Infection Structure Differentiation by Wheat Stem Rust Uredospores in Suspension

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ABSTRACT

A procedure is described for the induction of differentiation of infection structures (appressorium, vesicle, and infection hypha) by wheat stem rust uredospores suspended in a Tween-buffer-nonyl alcohol medium. Critical factors involved in infection structure formation, induced by a heat shock of 30 C, include the spore:liquid ratio, the duration of heat shock, and the composition of the suspending medium present during the induction period. Endogenous metabolites which are apparently different from the germination inhibitor and volatile stimulant influence the progress of differentiation. Phytopathology 61:649-652.

Uredospores of the rust fungi can be induced in vitro to differentiate infection structures, consisting of an appressorium, vesicle, and infection hypha, analogous to those formed during the early stages of parasitic invasion of the host plant. A variety of techniques has been employed with different rust fungi to elicit this normal course of germ tube development. Among the most effective procedures for the wheat stem rust fungus *Puccinia graminis* f. sp. *tritici* are (i) exposures at a sequence of different temperatures including a heat shock period after germination (7); and (ii) exposure to a volatile stimulant produced by steam-distillation of substances released into water from ungerminated uredospores (2, 7). These procedures, however, have been used primarily with small quantities of spores and are less effective with larger quantities. Some of the problems and inadequacies encountered have been discussed and examined by Wiese & Daly (12) and by Schipper et al. (10).

Germination of uredospores in suspension was accomplished by Caltrider & Gottlieb (4) and Staples et al. (11), but the percentage of spores germinating within a few hours was low. Maheshwari & Sussman (8) obtained considerable improvement by including nonyl alcohol in the germination medium. With these studies as a background, attempts were made with suspended spores to induce a high percentage of differentiation, using the temperature program effective with trace quantities of floating spores (7).

It is the purpose of this report to describe a method by which large quantities of wheat rust uredospores can be conveniently employed and the majority of the population induced to differentiate infection structures at relatively high spore:liquid ratios without the need for removing the endogenous germination inhibitor (1) prior to germination. The entire population of uredospores is exposed to identical environmental conditions, and germination proceeds in synchrony and is completed within 2 hr (8). Evidence is presented which suggests that spore metabolites, different from the germination inhibitor or volatile stimulant, exert a regulatory effect on the differentiation process.

Materials and Methods.—Recently harvested spores (less than 1 week old) were placed in 50-ml Erlenmeyer flasks containing 20 ml of suspending medium (TBN) composed of 0.01% Tween 20 (polyoxyethylene sorbitan monolaurate) in Ca/K phosphate buffer, pH 7.0 (7), and 10^{-4} M n-nonyl alcohol. The spores were suspended by vigorously shaking for 1 min. The flasks were tightly covered with Parafilm and incubated for 2 hr in a reciprocal shaker at 18 to 20 C while shaking at a rate of 150 to 170 two-cm strokes/min. After this period, the flasks were transferred to a temperature-controlled reciprocal shaker at 30 C for 3 hr. They were then returned to 18 to 20 C and shaken at the above rate for 16 to 20 hr. Samples of the spore suspension were removed to a glass slide, and the proportions of germinated and differentiated spores determined by counting 200 spores. A spore was considered germinated when the germ tube was at least as long as the spore diam. Terminal or intercalary swellings were counted as appressoria, whereas germ tubes having complete infection structures (appressorium, vesicle, and infection hypha) were scored as differentiated.

Results.—Infection structures produced in response to a 3-hr heat shock are shown in Fig. 1. During the heat shock period, the germ tube stops elongating and forms the appressorium (Fig. 1-A). By 12 hr after heat shock treatment, the infection hypha is initiated but has not elongated (Fig. 1-B). Infection structure formation is completed by 16 to 20 hr, and the infection hypha attains a length of 25 to 50 μ (Fig. 1-C). At lower spore densities (1 to 2.5 mg/ml), infection structures formed at right angles to the axis of germ tube growth (Fig. 1-B, C) as they apparently do on the host plant (9). With an increase in concentration of spores, however, the vesicle frequently elongated (Fig. 1-D), and growth of the infection hypha continued along the axis of the original germ tube. Length of the infection hypha was always greater in the former, normal type of differentiation.

With shorter heat shock periods (2 to 2.5 hr), a considerable portion of the germ tubes formed appressoria but failed to develop complete infection structures. Frequently a branch was produced behind the appressorium at right angles to the germ tube, and growth of the branch was extensive. However, as the heat shock period was increased to 3 hr, differentiation in-

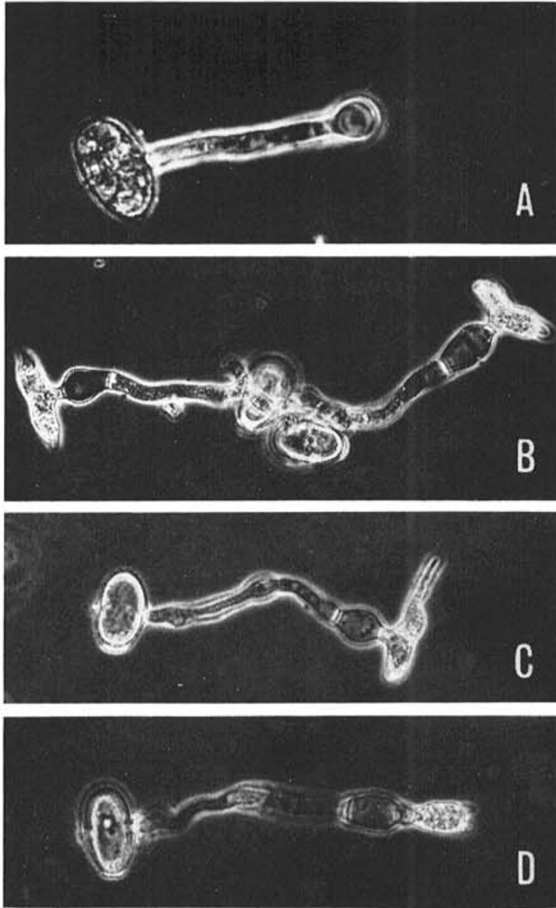

Fig. 1. Infection structures of *Puccinia graminis* f. sp. *tritici* induced in suspension by a heat shock of 30 C for 3 hr. **A)** Appressorium formed during heat shock. **B)** Infection structures formed 12 hr after heat shock. Little growth of the infection hypha has yet occurred. **C)** Complete infection structures formed 16 to 20 hr following heat shock. Growth of vesicle and infection hypha is at right angles to the direction of germ tube elongation. **D)** Infection structures formed at high spore density 12 hr after heat shock showing elongation of vesicle and initiation of infection hypha along the axis of the original germ tube.

creased and the abnormal form of branching was eliminated. Increasing the duration of heat shock to 4 hr did not further improve differentiation.

The proportion of germ tubes differentiating complete infection structures decreased with an increase in spore density (Fig. 2), suggesting that diffusible inhibitory metabolites are released from the spores during the germination period and become concentrated in the suspending medium. Differentiation was increased two- to fourfold when the germination medium was removed prior to the heat shock by centrifuging the spore suspension at 200 *g* for 2 min, decanting the germination solution, and resuspending the spores in fresh suspension medium with (TBN) or without (TB) nonyl alcohol (Fig. 2). Thus, it is evident that nonyl alcohol, which overcomes the action of the inhibitor and stimulates germination of spores en masse, is not required

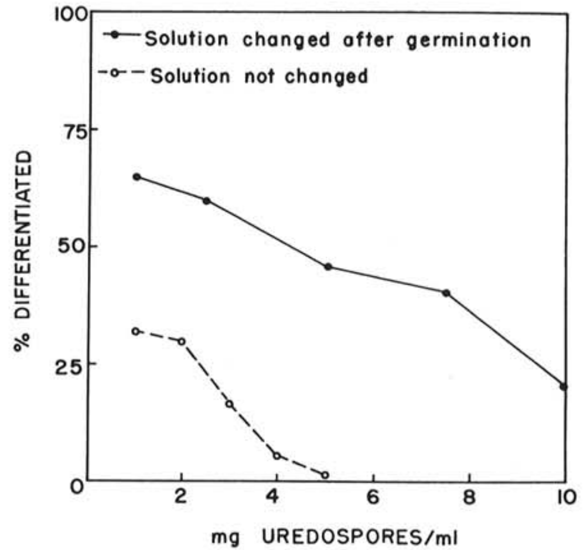

Fig. 2. The effect of spore concentration on differentiation of infection structures by suspended spores of *Puccinia graminis* f. sp. *tritici*. Each sample was incubated 2 hr at 18 C, then at 30 C for 3 hr, and returned to 18 C for an additional 16 hr before scoring differentiation. Data for the upper curve are from a series in which the suspending medium (TBN) was replaced with fresh buffer (TB) following the 2-hr germination period. Values plotted represent the percentage of germinated spores which formed complete infection structures. Germination was greater than 85% in all but the highest spore concentration.

for response of germ tubes to heat shock, as it can be removed prior to the inductive treatment without negative effects. As a germination stimulant, however, it is superior to a number of other agents which have been used (6, 12). For example, neither Triton WR-1339 (13) nor a factor from filter paper (3, 12) gave comparable germination levels. Spores which were prehydrated overnight in TB at 4 C germinated better than nonhydrated controls following their removal to fresh TB, but the addition of nonyl alcohol still further improved the extent and rate of their germination, and resulted in abundant appressorium formation.

The substances released into the germination medium are more inhibitory to appressorium formation than to subsequent stages of development, since replacing the medium after heat shock (i.e., after appressorium formation) has little effect, whereas renewal of the medium before heat shock markedly increases the per cent of spores forming both appressoria and infection structures.

The population effect on differentiation (Fig. 2) is strikingly similar to that on germination, but occurs at spore concentrations well above those at which germination is inhibited (1). If the same inhibitor is responsible for both effects, suppression of differentiation would require about 5 times as high a concentration of the material. Purified germination inhibitors at 4.5 times the concentration required to give 95% inhibition of germination had, however, no effect on the percentage of spores forming complete infection structures

TABLE 1. Effect of partially purified germination inhibitor on differentiation of infection structures by wheat stem rust uredospores in suspension[a]

mg spores/ml	Germination medium[b]	Heat shock medium[b]	% Germination	% Appressoria[c]	% Differentiated[c]
1.0	TBN	TB	94	21	57
1.0	TBN	I-TB	85	20	61
1.0	I-TB	I-TB	0	0	0
2.0	TBN	TB	96	50	39
2.0	TBN	I-TB	90	36	43

[a] All values were obtained after 24 hr. The germination inhibitor was prepared in Tween buffer (I-TB) to give 90 units/flask (4.5 units/ml). One unit of inhibitor is the concentration required to inhibit germination by 95% in micro-Conway germination assays (P.J. Allen, *unpublished data*). TBN was composed of 0.01 Tween 20 (polyoxyethylene sorbitan monolaurate) in Ca/K buffer, pH 7.0, and 10^{-4} M n-nonyl alcohol.

[b] The suspending medium was changed after 2 hr germination at 18 C to Tween buffer (TB) or to a solution of inhibitor in Tween buffer (I-TB) prior to incubation at 30 C for 3 hr.

[c] Values are the percentage of germinated spores which had formed appressoria only or complete infection structures.

(Table 1). Furthermore, measurements of the length of the infection hypha in the presence of germination inhibitor indicated that there was no inhibitory effect on growth.

A volatile substance which stimulated infection structure formation in floating spores (2, 7) did not induce differentiation in suspended spores. The active material was prepared by steam-distillation of an aqueous extract from nonviable *Puccinia helianthi* uredospores according to the procedure described by Maheshwari et al. (7). When tested with floating spores, this preparation induced 50 to 60% differentiation, but had no effect on the differentiation of a suspension of prehydrated spores (1 mg/ml) in TB. Thus, the volatile stimulant which is active with floating spores is apparently not involved in the regulation of differentiation of infection structures by suspended spores.

DISCUSSION.—The procedure described offers several advantages over previous methods, and affords the possibility of more accurate studies of metabolism during the differentiation of infection structures. For example, investigations of uptake and incorporation of labeled substrates, of respiratory activity during differentiation, and of the effects of exogenously supplied nutrients on germ tube growth and differentiation could easily be conducted. It may thus be possible to determine the contribution of infection structure formation to the initiation and progress of vegetative growth in the absence of the host. It is apparent from the present studies that, once the expression of specific genetic information is triggered, infection structure differentiation by wheat stem rust uredospores can proceed independent of a physical surface such as a leaf cuticle or air-water interface.

Several lines of evidence suggest that endogenous materials influence the capacity of a population of uredospores to differentiate infection structures in suspension. Removal of the materials released from the spores during the germination period increased the percentage of germ tubes which formed appressoria and infection structures in response to a subsequent inductive heat treatment. But replacing the germination medium of nonhydrated spores with the materials released into buffer during hydration of nongerminating spores had no effect, indicating that the release is not dependent upon germination. The inhibitory materials apparently specifically block appressorium formation. Since each stage of infection structure differentiation is dependent upon prior completion of the immediately preceding stage, inhibition of appressorium formation suffices to eliminate the remainder of the differentiation process even if the inhibitory agent is removed after the heat shock, and before the major phases of differentiation.

No evidence has yet been obtained concerning the nature of the inhibitory compounds. Numerous substances in addition to the germination inhibitor (1) and volatile stimulant (2) are released from spores upon contact with aqueous solutions. For example, Daly et al. (5) detected large amounts of carbohydrates (80% ethanol-soluble fraction) in the medium of germinating wheat rust uredospores, and we have found nucleotides, amino acids, and polyols in considerable quantity. Lipoidal materials forming surface films are also released rapidly from the uredospores (14). The role of the latter materials in differentiation of infection structures has not been investigated, but it is possible that they are determinants of the physical microenvironment of the germ tubes and, thus, exert a regulatory influence.

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