Induction of Secondary Fluorescence in the Resting Sporangium of the Potato Wart Disease Fungus, Synchytrium endobioticum (European race 2)

Michael C. Hampson

Research Station, Agriculture Canada, P.O. Box 7098, St. John's West, Newfoundland, Canada.
The author gratefully acknowledges the able technical assistance of Dominic Johnson, Student Assistant.
Accepted for publication 3 May 1974.

ABSTRACT

Strong secondary fluorescence was induced in resting sporangia of *Synchytrium endobioticum* under long-wave ultraviolet radiation. Sporangia were treated with 0.85% NaCl, phosphate-buffered at pH 7.4, and stained with 1×10^{-4} M solutions of basic arylmethane, acridine, or quinone-imine dyes. Secondary fluorescence was greatest in the walls of sporangia with organized contents, and in the internal regions of sporangia which had hyaline or disorganized contents. The ratio of wall-fluorescnce only to content-fluorescence decreased with increase in sporangial age. It was hypothesized that infectivity would fall with age. Pot experiments with sporangia of different ages at several concns supported this hypothesis; age was a more influential factor than was inoculum density. Care was taken to develop a method for extracting sporangia from gall tissue to yield very low backgrounds of stainable debris. It is suggested that the secondary fluorescence technique can be used as a novel basis for bioassay of the comparative activities of sporangial germinants; and, *inter alia*, for identifying viability in single sporangium samples.

Phytopathology 65:374-379

Additional key words: acridines, sporangial viability, bioassay.

The problem of sporangial viability is a central one in the study of potato wart disease caused by *Synchytrium endobioticum* (Schilb.) Perc. The fungus is obligate, the meiosporangium can remain dormant in soil for 30 yr or more, traditional tests for infectivity are lengthy and uncertain, and the disease has a quiescent period of several weeks (10).

Glynne (3) obtained a correlation between infectivity and the ability of the resting sporangium to stain when sporangial contents were pressed out in acid fuchsin. Nelson and Olsen (7) found that viable resting sporangia reacted positively to 2,3,5-triphenyl tetrazolium chloride. These tests, however, either destroyed sporangia or took several weeks to complete. Moreover, neither work indicated the fungal race used, and it is possible that results obtained with European race 2, the most widespread Newfoundland race, were influenced by physiological characteristics of that race (13). Indeed, Nelson and Olsen were only able to partially stain heat-killed race 2 sporangia with acid fuchsin.

Noble and Glynne (8) cite a technique for establishing resting sporangia viability in which sporangial walls fluoresced red and contents fluoresced blue-green when viable resting sporangia were mounted in lactophenol and irradiated with long-wave ultraviolet radiation. Dead or empty sporangia fluoresced dull red only. I was unable to repeat the technique, since I found that race 2 sporangia mounted in lactophenol (itself autofluorescent) glowed golden-yellow when irradiated (Fig. 1-A). Sporangia were only weakly autofluorescent in buffer (Fig. 1-C).

This paper describes an experience with fluorescent staining for determining sporangial viability in *S. endobioticum*.

MATERIALS AND METHODS.—*S. endobioticum* was grown on *Solanum tuberosum* L. 'Arran Victory'. Gall tissue was harvested, dried, and stored at 4 C until used.

To extract sporangia from gall tissue, gall samples were subjected to 55 kHz sonification for 6 min in 0.1% Triton X-100 phosphate-buffered saline, then homogenized in buffered saline for 4 min (Virtis Homogenizer Model 23, scale setting = 15). The homogenate was passed successively through 149, 105, and 37-μm nylon screens (Spex Industries, Inc., Metuchen, N.J.), the last under vacuum; sporangia were collected by washing this screen.

In fluorescent staining, sporangia were concd by centrifuging for 2 min at ca. 500 *g*, decanting, adding 25 ml fresh buffered saline and a known volume of stock fluorochrome solution and mixed by swirling.

To prepare sporangia for microscope examination, fluorochromes were decanted following centrifuged sporangial repacking, one or two ml buffered saline were added to each tube and sporangia allowed to settle in the tips of pipets into which sporangial suspensions were drawn. Sporangia were transferred to nonfluorescent, acid-alcohol-washed microscope slides, and irrigated with 10% aqueous glycerol (Fig. 1-D).

Stock solutions of dye stuffs (5×10^{-3} M) were stored in brown glass bottles at 4 C. Fluorescent stainings were carried out with freshly mixed phosphate buffers to give pH levels 3.5, 4.8, 5.8, 7.1, 8.2, 10.2, and 11.1 (1).

At least 100 sporangia were examined from each sample using a Zeiss Standard RA microscope fitted with a No. II Zeiss Fluorescent Illuminator and a HBO 200 W/4 light source. Observations were made with exciter filters BG 38 combined with BG 12 or UG 1, and a blue filter in the light train provided normal illumination. Most observations were carried out with a ×16 objective and ×8 ocular. All photomicrographs were taken at a magnification of ×128 and 0.5 s exposure on daylight type Kodak Kodachrome II film (5) and prints were enlarged ×2.

To determine levels of infectivity of sporangia at different times after harvest, pregerminated tubers were planted in sporangia-infested potting mix (perlite-peat moss; 1:1, v/v). The pots were placed in a controlled environment room (4) at 21 C (70 F) and 14 h day, and watered daily to field capacity. The plants were harvested at 8 wk. The rates of mix infestation were 150, 300, and

600 sporangia/g. Symptom expression was measured as the product of percent infection and d wt gall tissue/d wt plant (i.e., severity of attack) and termed the Wart Index.

RESULTS.—Twenty-five representatives of dye groups (6) were tested.

Whenever secondary fluorescence occurred it was depressed at acid pH values. Optimum staining pH was found to be 7.4.

Internally located secondary fluorescence indicated sporangial wall permeability and in testing for artifacts which might have developed during staining procedures resting sporangia were extracted from gall tissue in 0.85% NaCl. NaCl produced a remarkable effect, inducing a dramatic increase in fluorescent intensity. Similar increases occurred with $CaCl_2$, $MgCl_2$ and KCl but diminished with increase in molarity. Neither 1 N HCl, 1 N NaOH, Triton X-100, Calgon, $Ba(OH)_2$ and Javelle Water (0.525% available Cl), nor preheating sporangia at 37 C for varying times in these fluids failed to affect fluorescent intensity and percent secondary fluorescence. Physiological saline, at pH 7.4 and room temp, was used for all subsequent work.

Stepwise experimentation indicated that 1×10^{-4} M dye dilution would serve most fluorochroming requirements. Fluorescent staining occurred instantaneously; increases in contact time did not influence markedly fluorescent intensity.

Anionic dyes acid fuchsin, congo red, cotton blue, eosin Y, fluoroscein, primulin, rose bengal, thiazol yellow, and uranin failed to induce secondary fluorescence although several dyes observed under normal illumination stained walls and entered sporangia. The cationic dyes acetin blue, crystal violet, methylene blue, rhodamin B, and thionin also failed to induce secondary fluorescence, and the pyronins did so only faintly. Very high levels of fluorescence, however, followed staining with cationic arylmethane dyes auramine 0 and basic fuchsin; acridine dyes acridine orange, acridine yellow, phosphine, and acriflavine HCl; and quinone-imine dyes neutral red, rhoduline violet and magdala red (Table 1).

Some resting sporangia fluoresced due to staining of the walls only; while in others, fluorescence was also clearly located in the central part of the sporangium. Generally, all the successful fluorochromes induced secondary fluorescence of walls on irradiation, irrespective of the state of the contents. Least fluorescence intensity was displayed by thick walls of sporangia with organized contents.

Under normal illumination, three different types of resting sporangia were found in any one population: (i) thick-walled with contents differentiated into immature zoospores (organized) (A) (Fig. 1-D), (ii) thick-walled with contents either not differentiated or reticulated and vacuolated (disorganized) (B), (iii) thin-walled with hyaline contents (C). Group C will not be considered further. It was clear that the proportion of A to B varied with age of gall. With increasing age, group A members were less frequent, whereas group B members increased in number (Table 2).

When the sporangial populations were examined under fluorescent-staining conditions a high proportion of fresh resting sporangia displayed secondary fluorescence in the walls only. This number decreased with time, whereas the number of sporangia with brightly fluorescing contents increased with time (Table 3).

To test the hypothesis that resting sporangia with brightly fluorescing contents were nonviable, tubers were inoculated with sporangia of different ages at different levels of inoculum density.

Infectivity of the samples decreased with time (Table 4). The highest Wart Indices were recorded for sporangia harvested from infected plants within 5 wk.

DISCUSSION.—Several physical and chemical (enzyme digestion) methods were tried for obtaining sporangial suspensions suitable for staining, because fluorescent dyes taken up by accumulated plant and soil debris increased unwanted background and nonspecific

TABLE 1. Cationic dyes which induced secondary fluorescence in resting sporangia of *Synchytrium endobioticum*

Dye	C.I. No.	Sup.[a]	Secondary fluorescence colors
Fluorene dyes			
Pyronin Y	45005	EL	Faint pink
Pyronin B	45010	NA	Faint pink
Acridine dyes			
Acridine yellow	785	HL	Yellow, orange, green
Acriflavine-HCl	46000	HL	Red, orange, yellow, green
Acridine orange	46005	HL	Red, orange, yellow, green
Phosphine	...	HL	Red, orange, yellow, gold, green
Quinone-Imine dyes			
Neutral red	50040	HL	Red, orange, gold, yellow, green
Rhoduline violet	50215	EL	Red, orange, gold-yellow, green
Magdala red	...	EL	Faint red, yellow
Arylmethane dyes			
Auramine 0	41000	HL	Gold-yellow
Basic fuchsin	677	NA	Gold-red, yellow, green

[a]Suppliers: EK = Eastman Kodak Co., Rochester, N.Y.; EL = ESBE Laboratory Supplies, Toronto, Ont.; FS = Fisher Scientific Co., Fairlawn, N.J.; HL = Hartman-Leddon Co., Philadelphia, Pa.; NA = National Aniline Division of Allied Chemical and Dye Corp., New York, N.Y.

TABLE 2. Percentages[a] of resting sporangia of *Synchytrium endobioticum* with 'organized' (A)[b] or 'disorganized' (B)[c] contents in populations of different ages

Age of gall[d] (days)	Sporangial type	
	A (%)	B (%)
14	86	6
45	41	18
84	8	43

[a]Counted under normal illumination.
[b]Contents rounded off into immature zoospores.
[c]No differentiation into zoospores, contents reticulated or vacuolated.
[d]Sporangia extracted from galls grown on potato variety 'Arran Victory', harvested and stored at 4 C until used.

fluorescence. Ultrasonic energy did not appear to influence dye uptake, therefore it was used to effectively clean gall tissue of adhering soil particles; low speed homogenization released sporangia from gall tissue without excessive tissue shearing. Many microscope mounts were obscured by populations of starch grains, some of which are evident in Fig. 1-D.

Under the specified conditions populations of resting sporangia of *S. endobioticum*, European race 2, were fluorescent-stained by cationic dyes. Wittekind (12) comments that low molecular weight acidic dyes have no special affinity for cell structures, and this would seem to be the case for *S. endobioticum*. The basic dyes were of low molecular weight (ca. MW 330), possessed related molecular configurations, and yielded high percentages of brightly fluorescing sporangia under excited irradiation.

Other races of the fungus in Newfoundland (races 1, 6 and 8) will be examined for fluorescent behaviour and will be the subject of another paper.

The extent of fluorochrome penetration in the sporangia was associated with the dye class (Table 1). Generally, arylmethanes induced superficial fluorescence, quinone-imine dyes permeated wall structures more thoroughly, and acridines permeated walls and induced fluorescence of contents (Fig. 1-B).

TABLE 3. Percentages[a] of fluorescent stained resting sporangia of *Synchytrium endobioticum* in populations of different ages displaying most intense fluorescence of walls or contents in populations of different ages

Age of gall[b] (days)	Dyestuff	Part of sporangium with most intense fluorescence	
		walls (%)	contents (%)
14	acridine orange	86	12
45		49	50
14	phosphine	87	11
45		55	44
14	acridine yellow	92	8
45		60	42
14	acriflavine-HCl	86	14
45		54	45

[a]Counted under long-wave ultraviolet irradiation.
[b]Sporangia extracted from galls grown on potato cultivar 'Arran Victory', harvested and stored at 4 C until used.

TABLE 4. Infection intensities (expressed as Wart Indices) of potato variety 'Arran Victory' inoculated with varying population densities of *Synchytrium endobioticum* resting sporangia of different ages

Age of inoculating[a] sporangia (days)	Plants inoculated (no.)	Inoculum density (spores/g mix)	d wt gall (G)	d wt plant (P)	G/P	% I	Wart index[b] (WI)	Mean WI
11	4	300	7	19	0.37	100	36.8	27.6
	4	150	5	27	0.19	100	18.5	
15	9	200	3	26	0.15	100	15.0	
	2	100	3	14	0.21	100	21.4	16.7
	4	50	4	22	0.18	100	13.6	
36	9	200	4	28	0.14	100	14.0	
	4	100	5	21	0.24	50	11.9	17.6
	3	50	4	10	0.40	67	26.8	
54	10	200	0.3	26	0.01	80	0.9	
	2	100	0	15	0	0	0	2.2
	4	50	2	27	0.07	75	5.6	
63	4	300	2	23	0.08	100	8.7	5.5
	4	150	1	29	0.03	75	2.3	

[a]Sporangia extracted from galls grown on potato variety 'Arran Victory', harvested and stored at 4 C until used.
[b]Wart index (WI): G/P × % I = WI.

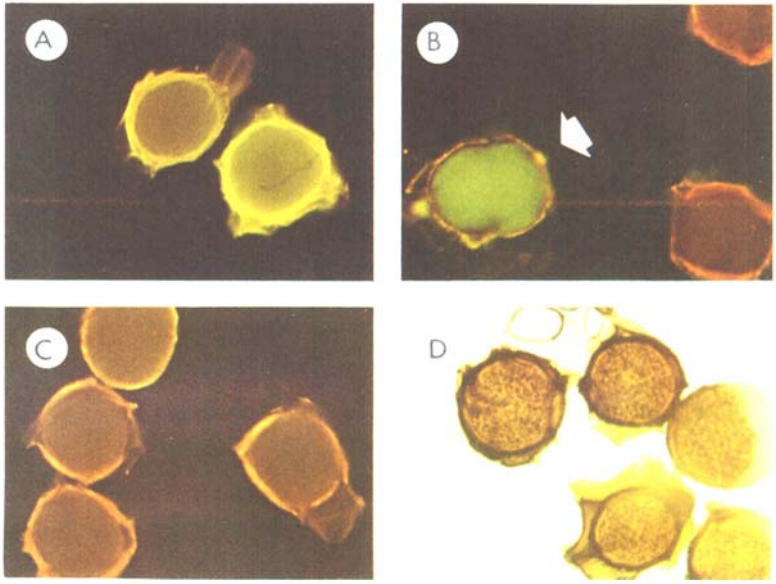

Fig. 1 – (**A** to **D**). Resting sporangia of *Synchytrium endobioticum* taken under (A-C) long-wave ultraviolet radiation, (D) normal illumination. Mounted in **A**) lactophenol, **B**) acriflavin – HCl, and **C**,**D**) buffer. Arrow indicates nonviable sporangium.

Acridine dyes displayed distinct metachromaticism; they have long been associated with vital staining procedures (2, 9, 11). The colour change (red to green) has been explained on the basis of increase in dye concn. Thus the apple-green internal fluorescence (Fig. 1-B) of a sporangium was very likely due to high accumulation of dye molecules. That the sporangial contents fluoresced green, suggests that changes have occurred in the permeability of the sporangial membrane. This variation in membrane condition is assumed to be due to distinct physiologic differences between group A and group B sporangia. Thus the association of organized contents and low levels of internal fluorescence suggest that the chief physiologic difference between group A and B members is one of viability.

This argument is taken up on inoculating tubers with sporangia extracted from gall tissue at different times after harvesting the galls. Variations in the numbers of sporangia per gram of potting mix suggest that the reduction in infectivity obtained was a function of the age of the inoculum and was not related to inoculum density at the levels examined, because in some instances lower densities gave higher Wart Index figures than did higher densities. Most likely the percent viability of the population decreased with age.

The visual relation between internal secondary fluorescence and disorganized sporangial contents, the increase in numbers of such sporangia with time, and the reduction in infectivity with time, support the hypothesis that acridine dyes differentiate between viable and nonviable resting sporangia of *S. endobioticum*.

Thus, an extension of this study, and one that has implications in estimating numbers of viable sporangia in potato growers' fields, would be to determine the lowest level of inoculum density that will bring about infection. It would also be interesting to examine the shift in population viability over a longer time base, because *S. endobioticum* is known to retain viability in soil for at least 37 years (M. E. Gallegly, *personal communication*).

A novel use of the fluorescent technique would be as a tool in the bioassay of compounds activating dormant sporangia, at present under study in this laboratory. A successful compound should reduce the number of viable sporangia in a sample. The technique could be applied without much difficulty to determine the viability of single sporangia isolated from fields suspected of harbouring the pest.

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