Techniques

A Fluorescent Technique for Studying Growth of *Peronospora tabacina* on Leaf Surfaces

Yigal Cohen, Sara Pe'er, Orly Balass, and Michael D. Coffey

First three authors: professor and graduate students, respectively, Department of Life Sciences, Bar-Ilan University, Ramat-Gan 52 100, Israel. Fourth author: professor, Department of Plant Pathology, University of California, Riverside
Accepted for publication 8 July 1986.

ABSTRACT

Cohen, Y., Pe'er, S., Balass, O., and Coffey, M. D. 1987. A fluorescent technique for studying growth of *Peronospora tabacina* on leaf surfaces. Phytopathology 77:201-204.

Calcofluor was used as a fluorescent nontoxic stain to study germination, appressorial formation, vesicle production, and sporulation of *Peronospora tabacina* on leaf surfaces. Fungal germination proceeds equally well on leaves of tobacco, pepper, tomato, and potato but not on *Nicotiana debneyi* in which germ tubes were abnormal and appressorial formation was inhibited. In tobacco, appressorial formation decreased with increasing plant age and gradually decreased in younger leaves of older plants. In tobacco, appressorial formation was not affected as long as 21 days after stem inoculation with *P. tabacina* but was inhibited at 30 days. Germination and appressorial formation in tobacco were not affected by metalaxyl but were strongly inhibited by β-ionone. The technique is simple, rapid, and reliable and can be used to study effects on fungal ingress in situ of plant genotypes, toxicants, and environmental factors.

Additional key words: microscopy, resistance.

In vitro studies with *Peronospora tabacina* have shown that conidial germination depends on biotic and abiotic factors such as age, pigmentation and source of conidia, temperature, relative humidity, CO_2, sugars, and toxicants (2,3,5,11,14,15). Unlike in vitro assays, in vivo tests with susceptible tobacco leaves showed very low (about 1%) germination of conidia (3,13,16). Percentage germination on leaves of the resistant *Nicotiana debneyi* is much higher than on susceptible tobacco (3,13).

Various techniques have been used to observe germination of *P. tabacina* on leaf surfaces. Shepherd and Mandryk (13) and Cruickshank (3) used one of the following: transfer of the conidial suspension from an inoculated leaf surface to a microscope slide with a micropipette, application of 1% cotton blue to conidial suspension on leaf surface and examination in situ, or removal of conidia from leaf surface by a collodion film and application of 1% cotton blue to conidial suspension. McKeen and Svirčev (6) fixed inoculated tissue in 4% glutaraldehyde, dehydrated in alcohol, and stained with aqueous acidified Lacmoid or cleared the tissue with methyl alcohol, chloroform, and lactophenol (1:1:1) for 1–2 hr and then stained it with acid fuchsin in lactophenol. Trigiano et al (16) cleared tissue in hot (80 C) 50% chloral hydrate and stained it with 0.1% aniline blue. These methods are laborious and slow and may be inaccurate due to removal and distortion of fungal structures. In

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situ staining techniques therefore have a great advantage when available. In this paper we report such a technique employed for *Peronospora hyoscyami* f. sp. *tabacina* Adam (= *P. tabacina*), the causal agent of blue mold in tobacco.

MATERIALS AND METHODS

Plants and pathogen. We used whole leaves or leaf disks (1 cm) of the following: *Nicotiana tabacum* L. cv. Ky 14 and Ky 16 (tobacco); *N. debneyi* (from C. C. Litton, University of Kentucky, Lexington); *Lycopersicon esculentum* 'Casino Royal' and 'Hosen-Ayalon' (tomato); *Solanum tuberosum* 'White Rose' and 'Alpha' (potato); and *Capsicum annuum* 'Yellow Wonder' (pepper). Plants were grown in the greenhouse.

Two isolates of the pathogen, *P. tabacina*, were used: in

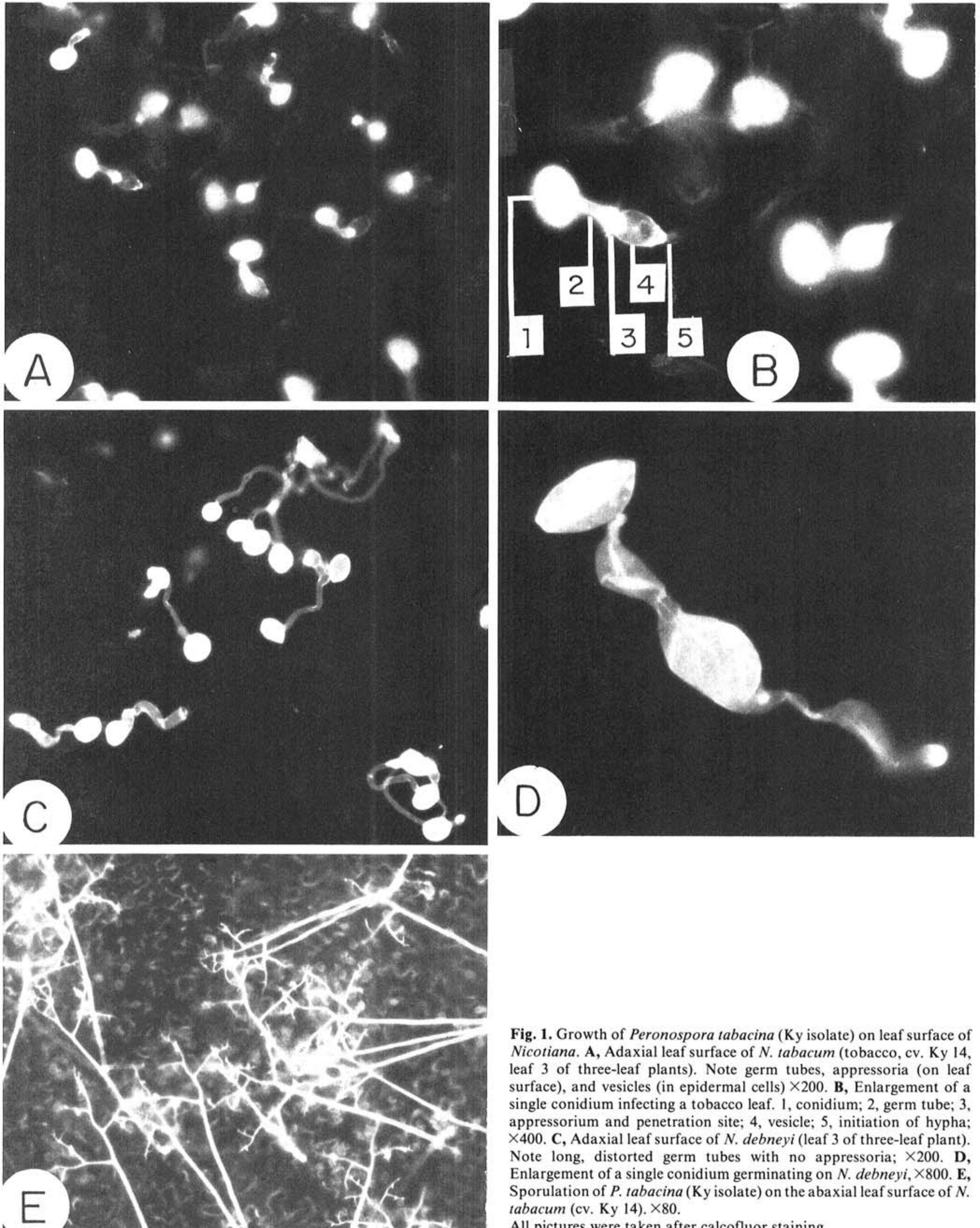

Fig. 1. Growth of *Peronospora tabacina* (Ky isolate) on leaf surface of *Nicotiana*. **A,** Adaxial leaf surface of *N. tabacum* (tobacco, cv. Ky 14, leaf 3 of three-leaf plants). Note germ tubes, appressoria (on leaf surface), and vesicles (in epidermal cells) ×200. **B,** Enlargement of a single conidium infecting a tobacco leaf. 1, conidium; 2, germ tube; 3, appressorium and penetration site; 4, vesicle; 5, initiation of hypha; ×400. **C,** Adaxial leaf surface of *N. debneyi* (leaf 3 of three-leaf plant). Note long, distorted germ tubes with no appressoria; ×200. **D,** Enlargement of a single conidium germinating on *N. debneyi*, ×800. **E,** Sporulation of *P. tabacina* (Ky isolate) on the abaxial leaf surface of *N. tabacum* (cv. Ky 14). ×80.
All pictures were taken after calcofluor staining.

California, the Georgetown isolate (= Ky) collected by Y. Cohen in Georgetown, KY, in 1979 from burley tobacco (supplied by M. Reuveni, Lexington, KY), and in Israel an isolate (= IL), collected in Maalot in 1976 from oriental tobacco. The pathogens were maintained on tobacco plants by repeated inoculations in growth chambers at 20 C. For experimentation, freshly produced conidia were harvested into distilled water, filtered through 8-μ Millipore membrane, washed twice with distilled water, and resuspended in cold distilled water. Usually 15 min lapsed between harvest and inoculation.

Inoculation. Ten-microliter droplets of a conidial suspension (100–200 conidia) were applied to whole leaves or to 1-cm leaf disks, floated (adaxial surface up, unless otherwise stated) on distilled water in 9-cm petri dishes. Dishes were incubated at 16 ± 2 C in the dark for 5–18 hr. Tobacco plants (cv. Ky 16) were protected against blue mold by stem injection, as described previously (1). At various intervals after stem injection, leaf disks were removed from leaf no. 11, floated on water, and inoculated on the adaxial surface. The effect of metalaxyl was observed in leaf disks floated on metalaxyl (25 WP) solutions of various concentrations. Toxicity of calcofluor and β-ionone was determined by placing 10-μl droplets of each compound on leaf disks floated on water immediately after inoculation.

Microscopic observations. A 5-μl droplet of aqueous 0.01% calcofluor (Calcofluor White M2R, disodium salt of 4,4′-bis(4-anilino-6-diethyl-amino-s-triazin-2-ylamino)-2,2′-stilbene disulfonic acid (Polysciences Inc., Warrington, PA) solution was applied to each inoculated site. Leaf disks or disks taken from whole leaves were covered with a cover glass and examined microscopically. In California, a fluorescent Zeiss microscope (photomicroscope III) equipped with an Osram halogen lamp (HBO 50W), a vertical illuminator (model III RS), and no. 18 filter set that included a BP395-425 excitation filter, an FT425 dichromatic beam splitter, and an LP450 barrier filter was used. In Israel, the studies were done with a fluorescent Zeiss standard microscope equipped with an IVFI epifluorescence condenser and no. 18 filter set. A Neofluar 16/0.40 objective was used, with a ×12.5 eyepiece. With this setup, conidia and infection structures fluoresced blue, while leaf tissue remained dark. Photographs were taken with an automatic camera using Plus-X Kodak black and white film.

Usually, 100 conidia were examined on each disk; they were divided into four classes: class A, nongerminating conidia; class B, conidia producing a short (about 5–25 μm) germ tube with an appressorium; class C, conidia producing a long (about 30–100 μm) germ tube with an appressorium; class D, conidia producing a long germ tube with no appressorium in its end.

RESULTS

Growth of Ky isolate on *Nicotiana* spp. In cotyledons of 19-day-old tobacco plants (cv. Ky 14), 80% of the conidia produced a short germ tube with appressorium (class B), 16% were nongerminating (class A), and 4% germinated with a long germ tube with no appressorium (class D). In cotyledons of *N. debneyi*, corresponding numbers were 47, 40, and 13%, respectively. The conidiophores produced per cotyledon 7 days after inoculation (determined by the calcofluor technique) was 49 for tobacco and 33 for *N. debneyi*. Both observations indicate a susceptibility of *N. debneyi* to blue mold at the cotyledon stage. In three-leaf seedlings (4 wk old), equally high percentage germination occurred on both species in all leaves; but although formation of appressoria and vesicles (inside epidermal cells) was abundant in tobacco (Fig. 1A), it was rare in leaves 1 and 2 and absent in leaf 3 of *N. debneyi* (Fig. 1C). Germ tubes on *N. debneyi* were mostly long and/or swollen and/or distorted (Fig. 1C, D). This was reflected in sporulation (Fig. 1E) on the fifth day after inoculation: numbers of conidiophores on lower surfaces (calcofluor assay) were 156, 216, and 312 per 6-mm-diameter leaf disk in leaves 1, 2, and 3 of tobacco, compared to 15, 0, and 0 in corresponding leaves of *N. debneyi*, indicating resistance developing in leaves 2 and 3.

Growth of Ky and IL isolates on other Solanaceae. Abundant germination occurred on pepper, potato, and tomato. On pepper (cotyledons and first leaf, about 2 wk old), germ tubes were short and produced appressoria. In tomato (cotyledons and young leaves, about 3 wk old) and potato (lower leaves of about 2-mo-old plants), about one-third of the germinating conidia produced short germ tubes with appressoria at their ends, one-third produced about 50-μm germ tubes with no appressoria, and about one-third produced coiled germ tubes about 100 μm long, with no appressoria. A strong hypersensitive reaction was observed in potato and tomato but not in pepper, 24 hr after inoculation.

Growth of the IL isolate on tobacco plants. Plant and leaf age had a profound effect on conidial germination and appressorial formation (Table 1). Percentage of nongerminating conidia (class A) increased and percentage of conidia germinating with a short

TABLE 1. Effect of plant's age, leaf age, and leaf surface inoculated on germination of *Peronospora tabacina* (IL isolate) on tobacco (cv. Ky 16)

Plant age (wk)	Leaf no.[b]	Percentage conidia[a]							
		Abaxial (lower) leaf surface				Adaxial (upper) leaf surface			
		A[c]	B	C	D	A	B	C	D
5	cotyledons	31 ± 7[d]	67 ± 6	1 ± 1	1 ± 1	30 ± 6	70 ± 5	0 ± 1	0
	1	30 ± 6	70 ± 6	0	0	39 ± 1	56 ± 9	5 ± 9	0
	2	32 ± 3	68 ± 4	0 ± 1	0 ± 1	46 ± 32	47 ± 34	4 ± 5	3 ± 3
10	5	31 ± 3	68 ± 3	1 ± 1	0	43 ± 14	39 ± 10	17 ± 4	1 ± 1
	6	28 ± 1	72 ± 1	0 ± 1	0	44 ± 26	42 ± 24	13 ± 10	1 ± 1
	7	26 ± 10	74 ± 9	0	0	28 ± 12	65 ± 14	7 ± 9	0
12	6	30 ± 3	68 ± 3	2 ± 1	0	29 ± 7	64 ± 8	7 ± 2	0
	7	35 ± 4	65 ± 4	0 ± 1	0	40 ± 20	48 ± 29	12 ± 9	0
	8	35 ± 2	65 ± 2	0	0	45 ± 21	51 ± 29	4 ± 3	0
	9	30 ± 2	69 ± 2	1 ± 1	0	46 ± 41	48 ± 40	6 ± 3	0
17	7	42 ± 9	56 ± 6	2 ± 2	0	33 ± 5	52 ± 15	14 ± 13	1 ± 2
	8	50 ± 11	50 ± 12	0 ± 1	0	51 ± 12	43 ± 10	6 ± 2	0
	9	58 ± 16	42 ± 15	0	0	60 ± 24	38 ± 22	2 ± 2	0
	10	73 ± 13	26 ± 12	1 ± 1	0	65 ± 28	32 ± 25	3 ± 3	0
	11	60 ± 7	39 ± 7	1 ± 0	0	94 ± 10	5 ± 9	0	1 ± 1
	12	92 ± 13	8 ± 13	0	0	100	0	0	0
	13	100	0	0	0	100	0	0	0
	14	100	0	0	0	100	0	0	0

[a] Three plants per treatment with four leaf disks (1-cm diameter) per leaf; 100 conidia counted per disk.

[b] From stem base.

[c] Classes A–D described in Materials and Methods.

[d] Mean of three replicates ± standard deviation of the mean.

TABLE 2. Germination of *Peronospora tabacina* (IL isolate) on adaxial leaf surfaces of tobacco (cv. Ky 16) protected against blue mold

Stem injection	Percentage conidia[a]			
	A[b]	B	C	D
Water (control)	53 ± 3	31 ± 7	14 ± 3	2 ± 1
P. tabacina (protected)[c]	82 ± 20	9 ± 11	6 ± 3	3 ± 2

[a]Ten leaf disks per plant, three plants per treatment.
[b]Classes A–D described in Materials and Methods.
[c]Protection done with 12-wk-old plants, according to Cohen and Kuć (1). Assay conducted 30 days after stem injection of leaf no. 11.

TABLE 3. The effect of β-ionone on conidial germination of *Peronospora tabacina* (IL isolate) on leaf surface of tobacco (cv. Ky 16)

Final β-ionone (μg/ml)	Germination on 2% agar (%)	Percentage conidia[a]			
		A[b]	B	C	D
0	73 ± 15	22 ± 2	55 ± 13	20 ± 16	3 ± 2
0.0005	63 ± 4	47 ± 4	29 ± 3	18 ± 10	6 ± 5
0.005	39 ± 6	55 ± 5	21 ± 7	15 ± 4	9 ± 2
0.05	24 ± 4	86 ± 12	3 ± 4	3 ± 2	7 ± 3
0.5	5 ± 7	90 ± 1	0.5 ± 0.7	0	10 ± 3
5	0	...	...	...	...

[a]Ten leaf disks per treatment.
[b]Classes A–D described in Materials and Methods. Assayed with leaf 2 or three-leaf (4-wk-old) tobacco seedling. β-ionone applied on top of inoculum droplets 0 hr after inoculation. Aqueous 0.01% calcofluor added 18 hr after inoculation.

germ tube and appressorium (class B) decreased with increasing plant age. This trend was observed for conidia germinating on either adaxial or abaxial surface. Leaf age had a minor influence in 5-, 10-, and 12-wk-old plants but a major effect in 17-wk-old plants in which class B germination gradually decreased in younger leaves and reached zero in the youngest. Although no major differences in germination were observed between adaxial and abaxial surfaces, variability (reflected by standard deviation) was greater on adaxial than on abaxial surfaces. These results support Shepherd and Mandryk (12), who showed that in 20-leaf tobacco (cv. Virginia Gold) plants, the concentration of substances inhibitory to germination of *P. tabacina* is highest on the upper leaves and lowest on the lower leaves and that this distribution inversely correlates with the relative susceptibility of such leaves to infection. No significant differences in fungal growth were observed between normal and protected plants 15, 17, and 21 days after injection. However, germination was inhibited and the percentage of conidia producing appressoria decreased at 30 days (Table 2). Protected whole plants developed many restricted lesions when spray-inoculated at 21 days but very few lesions or none if spray-inoculated at 30 days after stem inoculation. These results agree with our previous findings (1).

Growth of the IL isolate in the presence of toxicants. Calcofluor of 0.01% had no effect on conidial germination or formation of appressoria and vesicles on tobacco leaves, and it did not affect further fungal development. Metalaxyl, up to 100 μg a.i./ml had only a slight effect on germination and appressorial formation but completely inhibited fungal sporulation in leaf disks floated on 0.05 μg a.i./ml, indicating that the chemical inhibits postinfectional stages of fungal development (16). β-ionone at 0.00 and 0.5 μg/ml inhibited appressorial formation 50 and 99%, respectively, compared to a water control (Table 3), thus confirming previous findings (5).

DISCUSSION

The calcofluor epifluorescence technique is useful for comparative analyses of the early infection process with *P. tabacina*. Our observations indicated that resistance to *P. tabacina* may operate at pre- or postinfection stages of fungal development. Preinfection resistance was due to reduced appressorial formation (or absence thereof), whereas postinfection resistance was associated with collapse of epidermal plant cells. The former type of resistance was observed when conidia of *P. tabacina* were applied to the youngest leaves of normal 17-wk-old tobacco plants, in leaves of (protected) tobacco 30 days after stem-injection of *P. tabacina*, in leaf no. 2 and older leaves of *N. debneyi*, and in tobacco leaves treated with β-ionone. The latter type of resistance was observed when conidia of the pathogen were applied to leaves of tomato, potato, and metalaxyl-treated tobacco.

The new findings of this study are that: *N. debneyi* is susceptible to blue mold in cotyledons and first leaves; resistance to blue mold in leaves of stem-injected tobacco at 30 days, but not before, results from escaped infection due to inhibition of appressorial formation; and tomato and potato leaves are hypersensitive to *P. tabacina*.

The calcofluor technique was used by Ersek et al (4) to precisely locate fungal infection sites in the *Peronospora manshurica*-soybean system. Similar techniques have also been useful with other plant (8,10) and human (7) fungal pathogens. Our studies (Cohen and H. Eyal, *unpublished*) have shown that the calcofluor epifluorescence technique is useful for studying conidial germination and growth of *Sphaerotheca fuliginea* on susceptible and resistant genotypes of muskmelons.

The calcofluor fluorescence technique described in this paper is easy to handle, fast, and reliable. It provided comparative data on growth in situ of *P. tabacina* (germination, appressorial formation, vesicle production, and sporulation) on various leaf surfaces and was used to study the effect of toxicants on the early infection process. Calcofluor is a nontoxic cellulose marker that has been used as a main optical brightener for staining of microorganisms (9).

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