

Quantitative Inoculation of Eastern Cottonwood Leaf Tissue with *Melampsora medusae* Under Controlled Conditions

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ABSTRACT

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A procedure for quantitative inoculation of excised leaf tissue of eastern cottonwood with urediospores of *Melampsora medusae* is described. The procedure does not require large numbers of spores or elaborate apparatus. Ten- μ liter samples of a urediospore suspension in 0.1% water agar were placed on the underside of leaf disks (17 mm diameter) supported on 1% agar in petri dish incubation chambers. Favorable incubation conditions were a 14-hr photoperiod (10,000 lux) at 18 C. Uredia usually appeared within 6 days after inoculation and most were erumpent within 8 days. Disks from leaf quadrants that had been washed briefly with 52% ethanol prior to inoculation had significantly greater infection than

unwashed or water washed disks from quadrants of the same leaf. A linear relationship was obtained by plotting $\sqrt{1+x}$ transformations, in which $x =$ infections/disk, against \log_2 of the initial inoculum density. The range of most efficient inoculum density was 1,250–5,000 urediospores per disk, which indicated that 25–29 urediospores could cause a single infection. Leaves of about the same size and position on the stem from the same clone sometimes differed significantly in susceptibility to rust. This intracolonial variation can be accommodated statistically by distributing disks from the same leaf among experimental treatments.

Additional key words: cottonwood leaf rust, *Populus deltoides*.

Leaf rust caused by *Melampsora medusae* Thuem. is an important disease of cottonwood (*Populus deltoides* Bartr.) and some poplar hybrids. Severe attack can lead to premature defoliation and reduction in growth (13,15).

Cottonwood clones resistant or susceptible to this pathogen have been identified. In addition, vegetative propagation of the host by stem cuttings is accomplished readily. These qualities make this host-pathogen system a desirable candidate for studies in disease physiology.

A reliable technique for the quantitative inoculation of the host was required. Various techniques have been developed for the inoculation of leaf tissue with spores of rust fungi under controlled conditions; these include the use of settling towers (2,7) and various devices that mechanically spray a given leaf-target area with a spore suspension for a set period of time (6,8).

The objective of the present study was to develop a reliable, economical, and quantitative technique for inoculating cottonwood leaf tissue that would not require an elaborate apparatus or large numbers of spores. A preliminary report of this work has been published (9).

MATERIALS AND METHODS

General procedures. Leaves of about the same size and position on the stem (6–9 below the apex) from the same clone were selected for individual experiments. Leaf disks were cut with a No. 12 cork borer (about 17 mm in diameter) from greenhouse or field-grown plants; major leaf veins were avoided. Ten leaf disks were placed, abaxial side up, around the periphery of a 90-mm diameter petri dish containing 15 ml of 1% water agar (Fig. 1).

For inoculation, a 10- μ l drop of a well-dispersed urediospore suspension was placed on each leaf disk. A fixed-stroke micropipette (eg, Eppendorf) was particularly useful for this purpose. The urediospore suspension then was spread over the leaf disk with a glass rod (about 1.5 mm in diameter). The inoculum drop was dispersed easily on field-grown leaves and it was spread to within 1 mm of the edge of the leaf disk. The area inoculated on

each leaf disk, therefore, was about 1.75 cm². The inoculum drop was difficult to disperse on leaf tissue from greenhouse-grown plants. Therefore, a glass rod was used to separate the inoculum drop on each leaf disk into about 10 droplets. The concentration of urediospores in the inoculum was determined with an AO Bright Line (American Optical Co., Buffalo, NY 14215) hemacytometer.

Uredia usually appeared within 6 days after inoculation and most were erumpent at 8 days. Final counts of uredia produced were made 9–11 days after inoculation. Sometimes telia were produced with uredia, particularly on greenhouse-grown leaves inoculated during autumn and winter. When this occurred, telia were included with uredia in final counts representing degree of infection.

Urediospore suspension media. In initial experiments, Tween-20 (1–2 drops/100 ml of water) was used to disperse the hydrophobic urediospores in aqueous suspension (9). Urediospores, however, settled out quickly when suspended in this medium; it was necessary to agitate the suspension before each aliquot was withdrawn. Urediospores, however, remained in suspension when dispersed in 0.1% water agar. Dilute agar has been used previously to prepare urediospore suspensions of other rust fungi (8,12).

An experiment was conducted to compare the efficiency and reproducibility of inoculation via the 0.1% water agar and aqueous Tween-20 inoculum suspension media. Ten disks were cut from each of four leaves from the same clone. Five disks from each leaf were inoculated with a pre-agitated urediospore suspension in aqueous Tween-20 (1 drop/100 ml); the other five disks from each leaf were inoculated with a separate urediospore suspension in 0.1% water agar. The age, source, and number of urediospores in both suspensions were similar.

Leaf washing. To test whether substances on leaf surfaces enhance or retard the infection process, leaves were washed with solutions varying in dielectric constant (ϵ). Quadrants of field-grown leaves of the same susceptible clone were given one of the following treatments: unwashed, washed with deionized water ($\epsilon =$ about 80), washed with 35% ethanol ($\epsilon =$ about 60), and washed with 52% ethanol ($\epsilon =$ about 50).

Leaves were washed by wetting the lower leaf surface with the test solution for a total of 30 sec (sprayed twice for 5 sec with 20 sec between sprays). Leaf quadrants that received alcoholic washes

were given a final rinse with deionized water. In previous tests, disks from leaves treated in this manner with 70% ethanol ($\epsilon =$ about 40) sometimes became necrotic within a few days; that treatment, therefore, was eliminated. Eighty leaf disks (five per quadrant from each of four leaves) then were inoculated with 10 μ l of the same urediospore suspension prepared in 0.1% agar.

Incubation conditions. The temperature optimum for germination of *M. medusae* urediospores was 18 C (13). Temperature and light have been shown to influence the development of other rust diseases (11,16).

An experiment was conducted to determine the effects of an initial period of light or darkness at two temperature levels on subsequent infection. Twenty disks were cut from each of four leaves from the same clone. After inoculation, five disks from each leaf were exposed to one of the following conditions for 12 hr: light at 18 \pm 1 C, dark at 18 \pm 1 C, light at 23 \pm 1 C, and dark at 23 \pm 1 C. Light (fluorescent, cool-white) intensity was 10,000 \pm 1,000 lux. All leaf disks then received a 12-hr dark period at 18 \pm 1 C followed by a 14-hr photoperiod (10,000 lux) at 18 \pm 1 C for the remainder of the incubation period (10 days).

Inoculum density. This study consisted of 120 disks (30 from each of four leaves from the same clone) inoculated in groups of 20 (five disks per leaf) with one of six suspensions obtained by serial dilution of a suspension containing about 2×10^6 viable urediospores/ml of 0.1% agar (about 9 mg of spores with 70% viability per ml). A 10- μ l sample of inoculum from each of these six serially diluted suspensions thus contained about 2×10^4 , 1×10^4 , 5×10^3 , 2.5×10^3 , 1.25×10^3 , or 6.25×10^2 urediospores, respectively.

RESULTS

The coefficient of variation (C.V.) for infection of leaf disks inoculated with urediospores suspended in 0.1% agar was consistently lower and numbers of infections were greater than those suspended in aqueous Tween-20 (Table 1). Reproducibility of infection within five-replicate disks of the same leaf as well as infection efficiency, therefore, was greater with inoculum suspended in 0.1% agar.

Even though leaves used in this experiment were of about the same size and position on the stem and from the same clone, significant differences were observed in infection among some leaves inoculated with the same urediospore suspension (Table 1).

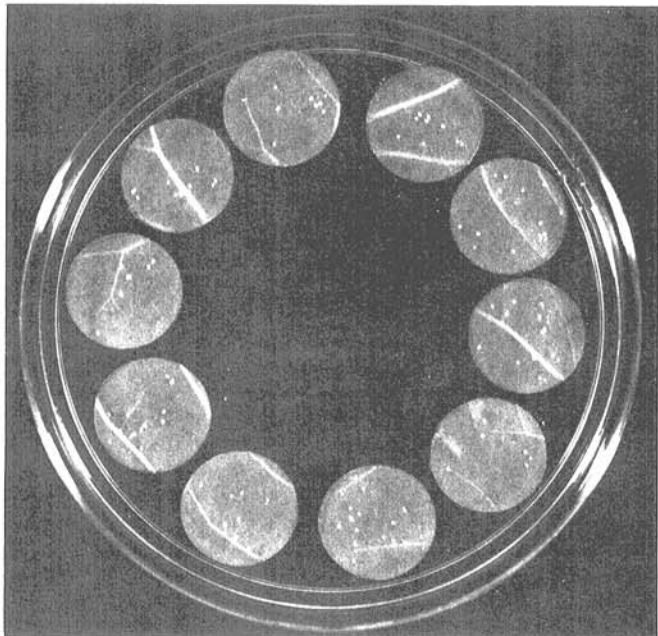


Fig. 1. Petri dish containing cottonwood leaf disks with uredia after inoculation with urediospores of *Melampsora medusae*. Leaf disks are supported on 1.0% water agar.

This phenomenon was observed also in other experiments (Tables 2 and 3).

Disks from leaf quadrants washed with 52% ethanol prior to inoculation had a significantly greater degree of infection than did unwashed leaf quadrants or those washed with water. The amount of infection was inversely proportional to the dielectric constant, or polarity, of the wash solution (Table 2).

Leaf disks that had received an initial light period after inoculation had greater infection than those that had received an initial dark period. This difference was large and statistically significant at 18 C but small and nonsignificant at 23 C. Initial periods of light or darkness at 18 C resulted in significantly greater infection than initial periods of light or darkness at 23 C (Table 3).

The effect on infection of successively halving a urediospore suspension initially containing about 2×10^6 urediospores/ml (2×10^4 urediospores/10 μ l of inoculum) is presented graphically in Fig. 2. A linear relationship was obtained by plotting $\sqrt{1+x}$ transformations, in which $x =$ infections per disk, against \log_2 of initial inoculum density.

A measure of infective efficiency may be obtained by dividing the number of urediospores used at each inoculum density by the corresponding number of infections that occurred. The most

TABLE 1. Infection of cottonwood leaf disks by urediospores of *Melampsora medusae* suspended in two different media

Leaf ^a	Inoculum suspension medium			
	H ₂ O - Tween ^b		0.1% agar ^c	
	Uredia ^d (no.)	C.V. (%)	Uredia ^e (no.)	C.V. (%)
1	29.4 x	51	93.4 s	48
2	28.8 x	42	90.0 s	20
3	15.6 xy	38	27.8 t	26
4	11.6 y	81	56.6 t	19

^aLeaves of about the same size and position on the stem from the same clone were used.

^bUrediospore inoculum was suspended in deionized water containing one drop of Tween-20 per 100 ml.

^cUrediospore inoculum was suspended in 0.1% water agar (Difco).

^dAverage number of uredia produced on each of five disks from the same leaf. Averages followed by the same letter are not significantly different from each other, LSD ($P = 0.05$) = 15.0.

^eAs in footnote d, LSD ($P = 0.05$) = 33.4.

TABLE 2. Effect of pre-washing cottonwood leaves with different solutions on the incidence of infection by *Melampsora medusae*

Wash solution ^a	Dielectric constant ^b	Uredia ^c per Leaf ^d in:				Avg ^e
		1 (no.)	2 (no.)	3 (no.)	4 (no.)	
Unwashed	...	3.4	2.2	4.0	2.4	3.0 y
Deionized water	80	4.0	6.8	3.6	2.8	4.3 y
Ethanol (35%)	60	17.8	6.2	4.0	1.8	7.5 yz
Ethanol (52%)	50	18.5	13.0	6.0	5.2	10.6 z
Avg ^f		10.9 r	7.1 s	4.4 t	3.1 t	

^aLeaves were washed with solutions for a total of 30 sec.

^bApproximate dielectric constant of wash solutions at room temperature.

^cAverage number of uredia produced on each of five disks from the same leaf.

^dLeaves of about the same size and position on the stem from the same clone were used.

^eAverage number of uredia produced on 20 disks (five disks from each of the same four leaves) having the same wash treatment prior to inoculation. Averages followed by the same letter are not significantly different by the Waller-Duncan K-ratio t -test (14); K-ratio = 100.

^fAverage number of uredia produced on 20 disks of the same leaf having four different wash treatments prior to inoculation. Averages followed by the same letter are not significantly different by the Waller-Duncan K-ratio t -test; K-ratio = 100.

efficient inoculum density range was 1,250–5,000 spores/disk indicating that about 25–29 urediospores were required to cause an infection. The highest (2×10^4 /disk), lowest (625/disk), and next to highest (1×10^4 /disk) inoculum densities were less efficient because about 54, 45, and 38 urediospores, respectively, were needed to cause a single infection. These values compare favorably with those obtained for the infection of white pine by basidiospores of *Cronartium ribicola* (830/needle spot) and wheat by urediospores of *Puccinia graminis* var. *tritici* (100/uredium), but less favorably with infection of bean by urediospores of *Uromyces phaseoli* (10/lesion) (4,7,8).

TABLE 3. Effects of an initial light or dark period at two temperature levels on infection of cottonwood leaf disks by *Melampsora medusae*

Leaf ^a	Infection ^b at:				Avg ^f
	18 C ^c		23 C ^c		
	Light ^d	Dark ^e	Light ^d	Dark ^e	
1	173.8	90.5	47.8	42.4	88.6 x
2	105.2	78.2	45.2	26.6	63.8 y
3	108.6	28.8	21.0	6.0	41.1 z
4	64.6	33.2	27.6	16.8	35.6 z
Avg ^g	113.1 r	57.7 s	35.4 t	23.0 t	

^a Leaves of about the same size and position on the stem from the same clone were used.

^b Average number of infections (uredia and telia) produced on each of five disks from the same leaf.

^c Temperature (± 1 C) for first 12 hr after inoculation. Remainder of incubation period (10 days) was at 18 C.

^d Lighting (10,000 \pm 1,000 lux) for first 12 hr after inoculation. All leaf disks then received 12 hr of darkness followed by a 14-hr daily photoperiod for the remainder of incubation period.

^e Darkness for first 12 hr followed by same incubation conditions as in footnote d.

^f Average number of infections produced on 20 disks of the same leaf after four different initial incubation conditions. Averages followed by the same letter are not significantly different by the Waller-Duncan K-ratio *t*-test (14), K-ratio = 100.

^g Average number of infections on 20 disks (five disks from each of the same four leaves) having the same incubation conditions. Averages followed by the same letter are not significantly different by the Waller-Duncan K-ratio *t*-test; K-ratio = 100.

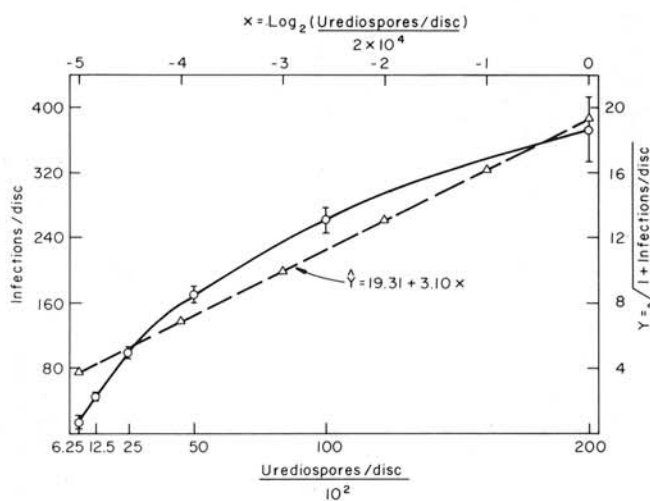


Fig. 2. The effect of successively halving inoculum of *Melampsora medusae* containing 2×10^4 urediospores per $10 \mu\text{l}$ on the number of infections/cottonwood leaf disk (about 1.75 cm^2). Each point represents an average of 20 disks (five from each of four leaves from the same clone). Horizontal bars represent 95% confidence limits around points on the arithmetic scale (left and lower axes). The dashed straight line was obtained by plotting $\sqrt{1+x}$ transformations, where x = infections per disk, against a \log_2 scale of initial inoculum density (right and upper axes).

DISCUSSION

It was surprising that leaves from the same clone and of about the same size and position on the stem sometimes differed significantly in susceptibility to rust. This may reflect relatively slight differences in leaf physiology and development; ie, leaf plastochron index (5). Such leaf-to-leaf variation should be recognized as a source of error. An added advantage of the inoculation technique described is that intracolonial variation can be accommodated statistically by distributing disks from the same leaf among experimental treatments.

The highest inoculum density used (about 9 mg containing 2×10^6 viable urediospores/ml) was least efficient in causing infection. This may reflect self inhibition of spore germination, which is common among rust fungi. Consistent evidence for this, however, was not obtained and self inhibition of other rust fungi occurred at substantially lower inoculum densities. For example, the ED_{50} was $< 0.5 \text{ mg/ml}$ for urediospores of *P. graminis* var. *tritici* (1) and $< 2 \text{ mg/ml}$ for aeciospores of *C. commandrae* (3). Perhaps more significant factors were urediospore crowding, coalescing of infections, and the limited number of stomata available for infection. Our estimate of the total number of stomata per disk for this clone was about 3.5×10^4 , or less than two stomata per urediospore.

The enhanced infection of leaves washed with 52% ethanol prior to inoculation may be due to an inhibitor on unwashed leaf surfaces. The isolation and biological activity of such an inhibitor will be presented in a later communication.

When this inoculation technique was employed to compare interclonal differences in susceptibility of cottonwood leaves to *M. medusae*, the results obtained were in good agreement with field observations (10).

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