

Oospores Associated with Tomato Seed May Lead to Seedborne Transmission of *Phytophthora infestans*

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Tomato fruits at the green mature stage were inoculated with a mixed sporangial suspension of A₁ and A₂ isolates of *Phytophthora infestans*. Other fruits were inoculated with either A₁ or A₂ sporangia. Seeds were extracted from the blighted fruits and sown in soil or on agar media to test for the transmission of late blight to the emerging seedlings. Only 23 (0.09%) of approximately 25,000 seedlings developed symptoms. All blighted seedlings originated from fruits inoculated with mixed A₁ + A₂ sporangia. Isolates of *P. infestans* recovered from the emerging blighted seedlings were seemingly of oosporic origin, as they differed phenotypically (mating type, virulence, sensitivity to metalaxyl) from the parent isolates used to inoculate the fruits. The results suggest that transmission of *P. infestans* might occur by seeds extracted from fruits carrying oospores and less probably by seeds extracted from fruits having no oospores.

KEY WORDS: Potato late blight; epidemiology; genetics; seed pathology; *Lycopersicon esculentum*.

INTRODUCTION

The oomycete *Phytophthora infestans* (Mont.) DeBary, the causal agent of late blight in potato (*Solanum tuberosum* L.) and tomato (*Solanum lycopersicon* L.), has become increasingly more aggressive to tomato in recent years in various parts of the world including Israel (2,5-7,9,11,14,15,17,19,20,24,25,27). The reasons for the increased aggressiveness of *P. infestans* to tomato are not clear. One possibility is that abundant oospores are produced in tomato fruits (21,24) relative to potato tubers (3,16,18) and thus enhance the emergence of new, recombinant, aggressive isolates.

In a previous study (21) we showed that tomato fruits at the mature green stage are highly susceptible to infection by *P. infestans*. Moreover, the pathogen produced large numbers of oospores in all parts of the blighted fruit, including on the seed coat. A few seeds were shown to carry mycelia and oospores also in the embryo (21). In commercial tomato production, blighted fruits are often discarded in close vicinity to the production area and thus may serve as inoculum source for that crop or other tomato or potato crops growing in neighboring areas. Such a source of inoculum may contain recombinant isolates emerging from the oospores harbored by the fruits. Seeds from dumped, blighted tomato fruits were shown to germinate into blighted seedlings (26), thus serving as a potential source of inoculum to adjacent potato and tomato crops.

The objective of this study was to determine whether seeds extracted from blighted tomato fruits carrying oospores, would produce blighted seedlings. To meet this objective,

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fruits were inoculated with A_1 , A_2 or mixed A_1+A_2 sporangial suspensions of various isolates of the pathogen, and seeds were allowed to produce seedlings, on which the development of the disease and the characteristics of *P. infestans* were monitored. The results showed that under certain circumstances, seeds from blighted tomato fruits carrying oospores may develop into blighted plants. An abstract reporting some of these results was published recently (22).

MATERIALS AND METHODS

Fruits Various cultivars of tomato were grown in shade houses (50 mesh = 0.08 mm²) during the spring and summer of the years 2001–2003. Fruits were harvested at the mature green stage, washed, blotted dry, and inoculated with *P. infestans*. One or more of the following 36 cultivars of tomato were used (according to availability) in various experiments: 1. ZH (own inbred); 2. Roter-Gnom (Syngenta Crop Protection, Stein, Switzerland); 3. HA-144, 4. HA-189, 5. HA-300, 6. HA-870 and 7. Brigade (Hazera Genetics, Brurim, Israel); 8. VF-36, 9. GI-15, 10. G-5108 (Zeraim, Gedera, Israel); 11. Pieralbo, 12. Macline and 13. Pieraline (carrying Ph-2 gene for resistance, from M. Pitrat, INRA, Monfavet, France); 14. Agata, 15. Allan, 16. Baily-Naliv, 17. De-Berao, 18. Drujok, 19. Flora, 20. Iskorka, 21. Logid, 22. Master, 23. Olga, 24. Zonen, 25. Persey, 26. Renny, 27. Sinbad, 28. Solnechny, 29. Talalychin and 30. Zakas (Sortsemouoch, Moscow, Russia); 31. CLN-1314 (AVRDC, Shanhua, Taiwan); 32. Fina-1 (Inst. Vegetable Research, Guandzhu, China); 33. Xiaosheng-nu (Ho-Huan, Puli Nantou, Taiwan); 34. Korsar, 35. Baby and 36. New-Yorker (own collection). All cultivars were susceptible to the isolates of *P. infestans* used in this study.

Pathogen and inoculation Seven isolates of *P. infestans* were used in all experiments (Table 1) except Exp. 2, in which additional isolates were used (not listed). Isolates were maintained on potato tuber slices at 15°C in the dark. Freshly produced sporangia were collected into cold (4°C) distilled water and the concentration was adjusted to 2000–3000 sporangia per ml. To allow for oospore production in the inoculated fruits, sporangia of A_1 (sensitive to metalaxyl) and of A_2 (resistant to metalaxyl) isolates were mixed at a 1:1 ratio before inoculation. Control fruits were either inoculated with A_1 or A_2 sporangia, or left untreated.

Fruits (4–12 per cultivar per isolate combination) were placed on a moist filter paper inside a box, with the stem scar upward. Filter paper discs (5 mm diam, Whatman no. 1) were dipped in the sporangial suspension and then attached to the stem scar of the fruit, two or three filter paper discs per fruit. Boxes were sealed with their lids and maintained at 15°C in the dark for 2–3 days to ensure infection. Lids were then removed and boxes were kept at 18–20°C (50–60% r.h., 12 h light) for 3 weeks or more, depending on the experiment, to allow for blight development (21).

Seed extraction, processing and culturing Blighted fruits inoculated with A_1+A_2 sporangia were examined for the presence of oospores as described previously (21). Briefly, a fruit was cut into two halves along the stem scar, a ~5 mm piece of tissue was removed with a scalpel from 1 cm below the stem scar, placed on a microscope slide with a few drops of 70% glycerol, and examined microscopically. Seeds were extracted from oospore-containing fruits (about two-thirds of the fruits inoculated) as well as from the A_1 -inoculated, A_2 -inoculated, and uninoculated control fruits, fermented in water on the

bench for 2–3 days, washed and allowed to dry on the bench. Seeds were then stored in paper bags on the bench or at 4°C for various periods (up to 88 days), depending on the experiment. Seeds were then sown in a pasteurized potting mixture (peat + perlite, 1:1 v/v) in 1-liter pots in the greenhouse, 20–50 seeds per pot. The temperature was 15–17°C during the night and 20–24°C during the day. Plants were examined for blight symptoms for 60 days after emergence. In other experiments, seeds were surface-sterilized with 2% sodium hypochlorite for 5 min, washed and placed on PARP-selective medium (25) or on 2% water and agar in 3-cm-deep petri dishes or in 20×20×3 cm dishes (Nunk). Dishes were kept in a growth chamber at 18–20°C with 12 h of light ($100 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Seedlings were examined for blight symptoms for 30 days after plating.

Seventeen experiments were conducted (see Table 2). When fruits were inoculated with mixed A₁ S + A₂ R sporangia, seeds were extracted from oospore-bearing tomato fruits. In six experiments (nos. 5, 10, 14–17) seeds were also extracted from fruits inoculated with only the A₁ or A₂ parent isolates. In Exp. nos. 1–5, 16 and 17, two to seven different A₁+A₂ isolate combinations were used, whereas in Exps. 6–15 a single pair of A₁ + A₂ isolates was used (TUR1 + HAM or 870 + 367). In nine experiments only one tomato cultivar was used, whereas in the other experiments two to 20 tomato cultivars were used. In ten experiments seeds were sown in pasteurized soil whereas in the seven other experiments seeds were surface-sterilized before being sown in agar plates. Seeds were extracted, in different experiments, at 21–41 days after fruit inoculation, when oospores were present inside the fruit pericarp (seeds were not examined for the presence of oospores). Seeds were dried, after fermentation, on the bench for 2–14 days before being used. In Exp. nos. 3 and 5, seeds were stored at 4°C for 88 and 60 days, respectively, before being used. In Exp. nos. 6 and 7, pots were kept at 100% r.h. in transparent rigid, plastic boxes, whereas in the other experiments pots were maintained at 60–70% r.h.

Disease assessment Emerging seedlings were examined daily (except Saturdays) for blight symptoms. Blighted seedlings were removed with a forceps, placed individually in a plastic bag, washed with water, blotted dry, and placed between two potato tuber slices (cv. ‘Alpha’ or ‘Mondial’). Each such potato tuber-slice sandwich, carrying one blighted seedling, was placed in a 9-cm petri dish on dry filter paper. Petri dishes were placed in dry plastic bags and maintained at 15°C in the dark to allow the pathogen to infect the potato tuber tissue. The sporangia produced between and on the tuber slices were removed into cold water, examined microscopically and used for inoculation. One or more of the following inoculation tests were conducted with each such isolate: sensitivity to metalaxyl, mating type, and virulence factor combination.

Sensitivity to metalaxyl was tested with tomato (cv. ‘ZH’) leaf discs as described before (12). Briefly, ten tomato leaf discs were placed in a 9-cm petri dish on a 7-cm-diam paper disc saturated with 1 ml of 25% WP (wettable powder) metalaxyl (0, 0.1, 1, 10 or 100 $\mu\text{g ml}^{-1}$ a.i., Syngenta, Basel, Switzerland). Leaf discs were each inoculated with one 10- μl droplet of sporangial suspension (2000 per ml) of the test isolate. Dishes were kept at 18°C in the dark for 20 h and then at 18–20°C with a 12-h light photoperiod. At 7–8 days post-inoculation, disease development was assessed visually. An isolate was considered sensitive when sporulating on discs treated with 0 and 0.1 $\mu\text{g ml}^{-1}$ but not on discs treated with $\geq 1 \mu\text{g ml}^{-1}$; intermediately resistant when sporulating on 0–10 $\mu\text{g ml}^{-1}$ but not on 100 $\mu\text{g ml}^{-1}$ metalaxyl; and resistant when sporulating on discs treated with up to 100 $\mu\text{g ml}^{-1}$ of metalaxyl. Mating type determination was carried out with tomato leaflets, with

known reference (tester) A₁ and A₂ isolates, as described before (4). Briefly, two tomato (cv. ZH) leaflets were placed on wet filter paper in a petri dish. Three such dishes were used in each assay. Leaflets in the first dish were inoculated with the tested isolate by placing six 10- μ l droplets of sporangial suspension (2000 sporangia ml⁻¹) on each leaflet. Leaflets in the second dish were inoculated each with three droplets of the tested isolate and three droplets of an A₁ tester isolate (T04). Similarly, leaflets in the third dish were inoculated with the tested isolate and an A₂ tester isolate (367). After 10 days of incubation at 18–20°C, leaflets were clarified in boiling ethanol and examined microscopically for the presence of oospores. The tested isolate, by itself, never showed oospore production. Virulence phenotype was determined with leaflets of 12 potato differential cultivars in the manner described earlier (2). Potato differential cultivars, each carrying a single R-gene for blight resistance, were raised in pots in the greenhouse. For testing the virulence profile of an isolate, one leaflet was removed from each of the 12 cultivars, marked, and placed on wet filter paper in a 20×20 cm Nunk plate. Each leaflet was inoculated with six 10- μ l droplets of sporangial suspension (2000 sporangia ml⁻¹) of the tested isolate. After 7 days of incubation at 18–20°C, the compatibility of a tested isolate with each leaflet was determined. Compatibility was assigned when sporulation was seen on a leaflet, whereas incompatibility was assigned when a hypersensitive response (HR) was observed. Each isolate was given a virulence profile showing the R-genes that it overcame.

RESULTS

Of the 25,248 tomato seedlings that developed from seeds, only 23 (0.09%) were infected with *P. infestans* (Table 2). All infected seedlings emerged from fruits inoculated with A₁ + A₂ sporangia. No blight was observed in plants (a total of 3937) that developed from seeds extracted from fruits inoculated with either A₁ or A₂ sporangia, nor from seeds taken from uninoculated fruits (total of 1320 plants). If one considers only oospore-bearing fruits, then percent transmission was 0.12% (23 of 19,991 plants). Seventeen infected plants were recovered from agar plates and six from soil. Plants in soil were infected at the top of the hypocotyl hook at the interface with the cotyledons, whereas in agar the disease was noted in the hypocotyl and cotyledons. Disease was noted in these plants about 2 wk after sowing. No additional infected plants were seen throughout the observation period (60 days for pots and 30 days for agar).

TABLE 1. Isolates of *Phytophthora infestans* used in the present study

Isolate	Origin	Source	Mating type	Response to metalaxyl ^z	Virulence phenotype ^y	Used in experiment(s) ^x
T04	Switzerland	Tomato	A1	Sensitive	0 1 2 3 4 6 8 9 11	1–5,15–17
TUR-1	Turkey	Tomato	A1	Sensitive	0 1 3 4 6 7 8 9 11	1,3–13,16
870	Israel	Tomato	A1	Sensitive	0 1 3 4 7 9	14
SU	Israel	Tomato	A1	Sensitive	0 1 3 4 7 8 9	16, 17
HAM	Israel	Tomato	A2	Resistant	0 1 3 4 7 8 9 11	1,3–13
YAS	Israel	Potato	A2	Resistant	0 1 3 4 7 9	1–5,16–17
367	Israel	Potato	A2	Resistant	0 1 3 4 7 9	14–17

^zDetermined by the leaf disc bioassay according to Kadish and Cohen (12).

^yDetermined on 12 differential potato cultivars according to Cohen (2).

^xSee Table 2.

TABLE 2. Frequency of transmission of *Phytophthora infestans* via tomato seeds extracted from blighted fruits carrying oospores

Exp. no.	Cultivars inoculated ^z	No. of isolate pairs used for inoculation	Colonization period (days) ^y	Seeds drying period (days)	Growth substrate ^x	Total no. plants ^w	Blighted plants (no.)
1	1-4,7,9,10,12,13,14,18,19-23,26,27,30,36	4	31	un ^v	soil	9400	0
2	4	7	31	14	PARP agar	1400	0
3	1,12,14-16,22-24,20,29	2	29	88	soil	1000	0
4	4	4	25	0	PARP agar	400	0
5	1	4	37	60	soil	400	0
6	14,15	1	41	7	soil	1030	0
7	1	1	35	un	soil	500	0
8	1,15,27	1	35	un	water agar	540	0
9	11,13,31,32	1	32	8	soil	1570	1
10	1,14-16,23,27-29,34	1	22	2	soil	600	0
11	15	1	21	3	soil	250	0
12	8	1	28	un	soil	98	2
13	22	1	25	8	soil	600	3
14	5	1	26	8	water agar	200	0
15	33	1	28	7	water agar	584	2
16	1,33,35	3	27	14	water agar	3729	15
17	1,22	4	30	14	water agar	2727	0
Total						25,248	23 (0.09%)

^zSee Materials and Methods for names of cultivars.

^yPeriod between fruit inoculation and seed extraction.

^xSoil = pasteurized potting mixture; PARP = selective agar medium (25).

^wCounted up to 60 days after sowing in pots, or 30 days after sowing on agar media.

^vun = unknown.

TABLE 3. Phenotypic characterization of 21 *Phytophthora infestans* isolates recovered from tomato seedlings developed from seeds extracted from blighted tomato fruits carrying oospores

Exp. no.	Tomato cultivar	Parental isolates used for inoculating fruits ^z	Recovered isolates
12	VF-36	TUR1: A ₁ ; S; 0 1 3 4 6 7 8 9 11 HAM: A ₂ ; R; 0 1 3 4 7 8 9 11	A ₂ ; I A ₂ ; I
13	Master	TUR1: A ₁ ; S; 0 1 3 4 6 7 8 9 11 HAM: A ₂ ; R; 0 1 3 4 7 8 9 11	A ₁ ; S; 0 1 3 4 7 8 9 11 A ₁ ; S; 0 1 3 4 7 8 9 11 A ₁ ; S; 0 1 3 4 7 8 9 11
15	Xiangsteng-nu	TO4: A ₁ ; S; 0 1 3 4 6 8 9 11 367: A ₂ ; R; 0 1 3 4 7 9	A ₂ ; R; 0 1 2 4 9 11 A ₁ ; R; 0 1 3 4 7 9 10
16	ZH	SU: A ₁ ; S; 0 1 3 4 7 8 9 367: A ₂ ; R; 0 1 3 4 7 9	A ₁ ; S; 0 1 3 4 7 9 A ₁ ; S; 0 1 3 4 7 9 A ₁ ; S; 0 1 3 4 7 9 A ₁ ; S; 0 1 3 4 6 7 9 11 A ₁ ; S; 0 1 3 4 7 9 A ₁ ; S; 0 1 3 4 7 9 11 A ₁ ; S; 0 1 3 4 7 9 11 A ₁ ; S; 0 1 3 4 7 9 11 A ₁ ; S; 0 1 3 4 7 9 11 A ₁ ; S+R; 0 1 2 3 4 6 7 9 A ₁ ; S; 0 1 3 4 7 9 A ₁ ; S; Unknown A ₁ ; S; 0 3 7 A ₁ ; S; 0 1 3 4 7 A ₁ ; S; 0 1 3 4 7 9

^z A₁, A₂, A₁A₂ = mating type.

R, S, I = respectively, resistant, sensitive and intermediately resistant to metalaxyl (see Materials and Methods).
0–11 = virulence race structure.

Phytophthora infestans was successfully isolated from 21 of the 23 symptomatic plants, and the 21 isolates were characterized phenotypically (Table 3). The two isolates of Exp. no. 12 belonged to the A₂ mating type and both were intermediately resistant to metalaxyl; their virulence phenotype was not determined. The three isolates of Exp. no. 13 were A₁S and carried virulence factors 0, 1, 3, 4, 7, 8, 9, 11, which belong to the A₂R parent, HAM. Of the two isolates of Exp. no. 15, one was A₂R with virulence factors 0, 1, 2, 4, 9, 11 and the other was A₁A₂R with virulence factors 0, 1, 3, 4, 7, 9, 10. All 14 isolates of Exp. no. 16 were A₁ mating type; 13 were sensitive to metalaxyl and one yielded a mixture of sporangia, sensitive and resistant to metalaxyl. No isolate had a virulence structure identical to the A₁ parent. Six isolates had the virulence structure of the A₂ parent. All progeny isolates lost factor 8 of the A₁S parent. Two isolates gained factor 2 and four isolates gained factor 11, which were both absent in the parents.

DISCUSSION

The present work provides evidence that dry seeds extracted from blighted tomato fruits carrying oospores may transmit *P. infestans* to the next generation of tomato plants. It also corroborates previous findings (26) by showing that dry seeds extracted from fruits inoculated with either A₁ or A₂ sporangia, failed to produce infected seedlings.

Tomato seeds extracted from blighted tomato fruits harboring oospores (due to infection with mixed A₁ + A₂ sporangia), which were dried on the bench for 7–14 days, produced blighted seedlings. The proportion of such infected seedlings was low (0.12%), but the

phenotypic nature of the isolates recovered indicated a possible oosporic origin. Two such isolates showed intermediate resistance (I) to metalaxyl while their A_1 and A_2 parents were sensitive (S) and resistant (R) to metalaxyl, respectively. Resistance to metalaxyl in oomycetes is usually controlled by a single, semi-dominant locus [(17), and literature cited therein] and isolates with intermediate resistance were often obtained from a cross between S and R isolates (8). Other isolates had the mating type and sensitivity to metalaxyl of one parent (A_1 S) but a virulence profile of the other parent (A_2 R) or a new virulence profile, suggesting, possibly, oosporic origin. However, genetic instability of our isolates may have contributed to such changes. Groves and Champaco (10) reported that single-zoospore progeny obtained from four field isolates collected in Maine segregated for mating type and for self-fertility in culture.

Most infected plants emerging from fermented, air-dried seeds showed blight symptoms (and sporangia of *P. infestans*) at the hook of the germling while the seed coat was still attached to the cotyledons, suggesting that infection resulted from oospores either encrusted in the seed coat (21) or harbored in the embryo (21). Vartanian and Endo (26) showed that mycelia harbored by seeds failed to survive drying. In tomato fruits, *P. infestans* could survive in freshly extracted, wet seeds, but not in seeds that had been air-dried for 72 h following extraction (26). These studies were done with fruits infected by a single mating type and seeds were colonized only by mycelia. Our data therefore support the hypothesis that the transmission we observed was a result of oospores that had survived air-drying. The low rate of transmission derives mainly from the rare occurrence of oospores in seeds (21), but it may also result from poor compatibility of some crossings and/or poor viability of oospores of some combinations. The effect of host and pathogen genotypes on seed transmission merits further investigation.

One non-parental isolate recovered from seeds was A_1A_2 . It produced oospores with either A_1 or A_2 tester isolates, but not when cultured alone (self-sterile). Such a phenomenon was observed by H.S. Judelson (personal communication) and expected by the model of Ko [(13), and personal communication]. Savenkova and Cherepennikova-Anikina (23) observed it in cultures treated with chemicals. They postulated that such A_1A_2 mutants “either produce hormones that stimulate testers to form oospores or apprehend hormones from both testers.”

Another feature observed with isolates recovered from the blighted seedlings is a change in the virulence phenotype. This stands in accordance with Mayton *et al.* (17), who claimed that “sexual recombination might produce individuals with adaptability and virulence factors that are different than those of either parent.” A change in virulence might also evolve from a pre-existing virulence diversity in parental isolates. Abu-El Samen *et al.* (1) recently demonstrated a high level of virulence diversity in single-zoospore asexual progenies of US-8 isolates having intermediate resistance to metalaxyl. Goodwin *et al.* (9) showed rapid evolution of virulence within clonal lineages, presumably by mutation.

In Israel, heavy epidemics of late blight occurred in tomato during 1998–2003 [(2) and Y. Cohen, unpublished]. During 1998–2000, 25 races of the pathogen were identified, with 14 of them being specific to tomato (2). The reasons for the increased aggressiveness to tomato are not clear.

Of special significance was our finding (21) that oospores may be produced inside tomato seeds. The fact that *P. infestans* can be a seedborne pathogen is known [(26), and literature cited therein], but the epidemiological role of oospores harbored by tomato seeds

was never investigated. We have recently observed (21) that *P. infestans* may produce oospores not only in the pericarp of blighted tomato fruits but also on the surface of such fruits if kept in a moisture-saturated atmosphere. Oospore inoculum may thus become airborne and spread infection with recombinant genotypes during the season.

The abundant production of oospores in tomato fruits in nature (24,25) relative to potato tubers (16,18) may indicate the role of tomato not only in facilitating the establishment of sexually fit recombinant genotypes, but also in spreading the A₂ mating type to regions that have A₁ only. Mayton *et al.* (17) reported that after burial in soil in nature, oospores from tomato fruits were infectious to tomato and potato leaflets, whereas oospores from potato tubers did not cause infection. Gavino *et al.* (7) supplied evidence that the US-11 lineage represents a sexual progeny of US-6 and US-7, and because it is especially fit on tomato, it may dominate populations over large areas.

In conclusion, our results suggest a very low probability of transmission of *P. infestans* via tomato seeds extracted from blighted fruits carrying oospores. No such transmission was observed in seeds extracted from fruits inoculated with a single isolate, A₁ or A₂. The major changes observed in the phenotype of the recovered isolates and the fact that transmission occurred after seed drying, indicate that oospores may have a role in transmission of late blight in tomato. Seed transmission of late blight may represent a threat to commercial or home tomato production systems.

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