mM EDTA, 2% CTAB, 0.5% mercaptoethanol) was added, and the tubes were vortexed and incubated at 65 °C for 60 min. Chloroform-isoamyl alcohol (400 µL, 24: 1) was added, the tubes were vortexed for 1 min and centrifuged at 11,200 rpm for 15 min. The supernatant was transferred into a new 1.5 mL Eppendorf tube, 500 µL of chloroform-isoamyl alcohol (24: 1) and 400 µL of CTAB extraction buffer were added, the tubes were vortexed for 1 min and centrifuged at 11,200 rpm for 15 min for the second time. The supernatant was transferred into a new 1.5 mL Eppendorf tube, 500 µL of isopropanol was added, the tubes were vortexed and placed in −20 °C overnight. The tubes were centrifuged at 11,200 rpm for 15 min, and the DNA pellet was rinsed with 500 µL ice-cold 70% ethanol for 5 min in room temperature. The tubes were centrifuged at 11,200 rpm for 15 min, and the DNA pellet was dissolved in 50-µL HPLC water, incubated at 65 °C for 5 min and placed in −20 °C. DNA was similarly extracted from sporangia of *P. belbahrii.*

2.7. PCR

Detection of the pathogen was done with the following specific primers of the genomic ribosomal DNA (ITS1) of *P. belbahrii* [3]:

Bas-F: CCGTACAACCCAATAATTTGGGGGTTAAT
Bas-R: TTCAATTAGCTACTTGTTCAGACAAAG)

This pair produces a single DNA band of approximately 134 base pairs in size. The total volume of the reaction mixture was 15 µL and contained 7.5 µL Hy-Taq Ready Mix (2×), 0.3 µl of each Bas primer, 5.9 µL of nuclease-free water, and 1 µL of DNA sample (80–100ng/µL) or HPLC water for negative controls. PCR amplification was performed in the MultiGene^{TM} OptiMax Thermal Cycler (Labnet International Inc., Edison, NJ, USA) with the following cycle parameters: Initial denaturation at 95 °C for 5 min, 30 cycles of denaturation at 95 °C foe 30 sec, annealing at 63 °C for 30 sec, extension at 72 °C for 30 sec and termination at 72 °C for 5 min. The PCR products were separated by 1% agarose gel electrophoresis in TBE buffer (40 mM of Tris-borat, pH 8.0, 1 mM of EDTA) at 130 V for 40 min and stained with ethidium bromide. The Gene Direx (Taoyuan, Taiwan) 100 bp DNA ladder was run on the same gel. The gel was placed under UV light in order to identify the DNA bands.

2.8. Direct Inoculation of Seeds and Roots

To examine the ability of sporangia to cause infection of seeds, commercial seeds ('Sweet Basil') were sown in pots containing moistened pasteurized potting mixture, 10 seeds per pot. Seeds were inoculated by placing a 50 µL droplet of fresh sporangial suspension (1×10^4 sporangia/mL) on each seed before covering with the potting mixture. To examine the ability of sporangia to cause infection of roots, 10 mL of fresh sporangial suspension (1×10^4 sporangia/mL) were drenched onto the soil surface of potted basil plants at the cotyledon or 2-leaf stage (2–5 plants per 250 mL pot). Pots and plants were incubated in a growth chamber at 20 °C (60%–70% RH, 14h light/day, 60 µmol m^2 s^{-1}) and seedlings were examined visually for disease symptoms during 4 weeks after inoculation. Thereafter, PCR assays were performed to determine whether the pathogen is present in the plants in a latent form.

2.9. Seed Infection and Transmission

Multiple experiments were conducted to study seed infection and disease transmission via basil seeds. Three cultivars of basil were studied during 2012–2017: 'Peri' (Genovese cutting type, Volcani Center for Agricultural Research, Israel), 'Sweet Basil' (Genovese type, Genesis Ltd., Ashalim, Israel), and 'Genoveser' (broad Italian leaf type, Ball Straathof, South Africa).

Two seed samples (Peri) were obtained in summer 2012 from Hishtil Nurseries Ltd. (Petach Tikva, Israel). The first sample was harvested from healthy basil plants and the other from BDM-infected basil plants. Seed samples were assayed by PCR (3 × 50 seeds per sample) for the presence of *P. belbahrii* and other seeds (~1000 seeds per sample) were grown in pasteurized soil in growth chambers to the 4-leaf stage, and examined for symptoms of BDM and for the presence of *P. belbahrii* by PCR.

In Mid-March of 2016 and 2017, about five hundred 12-leaf basil plants ('Sweet Basil') were planted in a 6 × 50 m net houses (covered with a 50 mesh white plastic net) located at BIU farm on campus. The plants were spray inoculated with spore suspension of isolate Knafo 3 of *P. belbahrii* in early April in both years, after they had reached the 18–20 leaf stage. Symptoms including sporulation were first observed at 7 and 9 days post inoculation in 2016 and 2017, respectively. Temperature was favorable for disease development [21] in both years. The disease reached the top of the plants in mid-June at about flowering time.

To examine seed infection and BDM transmission, seeds (including the petals and calyx) were harvested in July of both years. In 2016, about 5000 seeds were collected from about 100 infected plants. The floral parts were removed and two subsamples of 150 seeds were taken for DNA extraction. The other seeds (about 4700) were sown in pasteurized soil in pots, 10 seeds per pot. About 1000 commercial seeds (Sweet Basil and Peri) were similarly sown as controls. Plants were grown in a growth chamber at 22 °C (60%–70% RH, 14 h light a day, 60 μmole.m^{-2} s^{-1}). When they reached the 4-leaf stage, plants were examined for symptoms of BDM and then assayed by PCR, 15 samples of 10 plants per sample from each seed batch, for the presence of pathogen in their tissues.

In 2017, about 13,700 seeds were harvested from about 200 infected plants. They were divided into two batches: seeds of one batch (~9400 seeds) were separated from their floral parts (petals and calyx) and sown in pasteurized potting mixture without disinfection, 10 seeds per pot. Seeds of the other batch (~4300) were similarly sown, but with their petal and calyx (carrying sporangia) attached. About 1000 commercial seeds (Sweet Basil and Peri) were similarly sown as controls. Plants were grown as above and when reached the 4–6 leaf stage they were examined for symptoms and sporulation of BDM and then taken for PCR assays, 5–22 samples of 10 plants per sample from each seed batch.

In 2018, seeds were collected from the following five basil cultivars growing at Ashalim in southern Israel: Emily 42, Emily 47, Emily 201, Edwina 5 (broaden Italian leaf), and an experimental line 1241 (Genovese type). Plants were all heavily infected with an isolate called "M" of *P. belbahrii* at time of harvest. Unlike isolate Knafo 3, isolate M was pathogenic to also line 1241, which carries the *Pb1* gene for resistance.

To detect *P. belbahrii* in contaminated seeds (seeds collected from infected plants), two samples of 150 from each cultivar in each year were taken for DNA extraction. Seeds of one sample were disinfected to remove sporangia attached to their surface (one min soaking in 3% hypochlorite followed by washing with sterile water) before extraction. Disinfection did not affect seed germination. Seeds of the other sample were extracted with no previous treatment.

3. Results

3.1. Systemic Infection

Systemically infected plants were those that exhibit chlorosis and sporulation of the pathogen in their top leaves including the apex. The results showed a negative correlation ($R^2 = -0.9653$) between plant age (leaf number) at time of inoculation and systemic infection by the pathogen (Table 1). Almost all plants (97.3%) inoculated at the cotyledon stage became systemically infected at 7dpi. A gradual decrease in the proportion of systemically infected plants occurred in older plants. Thus, inoculation at the 2, 4, 6, 8, 10, and 12-leaf stage produced about 96, 88, 70, 62, 32, and 27% systemically infected plants, respectively (Table 1). Neither disease symptoms, nor sporulation was seen on the control plants inoculated with water. All plants that were inoculated at the 2–4 leaf stage also showed systemic infection in their cotyledons at 7 dpi.

Table 1. The relationships between plant age and systemic spread of *Peronospora belbahrii* in basil plants. For the method of inoculation, see Figure 1.

Plant Age at Time of Inoculation	Number of Inoculated Plants	Assessment Time	Number of Systemically Infected Plants	% Systemically Infected Plants
Cotyledons	147	7 dpi	143	97.28
2 leaves	156	7 dpi	149	95.51
4 leaves	159	7 dpi	140	88.05
6 leaves	113	10 dpi	79	69.91
8 leaves	117	10 dpi	72	61.54
10 leaves	229	14 dpi	73	31.88
12 leaves	117	14 dpi	31	26.50

3.2. Microscopy

Microscopic examination of free-hand stem sections revealed the presence of mycelia and haustoria of *P. belbahrii* in the systemically infected plants. The mycelium colonized the intercellular spaces of the vascular parenchyma adjacent to the xylem and the phloem vessels, and produced haustoria inside the parenchyma cells (Figure 2).

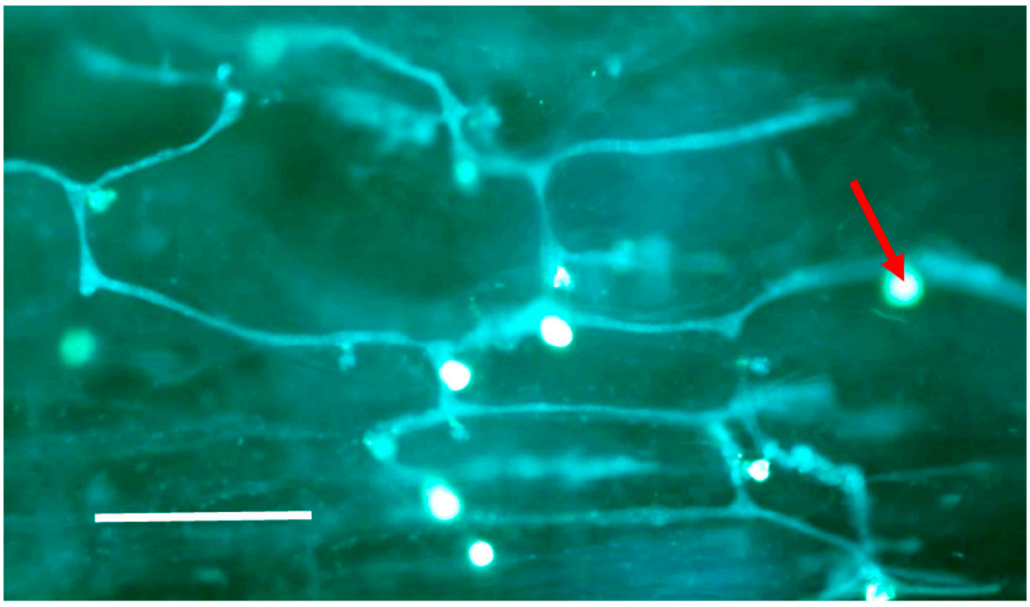

Figure 2. A photomicrograph showing mycelium with haustoria (arrow) of *P. belbahrii* inside the stem of a systemically infected basil plant. Bar = 50 µm.

3.3. PCR

DNA was extracted from the apex, stem, petiole and hypocotyl of eight systemically infected plants, which were inoculated at the of 4-leaf stage. The specific DNA fragment 134bp (3) was detected in all four organs of all eight plants (Figure 3). No such band was detected in the water-inoculated, healthy control plants. The presence of *P. belbahrii* in the apex, and surprisingly, in the hypocotyl, suggested that the pathogen grew systemically in both directions, acropetal and basipetal. We failed to detect the pathogen in the root of systemically infected plants by either microscopy or PCR.

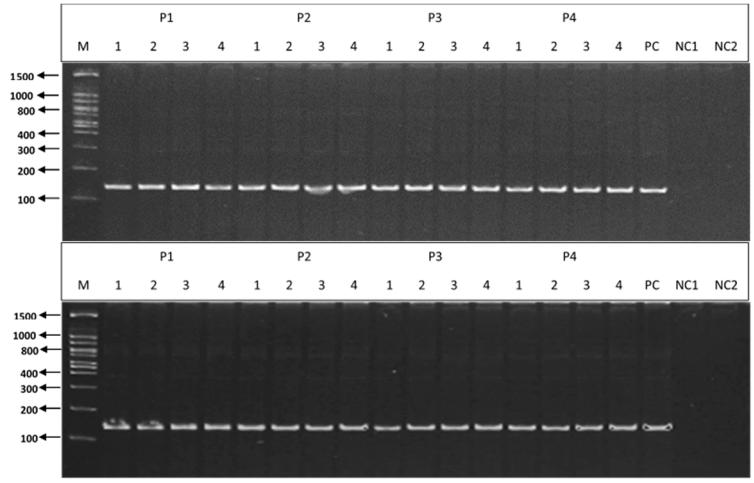

Figure 3. Detection of *P. belbahrii* in various organs of systemically infected basil plants that were inoculated at the 4-leaf stage. P1–P4: plant number. M—molecular markers (100–1500 bp). Lanes 1, 2, 3 and 4: PCR amplification products obtained from the apex, stem, petiole and hypocotyl, respectively. PC-positive control, amplification product (134bp) obtained from sporangia of *P. belbahrii.* NC1-negative control, amplification product obtained from leaves of control healthy basil plants. NC2-negative control-water.

3.4. Axillary Bud Infection

Plants that were inoculated at the 4–12 leaf stage and became systemically infected, produced systemic infection in the axillary buds sprouting either below (Figure 4) or above (not shown) the inoculated leaves. Plants that were inoculated at cotyledon or the 2-leaf stage were severely stunted and failed to produce axillary buds.

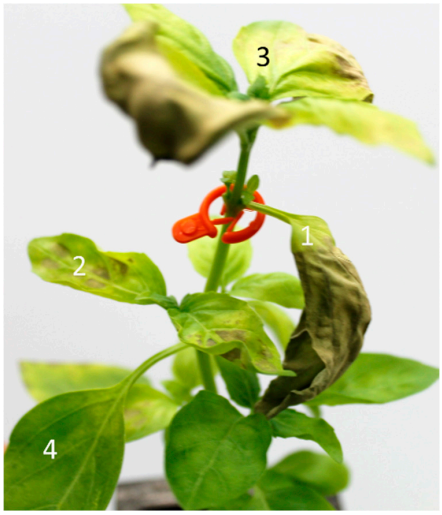

Figure 4. Symptoms of downy mildew in axillary buds of a basil plant. 1—Originally inoculated leaf (the other leaf was abscised). The red ring indicates the location of the originally inoculated leaves. 2—An infected axillary bud. 3—Infected apex. 4—Mild symptom on a leaf below the axillary bud.

3.5. Root Infection

Seeds or plants inoculated via the soil failed to produce BDM-symptomatic plants, nor did they exhibit, after disinfection with hypochlorite and washing with sterile water, the 134 bp DNA band of the pathogen, suggesting that the root system does not serve as an entrance court for the pathogen.

3.6. Seed Infection and Seed Transmission

Systemically infected plants failed to flower, and therefore produced no seeds (Figure 5). Hence, we studied seed infection and seed transmission in plants that grew from contaminated seeds. We harvested such seeds from BDM-infected plants growing in the field during the summer season of 2012–2018 (Table 2, Figure 6).

Table 2. The origin of basil seeds used in this study.

Experiment	Year	Cultivar	Supplier	Production	Harvest	Isolate	Calyx
1	2012	Peri	Volcani center	Petah Tikva, IL	healthy		
2	2012	Peri	Volcani center	Petah Tikva, IL	infected	Unknown	removed
3	2016	Sweet Basil	Genesis	Bar Ilan University, IL	infected	Knafo 3	removed
4	2016	Peri	Volcani center	Newe Yaar, IL	healthy		
5	2016	Sweet Basil	Genesis	Ashalim, IL	healthy		
6	2017	Sweet Basil	Genesis	Bar Ilan University, IL	infected	Knafo 3	removed
7	2017	Sweet Basil	Genesis	Bar Ilan University, IL	infected	Knafo 3	attached
8	2017	Peri	Volcani center	Newe Yaar, IL	healthy		
9	2017	Sweet Basil	Genesis	Ashalim, IL	healthy		
10	2017	Genoveser	Ball Straathof	South Africa	healthy		
11	2018	Five cultivars *	Genesis	Ashalim, IL	infected	M	removed

* Emily 42, Emily 47, Emily 201, Edwina 5 and 1241.

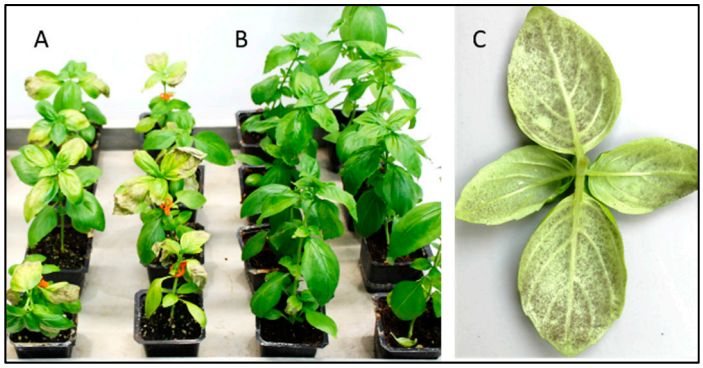

Figure 5. The effect of systemic infection with *P. belbahrii* on growth of basil plants. (**A**)—Systemically infected plants that were inoculated at the 4-leaf stage remained stunted. (**B**)—Healthy, water-inoculated control plants. (**C**)—Sporulation of *P. belbahrii* on the lower leaf surfaces of top leaves taken from a systemically infected plant shown in (**A**). The photos were taken at two weeks after inoculation.

(A) (B)

Figure 6. The appearance of downy mildew in flowering basil plants in the field. Seeds were harvested from such plants to examine seed infection and transmission of *P. belbahrii*. (**A**)—A flowering basil plant infected with downy mildew. (**B**)—A close-up photo of the apex, showing heavy sporulation of *P. belbahrii*.

Microscopic observations revealed many sporangia embedded in the mucin secreted by contaminated seeds upon instant imbibition in water. The DNA extracted from such contaminated seeds exhibited PCR amplification products of *P. belbahrii* (Figure 7). No such amplification products were produced by the DNA extracted from seeds harvested from healthy plants (Figure 7). Disinfection of contaminated seeds with 3% hypochlorite solution followed by washing with sterile water removed the sporangia attached to the contaminated seeds. The DNA extracted from such disinfected seeds exhibited no PCR amplification products of *P. belbahrii*.

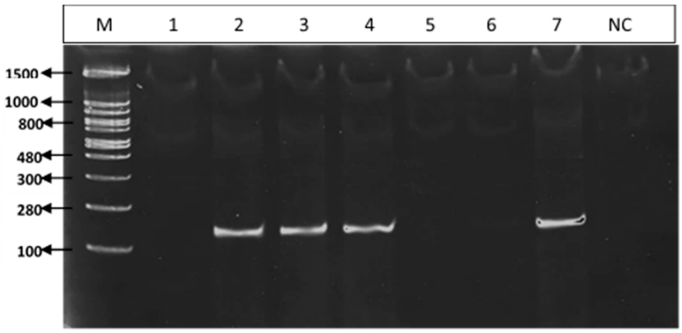

Figure 7. Detection of *P. belbahrii* in contaminated basil seeds. M—Molecular markers (100–1500 bp). Lanes 1–8: PCR amplification products obtained from: Lane 1—Seeds from healthy plants (Peri) harvested in 2012. Lane 2—Seeds from infected plants (Peri) harvested in 2012. Lane 3—Seeds from infected plants (Sweet Basil) harvested in 2016. Lane 4—Seeds from infected plants (Sweet Basil) harvested in 2017. Lane 5—Seeds from healthy plants (Sweet Basil) harvested in 2016. Lane 6- Seeds from healthy plants (Sweet Basil) harvested in 2017. Lane 7—Positive control, PCR amplification product (134 bp, arrow) obtained from sporangia of *P. belbahrii.* NC—negative control, water.

Contaminated seeds (not disinfected) were sown in pasteurized soil and grown for about 4 weeks in growth chambers until the 4-6 leaf stage in an environment conducive for colonization but not for infection or sporulation of *P. belbahrii* (1). Mean germination rate of these seeds exceeded 90%. None of the 900 contaminated seeds sown in 2012 produced plants with symptoms of BDM nor did these plants showed sporulation of *P. belbahrii* upon incubation in a dew chamber at 20 °C (Table 3). Similar results were obtained with 4700, 9400, 4300, and 2500 seeds sown in 2016, 2017, 2017 (sown with floral parts attached), and 2018, respectively (Table 3). From the 21,800 contaminated seeds sown, 19,620 plants developed. No plant show symptoms of BDM or sporulation of *P. belbahrii* (Table 3). Furthermore, none of the plant samples assayed with PCR at the 4–6 leaf stage showed the DNA band specific to the pathogen (Table 3), suggesting that seed transmission of *P. belbahrii*, either symptomatic or latent, does not occur in basil under Israeli conditions.

Table 3. Seed transmission of downy mildew caused by *Peronospora belbahrii* in basil plants.

Experiment	Year	Disease Transmission		Plants Assayed by PCR	
		Seeds Sown	Infected Plants	Total *	PCR Positive
1	2012	1000	0	50	0
2	2012	900	0	50	0
3	2016	4700	0	150	0
4	2016	1000	0	50	0
5	2016	1000	0	50	0
6	2017	9400	0	220	0
7	2017	4300	0	150	0
8	2017	1000	0	50	0
9	2017	1000	0	50	0
10	2017	900	0	20	0
11	2018	500/cultivar	0	50/cultivar	0

* Ten plants per sample.

4. Discussion

The major purposes of the present study were to examine the ability of downy mildew pathogen *Peronospora belbahrii* to: (i) Spread systemically in basil plants, (ii) reach the seeds, and (iii) be transferred by seeds. With some downy mildews, systemically infected plants may produce infected seeds [8,15]. Seed infection may also occur in plants showing latent infection [15]. *Pseudoperonopora cubensis*, the downy mildew agent of cucurbits, is seed transmitted without being systemic [16]. Its court of entrance is the pistil. Specific PCR primers and microscopy revealed its presence inside the embryo. A low proportion of the infected seeds produced infected plants [16].

Systemic infection with BDM was rarely seen in the field [1]. Here, we show that systemic infection can be readily produced in artificially inoculated basil plants under growth chamber conditions. When *P. belbahrii* was inoculated on a pair of young leaves of 2 or 12-leaf basil plants about 97% and 27% of the inoculated plants became systemically infected within 7 and 14 days after inoculation, respectively. Microscopic observations made with such plants detected the mycelium in the vascular parenchyma of the stem. PCR assays confirmed its growth from the inoculated leaf lamina to the petiole and then to the stem. In the stem, the mycelium could grow acropetally to the apex, basipetally to the cotyledons and hypocotyl below the inoculated leaves, and laterally to the axillary buds. Unlike *Plasmopara halstedii* in sunflower, *P. belbahrii* never reached the roots.

P. belbahrii can develop systemic infection in young basil plants. Systemically infected plants remained stunted (probably due to hormonal imbalance) [22]) and produced no seeds. Hence, no seed transmission could be studied with such plants. To study seed transmission, we used seeds of heavily infected plants growing in the field in various locations of the country. We found neither molecular nor phenotypic evidence for the occurrence of *P. belbahrii* in such seeds. The DNA that was extracted from such contaminated seeds exhibited the 134bp fragment of *P. belbahrii*. This was confirmed by microscopic examinations, which revealed sporangia embedded in the surface of such seeds. However, the likelihood that sporangia on seed surface retain viability is null due to the very low survivability of detached sporangia [21]. Of thousands of contaminated seeds (including seeds that were externally-contaminated with fresh sporangia of *P. belbahrii*) that were sown in pasteurized soil in growth chambers, all developed healthy plants. Furthermore, the DNA extracted from hundreds such plants proved no latent infection with *P. belbahrii*. These results corroborate with Wyenandt et al. [13] who showed by rqPCR the occurrence of *P. belbahrii* on seeds that were collected from infected basil plants in the field.

The data we present here do not corroborate with previous studies from Europe [6,7] that claimed seed transmission of *P. belbahrii* in basil. Our study showed that seed transmission does not occur under the dry summer conditions of Israel. It is logical to assume that seed infection/transmission may happen in Europe and other locations where conditions are wetter during summer. Exposure of basil plants to prolonged wetness periods at time of flowering and seed production may facilitate seed infection.

Taken together, this study proves that *P. belbahrii* can spread systemically in basil plants. Systemically infected plants fail to produce seeds. Seeds collected from heavily infected plants in Israeli fields exhibit the pathogen on their surface, but never produced BDM-infected plants.

Author Contributions: Conceptualization, Y.C., L.F.-B., Y.B.-N.; Methodology, Y.C., L.F.-B., Y.B.-N.; Writing-Original Draft Preparation, Y.C.; Project Administration, Y.C.

Funding: This research was funded by BARD, Project US-*4947-16 R*.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Cohen, Y.; Ben Naim, Y.; Falach, L.; Rubin, A.E. Epidemiology of basil downy mildew. *Phytopathology* **2017**, *107*, 1149–1160. [CrossRef] [PubMed]
2. Hansford, C.G. Annual report of the mycologist. *Rev. Appl. Mycol.* **1933**, *12*, 421–422.

3. Belbahri, L.; Calmin, G.; Pawlowski, J.; Lefort, F. Phylogenetic analysis and real time PCR detection of a presumbably undescribed Peronospora species on sweet basil and sage. *Mycol. Res.* **2005**, *109*, 1276–1287. [CrossRef] [PubMed]
4. Roberts, P.D.; Raid, R.N.; Harmon, P.F.; Jordan, S.A.; Palmateer, A.J. First report of downy mildew caused by a Peronospora sp on basil in Florida and the United States. *Plant Dis.* **2009**, *93*, 199. [CrossRef] [PubMed]
5. Cohen, Y.; Vaknin, M.; Ben-Naim, Y.; Rubin, A.E.; Galperin, M.; Silverman, D.; Bitton, S.; Adler, U. First report of the occurrence and resistance to mefenoxam of *Peronospora belbahrii*, causal agent of downy mildew of basil (*Ocimum basilicum*) in Israel. *Plant Dis.* **2013**, *97*, 692. [CrossRef] [PubMed]
6. Garibaldi, A.; Minuto, G.; Bertetti, D.; Gullino, M.L. Seed transmission of *Peronospora* sp. of basil. *J. Plant Dis. Prot.* **2004**, *111*, 465–469.
7. Farahani-Kofoet, R.D.; Roemer, P.; Grosch, R. Systemic spread of downy mildew in basil plants and detection of the pathogen in seed and plant samples. *Mycol. Progress* **2012**, *11*, 961–966. [CrossRef]
8. Cohen, Y.; Sackston, W.E. Factors affecting infection of sunflowers by *Plasmopara halstedii*. *Can. J. Bot.* **1973**, *51*, 15–22. [CrossRef]
9. Subbarao, C.S.; Anchieta, A.; Ochoa, L.; Dhar, N.; Kunjeti, S.G.; Subbarao, K.; Klosterman, S.J. Detection of latent infections of *Peronospora effusa* in spinach. *Plant Dis.* **2018**, *102*, 1766–1771. [CrossRef] [PubMed]
10. Wyenandt, C.A.; Simon, J.E.; Pyne, R.M.; Homa, K.; McGrath, M.T.; Zhang, S.A.; Raid, R.N.; Ma, L.J.; Wick, R.; Guo, L.; et al. Basil downy mildew (*Peronospora belbahrii*): Discoveries and challenges relative to its control. *Phytopathology* **2015**, *105*, 885–894. [CrossRef]
11. Gilardi, G.; Pintore, I.; Demarchi, S.; Gullino, M.L.; Garibaldi, A. Seed dressing to control downy mildew of basil. *Phytoparasitica* **2015**, *43*, 531–539. [CrossRef]
12. Pintore, I.; Gilardi, G.; Gullino, M.L.; Garibaldi, A. Detection of mefenoxam-resistant strains of *Peronospora belbahrii*, the causal agent of basil downy mildew, transmitted through infected seeds. *Phytoparasitica* **2016**, *44*, 563–569. [CrossRef]
13. Wyenandt, C.A.; Maimone, L.R.; Homa, K.; Madeiras, A.M.; Wick, R.L.; Simon, J.E. Detection of the downy mildew pathogen on seed of basil following field infection in Southern New Jersey. *Horttechnology* **2018**, *28*, 637–641. [CrossRef]
14. Spenser, D.M.E. *The Downy Mildews*; Academic Press: Cambridge, MA, USA, 1981.
15. Cohen, Y.; Sackston, W.E. Seed infection and latent infection of sunflowers by *Plasmopara halstedii*. *Can. J. Bot.* **1974**, *52*, 231–238. [CrossRef]
16. Cohen, Y.; Rubin, A.E.; Galperin, M.; Ploch, S.; Runge, F.; Thines, M. Seed transmission of *Pseudoperonospora cubensis*. *PLoS ONE* **2014**, *9*, e109766. [CrossRef] [PubMed]
17. Ben-Naim, Y.; Falach, L.; Cohen, Y. Transfer of downy mildew resistance from wild basil (*Ocimum americanum*) to sweet basil (*O. basilicum*). *Phytopathology* **2018**, *108*, 114–123. [CrossRef] [PubMed]
18. Ben-Naim, Y.; Falach, L.; Cohen, Y. Resistance against basil downy mildew in *Ocimum species*. *Phytopathology* **2015**, *105*, 778–785. [CrossRef] [PubMed]
19. Cohen, Y.; Eyal, H.; Hanania, J. Ultrastructure, autofluorescence, callose deposition and lignification in susceptible and resistant muskmelon leaves infected with the powdery mildew fungus *Sphaerotheca fuliginea*. *Physiol. Mol. Plant Pathol.* **1990**, *36*, 191–204. [CrossRef]
20. Doyle, J.J. Isolation of plant DNA from fresh tissue. *Focus* **1990**, *12*, 13–15.
21. Cohen, Y.; Rubin, A.E. Daytime solar heating controls downy mildew *Peronospora belbahrii* in sweet basil. *PLoS ONE* **2015**, *10*, e0126103. [CrossRef]
22. Cohen, Y.; Sackston, W.E. Disappearance of IAA in presence of tissues of sunflowers infected by *Plasmopara halstedii*. *Can. J. Bot.* **1974**, *52*, 861–866. [CrossRef]

© 2019 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).