Materials and Methods

Plants

Nine cucurbit species were grown in 4 net houses # 2, 3, 5 and 6 (6×50 m each) located on the north-east part of the campus, at Bar-Ilan University Farm, Israel in six seasons during 2012–2014. These species were also grown in a controlled glasshouse # 3 located in the south-west part of the campus. The species were cucumber (Cucumis sativum, cvs.SMR-18 and Nadiojn), melon (Cucumis melo var. reticulatus, cvs. Ananas Yokneam and Ein-Dor), pumpkin (Cucurbita maxima cvs. Tripoli and Armonim), squash (Cucurbita pepo cvs.Beruti and Arlika), butternut gourd (Cucurbita moschata cv.Waltham), watermelon (Citrullus lanatus cv. Mallali), bottle gourd (Lagenaria vulgaris, local cultivar), sponge gourd (Luffa cylindrica, local cultivar) and bitter gourd (Momordica balsamina, local cultivar). Planting took place on February (Spring season), August (Autumn season) and November (Winter season). Plants were fertilized weekly with 0.5% N:P:K and sprayed with fungicides against powdery mildew when required. Natural infection with downy mildew occurred in net-houses # 2, 3, 5 and 6 at all seasons on leaves of all cucurbits except watermelon, sponge gourd and bitter gourd. No downy mildew showed up in glasshouse # 3 due to the lack of free moisture on the leaves. Seeds for all experiments describe herein were collected from greenhouse # 3. Frequent PCR assays done with seeds samples taken from glasshouse # 3 were proved negative for P. cubensis.

The procedures describe below were all similarly applied to healthy plant material derived from greenhouse # 3 and infected (symptomless flowers, ovaries, fruits, seeds) plant material derived from net-houses # 2, 3, 5 and 6.

Recovery of P. cubensis from flowers, fruits, and seeds

Strict hygiene measures were undertaken while attempting to recover P. cubensis from flowers, ovaries, fruits and seeds. Female flowers for ovaries were taken from cucumber and squash. Fruits were collected from cucumber, butternut gourd, pumpkin, bottle gourd and squash. Some fruits carried empty seeds because of lack of adequate pollination. The ovaries and fruits were washed with excessive soap water, washed thoroughly with tap water; surface sterilized by dipping in 4% hypochlorite solution for 10 minutes; dipped momentarily in ethanol and washed with sterile water. All further processing steps, including PCR, were carried out under strict sterile conditions. Ovaries, fruit seed cavity, seed embryos and mature seeds were homogenized in sterile cold water and used for inoculation of healthy detached leaves (taken from greenhouse # 3). Mature seeds were sown (see below) in sterile soil mixture or
used for microscopy. Three 50 µl droplets were taken from each homogenate, placed on a glass slide, covered with a cover slip, and examined with a dissecting microscope for the presence of mycelia or sporangia of *P. cubensis*. Fifty droplets, 50 µl each, of each homogenate were pipetted onto the lower surface of a detached cucumber leaf laying on a wet sterile filter paper in a 14 cm diameter Petri dish, and fifty such droplets were similarly inoculated onto a detached leaf of butternut gourd. Inoculated leaves were incubated at 20°C under 12 h photoperiod for 3 days, then washed with sterile water to remove the homogenate droplets, and thereafter kept for three weeks at 20°C under 12 h photoperiod to allow for downy mildew development. Detached leaves inoculated with droplets of sterile water served as controls. Symptoms of downy mildew with sporulation of *P. cubensis* appeared on some detached leaves inoculated with plant material (fruit seed-cavity homogenates, fruit tissue slices, ovary homogenates, and leaf pieces taken from seedlings developed from seeds) derived from net-houses #2, 3, 5 and 6 but never in similar plant material derived from greenhouse #3. Symptoms of *P. cubensis* were propagated on detached leaves of cucumber laying on wet filter paper in 14 cm Petri dishes. The pathotype of the isolates was determined by inoculation of detached leaves of 9 cucurbits species as described before [7,8]. Mating type was determined by inoculation of melon and cucumber leaf discs with sporangial mixtures of the test isolate and a tester isolate of known mating type as described before [9].

**Recovery of *P.cubensis* from hypocotyls**

Seeds were surface sterilized and sown in sterile pots (60 ml) filled with a sterile soil mixture (peat: vermiculite, 1:1, v/v), 1 seed per pot. Plants were grown in greenhouse #3 and when reached the first true leaf stage (2 weeks after sowing) their hypocotyl was removed with a sterile scalpel, surface sterilized, placed in sterile water (1 hypocotyl/5 ml) and homogenized with a sterile blade (2 minute, 7000 rpm, 4°C). The homogenate was used for inoculation of detached leaves of cucumber and butternut as described above.

**Microscopy**

Free hand sections were taken with a sterile razor blade from surface sterilized ovaries or fruits. Slices were placed on detached leaves of cucumber or butternut gourd to allow infection. Other slices were boiled in ethanol for 10 minutes, placed for 24 h in basic amine blue solution (0.05%, pH 8.9) at 4°C, stained with 0.01% calcofluor (Sigma), and examined with Olympus A70 epifluorescent microscope for the presence of sporangia and mycelia [10]. A similar procedure was employed to embryos taken from mature seeds.

**Recovery of *P.cubensis* from Seedlings**

Seeds were surface sterilized, placed on sterile filter paper in sterile 14 cm petri dishes or 20×20×3 cm sterile plastic dishes (Nunk, Denmark) and incubated at 25°C under 12 h photoperiod. When cotyledons were produced (about 7 days), plants were transplanted into 0.5 L pots filled with sterile potting soil, while others were used for DNA extraction. Plants were maintained at 20°C at 12 h photoperiod to allow for downy mildew development. When symptoms appeared, plants were sealed in 1 L sterile plastic boxes (100% RH) for several days to enable sporulation of *P. cubensis*.

**DNA extraction from plants or sporangia of *P. cubensis***

The method of Tinker et al [11] was employed with modifications. Samples of approximately 100–500 mg leaf, hypocotyl, root, ovary, or fruit tissue, or a sample of about 1×10⁵ sporangia, were macerated in 1.5-ml micro-tubes using disposable pellet pestle grinders. Maceration was continued after adding 0.6 ml CTAB (hexadecyltrimethyl-ammonium bromide) buffer [1.4 M NaCl, 20 mM EDTA, 100 mM TRIS-Cl, 2% (W/V) CTAB pH 8.0], and the samples were incubated at 60°C for 45 min. The samples were then extracted with 0.6 ml chloroform/isoamyl alcohol (24:1) and centrifuged at 12000 g for 5 min. The aqueous phase was transferred to a 1.5-ml tube where the DNA was precipitated with an equal volume of cold (-20°C) isopropanol. DNA concentration was determined with a ND-1000 spectrophotometer (NanoDrop USA). DNA separation was done on a 1.2% agarose gel and staining with ethidium bromide.

**DNA extraction from seeds**

Dry seeds were placed in 2 ml tubes and rehydrated with sterile water for 15 minutes. After the water was removed, sodium hypochlorite solution (4%) containing 0.1% Tween 20 (to break surface tension) was added to the samples for 10 minutes. Seeds were rinsed with sterile water for 5 minutes. The embryo and the integument were separated and transferred to 96 well plates with 1.3 ml tubes. Care was taken not to cross-contaminate the samples and new gloves and sterile forceps were used for every seed. DNA extraction was conducted by using the BioSprint 96 DNA Plant Kit (Qiagen, Hilden, Germany) in combination with a KingFisher Flex (ThermoFisher Scientific, Waltham, USA) DNA extraction robot. The quality of the extraction was tested by conducting a PCR with the primers ITS1 and ITS4 developed by White et al. [12]. For all samples amplifiable DNA was obtained.

**Primer development and molecular detection**

Species-specific primers were developed based on cox2 sequences of *Pseudoperonospora humuli, Pseudoperonospora cubensis* and related species which were obtained from the database of the
Table 1. *Pseudoperonospora cubensis* was recovered from the reproductive organs of downy mildew-infected plants (A–D) but not from the reproductive organs of healthy plants (E–H).

| From Downy Mildew-Infected Plants | From Control Healthy Plants |
|-----------------------------------|----------------------------|
| **A** Ovaries                     | **E** Ovaries               |
| Host                              | Host                       |
| Examine                           | Examine                    |
| Infectious                        | Infectious                 |
| %                                 | %                          |
| Cucumber                          | Cucumber                   |
| 82                                | 40                         |
| 15                                | 0                          |
| 18.3                              | 0.0                        |
| Melon                             | Melon                      |
| 10                                | 10                         |
| 3                                 | 0                          |
| 30.0                              | 0.0                        |
| Squash                            | Squash                     |
| 37                                | 30                         |
| 14                                | 0                          |
| 37.8                              | 0.0                        |
| Total                             | Total                      |
| 129                               | 80                         |
| 32                                | 0                          |
| 24.8                              | 0.0                        |
| **B** Fruit seed cavity           | **F** Fruit seed cavity    |
| Host                              | Host                       |
| Examine                           | Examine                    |
| Infectious                        | Infectious                 |
| %                                 | %                          |
| Cucumber                          | Cucumber                   |
| 316                               | 100                        |
| 22                                | 0                          |
| 7.0                               | 0.0                        |
| Melon                             | Butternut gourd            |
| 116                               | 30                         |
| 8                                 | 0                          |
| 6.9                               | 0.0                        |
| Pumpkin                           | Pumpkin                    |
| 29                                | 100                        |
| 3                                 | 0                          |
| 10.3                              | 0.0                        |
| Squash                            | Squash                     |
| 49                                | 20                         |
| 1                                 | 0                          |
| 2.0                               | 0.0                        |
| Total                             | Total                      |
| 510                               | 160                        |
| 34                                | 0                          |
| 6.7                               | 0.0                        |
| **C** Seeds                       | **G** Seeds                |
| Host                              | Host                       |
| Sown                              | Sown                       |
| Infectected plants                | Infectected plants         |
| %                                 | %                          |
| Cucumber                          | Cucumber                   |
| 400                               | 200                        |
| 1                                | 0                          |
| 0.25                              | 0.0                        |
| Butternut gourd                   | Butternut gourd            |
| 400                               | 200                        |
| 4                                | 0                          |
| 1.0                               | 0.0                        |
| Squash                            | Squash                     |
| 400                               | 200                        |
| 6                                | 0                          |
| 1.5                               | 0.0                        |
| Total                             | Total                      |
| 1200                              | 600                        |
| 11                                | 0                          |
| 0.92                              | 0.0                        |
| **D** Hypocotyls                  | **H** Hypocotyls           |
| Host                              | Host                       |
| Examine                           | Examine                    |
| Infectious                        | Infectious                 |
| %                                 | %                          |
| Cucumber                          | Cucumber                   |
| 150                               | 50                         |
| 1                                | 0                          |
| 0.7                               | 0.0                        |
| Melon                             | Butternut gourd            |
| 150                               | 50                         |
| 2                                | 0                          |
| 1.3                               | 0.0                        |
| Squash                            | Squash                     |
| 150                               | 50                         |
| 4                                | 0                          |
| 2.7                               | 0.0                        |
| Total                             | Total                      |
| 450                               | 150                        |
| 7                                 | 0                          |
| 1.6                               | 0.0                        |

The downy mildew-infected plants were grown in net-houses #2, 3, 5 and 6 while the healthy plants were grown in greenhouse #3.

A, E  Infectivity of crushed ovaries to detached leaves of cucumber and/or butternut gourd.

B, F  Infectivity of crushed fruit seed-cavity tissue to detached leaves of cucumber and/or butternut gourd.

C, G  Vertical transmission of *P. cubensis* from seeds to the next plant generation.

D, H  Infectivity of crushed hypocotyls to detached leaves of cucumber and/or butternut gourd.

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National Center for Biotechnology (http://www.ncbi.nlm.nih.gov/). The primers for \textit{P. humuli} and the two clades of \textit{P. cubensis}, which had been reported in Runge et al. [13], as well as their specificity, are shown in Table S1. As described by Ploch et al. [14] two PCRs were carried out to detect the pathogens with high specificity and sensitivity. In the first PCR, 0.4 mM of Oomycete specific primers \textit{cox}2-F and \textit{cox}2-R [15] were used in a reaction mixture containing 1× Mango PCR Buffer, 0.2 mM dNTPs, 1 mM MgCl2, 0.8 mg/ml BSA and 0.5 U Mango Taq DNA Polymerase (Bioline, Luckenwalde, Germany). For all three primer combinations (Table S1) a separate nested PCR was conducted with a 1 to 10 dilution of the oomycete specific PCR. Cycling temperature and times are detailed in Table S2.

Results

Downy mildew in butternut gourd fruits

Butternut gourd fruits taken from downy mildew-infected plants revealed a dark seed cavity (Fig. 1a). Microscopic examinations of free-hand sections taken from the seed cavity revealed coenocytic hyphae and a few sporangia of \textit{P. cubensis} (Fig. 1b–d). No sporangiophores of \textit{P. cubensis} were seen. Between 0–10 sporangia per 50 μl droplet were detected in the seed cavity homogenate of various fruits. Butternut gourd fruits taken from healthy plants showed no discoloration of the seed cavity nor mycelia or sporangia.

Recovery of \textit{P. cubensis} from ovaries, fruits, hypocotyls and seeds

Table 1 summarizes the data obtained during 2012–14. A total of 129 ovaries of cucumber, melon or squash were collected from downy mildew-infected plants, surface sterilized, crushed and inoculated onto detached healthy leaves of cucumber and butternut gourd. No ovaries of butternut gourd were used. About 25% of the ovaries were infectious, capable of producing typical downy mildew symptoms on the detached leaves (Table 1A). The isolates recovered from ovaries of cucumber or melon belonged to pathotype 3 mating type A1, while those recovered from squash belonged to pathotype 6 mating type A2.

A total of 510 fruits of cucumber, melon, pumpkin or squash were collected during 2012–2014. Fruits were surface sterilized, cut open in two halves, seeds (when available) removed, and the seed cavity tissue crushed and inoculated onto detached leaves. Thirty four fruits (6.7%) were infectious, producing typical downy mildew symptoms on the detached leaves of cucumber and/or butternut gourd (Table 1B; Fig. 2). The isolates recovered from seed cavity of cucumber and melon belonged to pathotype 3 mating type A1, while those recovered from pumpkin and squash belonged to pathotype 6 mating type A2.

Mature seeds were taken from fruits of cucumber, butternut gourd or squash, surface-sterilized, planted (20 seeds per fruit, 20 fruits from each species) in sterile soil mixture in pots and grown for one month at 25°C with 12 h light/day. As shown in Table 1C, one cucumber, four butternut gourd and six squash plants developed downy mildew symptoms with sporulation of \textit{P. cubensis} (Fig. 3).

In a similar experiment, seeds were planted in sterile soil and grown in greenhouse #3. At 2 weeks after germination no disease was seen on the cotyledons. The hypocotyls were taken, surface-sterilized, homogenized in sterile water and inoculated onto detached leaves. Seven out of the 450 hypocotyls tested (1.6%) were infectious, producing downy mildew on detached leaves of cucumber and/or butternut gourd (Table 1D).

In parallel experiments, ovaries, fruits and seeds were collected during 2012–2014 from healthy plants growing in glasshouse #3 in which the controlled dry atmosphere prevented downy mildew development. None of the 80 ovaries, 160 fruits or 150 hypocotyls was infectious to detached leaves of cucumber or butternut gourd (Table 1E, F, H). No seed of the 600 seeds sown developed downy mildew symptoms (Table 1G).

Microscopy

A rare observation of \textit{P. cubensis} sporulating on embryo of cucumber is shown in Fig. 4a. Mycelium inside the embryo of
butternut gourd is shown in Fig. 4b. Sporangia inside ovaries of cucumber, adjacent to the ovules, are shown in Fig. 4c and 4d.

**PCR assays for *P. cubensis* in ovaries and seed cavity**

About 49% of the ovaries (39 out of 80) (Table 2A) and 55% of the flower peduncles (6 out 11) (data not shown) reacted positively when tested with primers Set 1. None of the 45 healthy ovaries has tested positive (Table 2B). Of the 301 fruits examined, 35 (11.6%) tested positive with primers Set 1 (Table 2C). All 120 healthy fruits from greenhouse #3 tested negatively to *P. cubensis* (Table 2D).

Interestingly, in 12 cucumber fruits whose proximal and distal ends were tested separately, 5 tested positive, all at the proximal end (stem end) of the fruit. Similarly, in 6 squash fruits all tested positively, all at the proximal end of the fruit. PCR assays conducted with pistil tissue of squash female flowers resulted with no signal, suggesting that the pathogen moves into the fruit from the stem side and not from the pistil side. Indeed, PCR assays revealed the occurrence of *P. cubensis* in stems of cucumber, melon, squash and pumpkin. Often, petioles and stem homogenates were infectious to detached leaves of cucumber and/or butternut gourd.

**PCR assays for *P. cubensis* in seed integuments and embryos**

PCR assays showed that *P. cubensis* occurs in seeds collected from downy mildew-infected plants (Fig. 5, Tables 3–5). In Fig. 5, three seeds per entry were used, one slot per seed. A positive reaction to primer Set 1 (detecting all 3 clades) was detected in 10 out of 15 seed samples. In 6 samples, only one seed responded positively whereas in 4 samples, 2 out of 3 seeds responded positively, indicating on the heterogeneity of infection in the seeds in a single fruit. The same number of samples responded to primer Set 2 (detecting clades 1 and 2), but with only one seed out of three responding positively. Eight samples responded to primer set 3 (detecting clade 2), one seed per sample. Sequencing of 6 randomly chosen PCR products confirmed the identity of the organism as being *P. cubensis*.

Table 3 presents the molecular detection analyses obtained for 10 seeds of 7 fruits: B2 of squash and D2–D7 of butternut gourd. The occurrence of *P. cubensis* was tested separately in the integuments and the embryo (see Methods and Material). Two embryos (D2/7, D5/4) and three integuments (D5/8, D6/1 and D6/3) were tested positive for *P. cubensis*. One seed B2/1 tested positive for primers Set 1 only.

In another assay (Table 4), the integuments and embryo of 70 individual seeds (from 15 fruits) of cucumber were analyzed with primer Set 3 that amplifies clade 1 of *P. cubensis*. The integuments of 5 seeds and the embryo of 6 seeds tested positive. In only one seed (C4/5) both the integuments and the embryo tested positive (Table 4A), suggesting that the pathogen may colonize the integuments, the embryo, or both. No amplicons of *P. cubensis* were detected in seeds taken from healthy plants (Table 4B).

Similar results were obtained with seeds of bottle gourd (*Lagenaria vulgaris*) (Table 5). The integuments and embryo of 100 individual seeds (10 seeds/fruit) were analyzed with all three primer sets of *P. cubensis*. Eight seeds tested positive with primer set 3 in both the integument and embryo, 6 seeds tested positive with primer set 2 in the integument but not the embryo, and no seed was positive with primer set 1 (Table 5A). No amplicons of *P. cubensis* were detected in seeds taken from healthy plants (Table 5B).
In pumpkin, we tested 42 embryos from 5 infected fruits with primer sets 2 and 3. Two out of 5 fruits had PCR-positive embryos. In one fruit, 4 out of 10, and in the second 1 out of 10, tested positive with both primers sets 2 and 3. Thirty embryos from 4 healthy fruits were all PCR-negative.

**Table 2. PCR assays showing the occurrence of *Pseudoperonospora cubensis* amplicons in ovaries and fruits of cucurbits.**

|                      | Ovaries | Primer Set 1 |
|----------------------|---------|--------------|
| **A. Ovaries from infected plants** |         |              |
| Cucumber             | 38      | 22           |
| Squash               | 37      | 16           |
| Melon                | 5       | 1            |
| **Total**            | 80      | 39           |
| **B. Ovaries from healthy plants** |         |              |
| Cucumber             | 15      | 0            |
| Squash               | 15      | 0            |
| Melon                | 15      | 0            |
| **Total**            | 45      | 0            |
| **C. Seed cavity of fruits from infected plants** |         |              |
| Cucumber             | 236     | 26           |
| Butternut gourd      | 28      | 4            |
| Pumpkin              | 37      | 5            |
| **Total**            | 301     | 35           |
| **D. Seed cavity of fruits from healthy plants** |         |              |
| Cucumber             | 80      | 0            |
| Butternut gourd      | 20      | 0            |
| Pumpkin              | 20      | 0            |
| **Total**            | 120     | 0            |

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**Discussion**

*P. cubensis* is a foliar pathogen of *Cucurbitaceae* with worldwide distribution. In the past decade major changes occurred in the population structure of this oomycete. In Israel, two major changes took place: in 2002 a new pathotype, number 6, appeared [8] and in 2010 a new mating type, A2, showed up [9]. In the

![Figure 5. PCR analysis of 16 seed samples, 3 seeds per sample, one slot per seed.](image-url)
Table 3. PCR assays showing the occurrence of Pseudoperonospora cubensis amplicons in individual seeds of cucurbits.

| Fruit Seed Name | IT5 - Test Integument | Embryo | Cox2 Integument | Embryo | Cox2 All Clades | Integument | Embryo |
|-----------------|------------------------|--------|-----------------|--------|-----------------|------------|--------|
| B2 1             | 1                      | 1      | 1               | 1      | 1               | 1          | 1      |
| B2 2 – 10        | 1                      | 1      | 1               | 1      | 1               | 1          | 1      |
| D2 1 – 6         | 1                      | 1      | 1               | 1      | 1               | 1          | 1      |
| D2 7             | 1                      | 1      | 1               | 1      | 1               | 1          | 1      |
| D2 8 – 10        | 1                      | 1      | 1               | 1      | 1               | 1          | 1      |
| D3 1 – 10        | 1                      | 1      | 1               | 1      | 1               | 1          | 1      |
| D4 1 – 10        | 1                      | 1      | 1               | 1      | 1               | 1          | 1      |
| D5 1 – 3         | 1                      | 1      | 1               | 1      | 1               | 1          | 1      |
| D5 4             | 1                      | 1      | 1               | 1      | 1               | 1          | 1      |
| D5 5 – 8         | 1                      | 1      | 1               | 1      | 1               | 1          | 1      |
| D5 9             | 1                      | 1      | 1               | 1      | 1               | 1          | 1      |
| D5 10            | 1                      | 1      | 1               | 1      | 1               | 1          | 1      |
| D6 1             | 1                      | 1      | 1               | 1      | 1               | 1          | 1      |
| D6 2             | 1                      | 1      | 1               | 1      | 1               | 1          | 1      |
| D6 3             | 1                      | 1      | 1               | 1      | 1               | 1          | 1      |
| D6 4 – 10        | 1                      | 1      | 1               | 1      | 1               | 1          | 1      |
| D7 1 – 10        | 1                      | 1      | 1               | 1      | 1               | 1          | 1      |
| H2O              | 1                      | 1      | 1               | 1      | 1               | 1          | 1      |

*Additional positive control “21226”

The seeds were taken from one fruit of squash (B) and 6 fruits of butternut gourd (D) (10 seeds per fruit). 1 = test positive; 0 = test negative.
Table 4. PCR assays showing the occurrence of *Pseudoperonospora cubensis* in individual seeds of cucumber.

### A. Seeds from downy mildew-infected plants

| Fruit Name | Seed Number | Clade 1 |   |
|------------|-------------|---------|---|
|            | Integument  | embryo  |   |
| C1         | 1           | 0       | 0 |
|            | 2           | 1       | 0 |
|            | 3–5         | 0       | 0 |
| C2         | 1–4         | 0       | 0 |
| C3         | 1–5         | 0       | 0 |
| C4         | 1           | 0       | 1 |
|            | 2–4         | 0       | 0 |
|            | 5           | 1       | 1 |
| C5         | 1, 2, 4     | 0       | 0 |
|            | 3           | 0       | 1 |
|            | 5           | 1       | 0 |
| C6         | 1, 2, 3, 5  | 0       | 0 |
|            | 4           | 0       | 1 |
| C7         | 1–5         | 0       | 0 |
| C8         | 1–5         | 0       | 0 |
| C9         | 1–5         | 0       | 0 |
| C10        | 1–5         | 0       | 0 |
| C11        | 1, 2, 3, 5  | 0       | 0 |
|            | 4           | 0       | 1 |
| C12        | 1, 3, 4, 5  | 0       | 0 |
|            | 2           | 1       | 0 |
| C13        | 1           | 0       | 1 |
|            | 2, 3, 4     | 0       | 0 |
|            | 5           | 1       | 0 |
| C14        | 1–3         | 0       | 0 |
| C15        | 1–3         | 0       | 0 |
| **Total**  | **70**      | **5**   | **6** |

### B. Seeds from healthy plants

| Fruit Name | Seed Number | Clade 1 |   |
|------------|-------------|---------|---|
|            | Integument  | embryo  |   |
| C1         | 1–5         | 0       | 0 |
| C2         | 1–5         | 0       | 0 |
| C3         | 1–5         | 0       | 0 |
| C4         | 1–5         | 0       | 0 |
| C5         | 1–5         | 0       | 0 |
| C6         | 1–5         | 0       | 0 |
| C7         | 1–5         | 0       | 0 |
| C8         | 1–5         | 0       | 0 |
| C9         | 1–5         | 0       | 0 |
| C10        | 1–5         | 0       | 0 |
| **Total**  | **50**      | **0**   | **0** |

A. Fruits taken from downy mildew-infected plants. B. Fruits taken from healthy plants.

B. 1 = test positive; 0 = test negative.

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Table 5. PCR assays showing the occurrence of *Pseudoperonospora cubensis* in individual seeds of bottle gourd (*Lagenaria vulgaris*).

| Fruit name | Seed Number | Integument | Embryo | Integument | Embryo | Integument | Embryo |
|------------|-------------|------------|--------|------------|--------|------------|--------|
| Lag1       | 5           | 0          | 0      | 0          | 0      | 0          | 0      |
| Lag2       | 2, 7        | 0          | 0      | 0          | 0      | 0          | 0      |
| Lag3       | 4, 7, 9     | 0          | 0      | 0          | 0      | 0          | 0      |
| Lag4       | 2, 5, 8     | 0          | 0      | 0          | 0      | 0          | 0      |
| Lag5       | 5, 8, 11    | 0          | 0      | 0          | 0      | 0          | 0      |
| Lag6       | 1-3, 6, 8   | 0          | 0      | 0          | 0      | 0          | 0      |
| Lag7       | 1-3, 6, 8   | 0          | 0      | 0          | 0      | 0          | 0      |
| Lag8       | 1-3, 6, 8   | 0          | 0      | 0          | 0      | 0          | 0      |
| Lag9       | 1-3, 6, 8   | 0          | 0      | 0          | 0      | 0          | 0      |
| Lag10      | 1-3, 6, 8   | 0          | 0      | 0          | 0      | 0          | 0      |
### Table 5. Cont.

**A. Fruits from downy mildew-infected plants**

| Fruit name | Seed Number | Integument | Embryo | Integument | Embryo | Integument | Embryo | Integument | Embryo | Integument | Embryo |
|------------|-------------|------------|--------|------------|--------|------------|--------|------------|--------|------------|--------|
|            | All 3 clades | Clade 1 and 2 | Clade 1 |
| Lag1       | 1–10        | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      |
| Lag2       | 1–10        | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      |
| Lag3       | 1–10        | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      |
| Lag4       | 1–10        | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      |
| Lag5       | 1–10        | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      |
| Lag6       | 1–10        | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      |
| Lag7       | 1–10        | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      |
| Lag8       | 1–10        | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      |
| Lag9       | 1–10        | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      |
| Lag10      | 1–10        | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      |
| Total      | 100         | 0          | 0      | 6          | 0      | 14         | 11     |

**B. Fruits from healthy plants**

| Fruit name | Seed Number | Integument | Embryo | Integument | Embryo | Integument | Embryo | Integument | Embryo |
|------------|-------------|------------|--------|------------|--------|------------|--------|------------|--------|
|            | All 3 clades | Clade 1 and 2 | Clade 1 |
| Lag1       | 1–10        | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      |
| Lag2       | 1–10        | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      |
| Lag3       | 1–10        | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      |
| Lag4       | 1–10        | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      |
| Lag5       | 1–10        | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      |
| Lag6       | 1–10        | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      |
| Lag7       | 1–10        | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      |
| Lag8       | 1–10        | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      |
| Lag9       | 1–10        | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      |
| Lag10      | 1–10        | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      |
| Total      | 100         | 0          | 0      | 0          | 0      | 0          | 0      | 0          | 0      |

A- Fruits taken from downy mildew-infected plants. B- Fruits taken from health plants.

1 = test positive; 0 = test negative.

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USA a new race appeared in 2004 capable of destroying long-lasting resistant cucumber cultivars [16]. In Italy, a new pathotype, number 5, appeared in 2003 [17], and in the Czech Republic many new pathotype combinations appeared recently [18].

Ruggeri et al [13] performed a molecular comparison between pre-2004 and post-2004 isolates of P. cubensis. They suggested that the new post-2004 genotypes have migrated (by man carrying leaf material) from South East Asia (Korea, Japan) to Europe and thereafter to the USA.

Another vehicle for such a migration could be a man carrying fruits or seeds of cucurbits, or commercial trade of fruits and seeds. We show here that fruits of cucurbits, collected from downy mildew-infected plants are symptomless but may carry mycelium and sporangia of P. cubensis. Fruit slices, or seed-cavity tissue homogenates made from such fruits, produced typical downy mildew symptoms with sporulation of P. cubensis when applied to detached healthy leaves of cucurbits. P. cubensis similarly occurs in symptomless stems, petioles and ovaries of infected plants. PCR assays showed that the pathogen occurs in peduncles of female flowers and at the stem end, not in the petal end, of the ovary or fruit, nor in the pistil of the flower. This suggests that penetration of the pathogen into the ovary, and thereafter into the fruit, occurs from the leaf into the stem and then through the peduncle of the female flower into the ovary.

P. cubensis was found to also occur in seeds of cucurbits. We confirmed it by microscopy, infectivity of crushed seeds to detached leaves, and species-specific PCR assays. All implicated that P. cubensis may be transmitted by seed. Indeed, seeds collected from infected plants produced infected plants with typical symptoms and sporulation of P. cubensis. Vertical seed transfer of downy mildew occurred in cucumber (0.25%), butternut gourd (1%) and squash (1.5%). Hypocotyls produced by such seeds were infectious (1.6%) when crushed and inoculated onto detached leaves of cucumber.

These findings may now offer a new explanation to the global structural changes in the pathogen population. Cucurbit fruits (probably ornamental squash or pumpkin) were imported for commercial purposes, or transported by man, from South East Asia into Europe and/or Israel. The fruits were collected from infected but produced a rather low incidence of infected plants. They suggested that the new post-2004 genotypes have migrated (by man carrying leaf material) from South East Asia (Korea, Japan) to Europe and thereafter to the USA.

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