Production of Pseudothecia and Ascospores by *Pyrenophora tritici-repentis* in Response to Macronutrient Concentrations

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ABSTRACT

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Pyrenophora tritici-repentis, causal agent of wheat tan spot, was grown on a medium consisting of purified cellulose moistened with an aqueous solution of nitrogen (urea or nitrate) and nutrient salts and incubated under conditions of light and temperature favorable for sexual reproduction. With adequate amounts of phosphorus and potassium, abundant pseudothecia formed at 900 ppm added N (micrograms of N per gram of dry cellulose), supplied as either urea or nitrate. At 90 ppm added N, pseudothecia production was reduced approximately 60%; at 9 ppm added N, only a few small pseudothecia formed. Ascospore production was proportional to N level, except for being inhibited by nitrate at 900 ppm. With urea at 900 ppm N, production of fertile pseudothecia responded to levels of P and K. With 200 ppm P and 500 ppm K, abundant fertile pseudothecia were produced. Reducing these levels 10-fold had no significant effect, but at 2 ppm P and 5 ppm K, few pseudothecia were produced and most were immature.

Tan spot of wheat (*Triticum aestivum* L. em Thell), a disease of increasing importance in wheat-growing states of the Central Plains, is caused by the ascomycete *Pyrenophora tritici-repentis* (Died.) Drechs. (7,8). Ascospores, produced in pseudothecia on straw from the previous year's crop, are the primary inoculum for tan spot epidemics (7,13). Severe epidemics are, therefore, commonly associated with conservation-tillage practices (2,7,13), since the straw remaining on the soil surface can support development of abundant primary inoculum.

Competition between saprophytic microorganisms in straw and *P. tritici-repentis* could decrease the pathogen's ability to sporulate. To assess the potential of nutrient competition as a biological control strategy, we investigated the nutritional requirements for formation of pseudothecia and ascospores by *P. tritici-repentis*.

For all fungi, nitrogen availability has a major influence on differentiation, with the amount and form of N often critical for sporulation (1). The N requirements for sexual reproduction by *P. tritici-repentis* have not been investigated, but ascospore production by several other fungi depends on N supply (5,6,14,15). Generally, there is a level of N above which ascospore production is inhibited. The minimum levels of N required for ascospore formation have seldom been determined but may range as low as 14 ppm N (4) or even 1 ppm N, depending in part on a favorable C:N ratio (15).

In this paper we report the quantitative effect of macronutrient levels on sexual reproduction by *P. tritici-repentis* when grown on a cellulose-based medium.

MATERIALS AND METHODS

The isolate of *P. tritici-repentis* used was obtained from infected wheat collected near Manhattan, KS. The isolate was stored on half-strength V-8 juice agar slants at 4 C and renewed at 6-mo intervals by obtaining reisolates from greenhouse-grown plants inoculated with the culture.

To test *P. tritici-repentis* under nutritional conditions similar to those in straw, we used a medium consisting of cellulose amended with selected nutrients. The basic medium was prepared by formulating a slurry of highly purified cellulose particles (Sigmacell type 100, Sigma Chemical Co., St. Louis, MO) in double-distilled water at a rate of 15 g of cellulose per 100 ml of water. The slurry was autoclaved for 15 min, then stirred vigorously with a magnetic stir bar as it cooled. Two-milliliter aliquots (0.3 g of cellulose) were transferred to sterile plastic petri dishes (60 × 15 mm) by means of a plastic pipette (cellulose adheres to the walls of a glass pipette). Each plate was swirled gently to coat the bottom evenly. The covered plates were then placed in an oven at 110 C, thus drying the cellulose to a thin, even layer. Finally, each plate was amended by applying 0.7 ml of sterile aqueous nutrient solution to the dried cellulose layer and rocking the plate to distribute the solution.

The aqueous solution for the "complete" medium contained the following nutrients per 100 ml: 83 mg of urea, 486 mg of K_2HPO_4, 213 mg of $MgSO_4 \cdot 7H_2O$, 0.7 mg of $FeCl_3 \cdot 6H_2O$, and 2 mg of $MnSO_4 \cdot H_2O$. Solutions of individual nutrients were prepared separately and autoclaved (except for urea, which was filter-sterilized) before being mixed to produce the amendment solution desired. When included in mixtures, $MgSO_4$ was added last to prevent precipitation of salts. The complete medium also included $CaCO_3$, incorporated at 1 mg ml^{-1} into the cellulose slurry before autoclaving. The complete-medium solutions were modified for various tests by reducing or deleting selected nutrients; in treatments requiring potassium or phosphorus individually, KCl or Na_2HPO_4, respectively, were used. Urea was replaced by $NaNO_3$ for some treatments in tests of N sources. Analysis of nonamended cellulose plates showed background levels of approximately 25 ppm N (dry weight basis). Neither the salts solutions nor the spore suspension added detectable N to the plates. The form and availability of background N in the cellulose are unknown.

The nutrient levels used in complete-nutrient treatments were chosen to approximate levels occurring in straw (16). Thus, the complete medium contained the following nutrient levels, expressed as percent dry weight of the cellulose: 0.09% N, 0.2% P, 0.5% K, 0.3% Ca, 0.06% S, 0.05% Mg, 0.001% Mn, and 0.005% Fe. The C:N ratio was 440. Published reports of N level in straw range from 0.3 to 0.9% dry matter (16). Thus, our levels of N were lower than the norm. In a preliminary replicated test, however, we found no significant difference in ascocarp production by *P. tritici-repentis* between 0.9 and 0.09% N (supplied as urea).

The pH of the moistened unamended cellulose was approximately 3.8. Because preliminary results indicated that pseudothecia formation can be inhibited by low pH, we adjusted the initial pH of all treatments to 7.0 ± 0.2. In some cases, the pH of the amendment solution had to be adjusted with NaOH to obtain this neutral pH. The pH of the amended medium was then verified on replicate plates (not used for fungal growth) with a surface

electrode (Combination X-EL, Corning Glass Works, Corning, NY) placed on the center of the plate. There was generally a gradient of pH from 7.0 in the center of the plate to a lower value (usually about 6.5 but in rare cases as low as 5.5) at the plate perimeter.

Each cellulose plate was inoculated with a small number of propagules of *P. tritici-repentis*, comprising conidia and conidiophores. The propagules were grown on plates of overlapping agar wedges (12) and collected in sterile distilled water. To minimize carry-over of nutrients from the agar plates, the propagule suspension was washed two times in sterile distilled water by centrifugation at 1,000 *g* for 10 min. The concentration was adjusted to 15 ± 3 propagules per 10 μl, and a 10-μl drop of suspension was placed in the center of each cellulose plate.

Each plate was sealed with Parafilm M, and the plates were placed inside a transparent plastic box to reduce evaporative water loss. The plates were incubated at 25 ± 1 C under 12-hr daylength (40W cool-white fluorescent bulbs placed 20 cm above the plates, providing 4,500 lx) for 4 wk to permit formation of pseudothecia. Because ascospores require cool temperatures to mature (11), the plates were moved to a 15-C incubator having 12-hr daylength provided by fluorescent lights. After 8 wk at 15 C, production of pseudothecia and ascospores was assessed. The number of pseudothecia on each plate was counted and the approximate sizes determined by means of a dissecting microscope with an ocular micrometer. Ten pseudothecia along a random diameter of each plate were removed and mounted in lactoglycerol on a glass slide with a cover glass. Each pseudothecium was crushed, and the contents were observed microscopically (160×). If a pseudothecium contained mature ascospores, the number of asci containing such spores was counted. These asci, which almost always contained eight ascospores, are hereafter designated "fertile asci." The total number of ascospores per plate was estimated by taking eight times the average fertility (number of fertile asci per ascocarp) for the plate. After sporulation was assessed, the pH of each plate was measured with a surface electrode.

RESULTS

P. tritici-repentis produced ascocarps and ascospores under the conditions used in these experiments, demonstrating its ability to use cellulose as a sole carbon source for growth and reproduction.

Ascocarps were classified into three sizes: <300 μm, 300–450 μm, and 450–1,100 μm. The smallest pseudothecia (<300 μm) were predominantly immature (infertile) and never contained more than 10 fertile asci. Fertile pseudothecia in the 300–450 μm class usually had <10 fertile asci but in rare cases contained as many as 40. The largest ascocarps (≥450 μm) contained as many as 100 fertile asci. The mean numbers (followed by standard deviation) of fertile asci per fertile ascocarp for the three sizes were, respectively, 3.1 (2.1), 7.0 (8.3), and 19.3 (18.2).

N level and form influenced the ability of *P. tritici-repentis* to sporulate on the cellulose medium. With either urea or nitrate, the number of pseudothecia produced was proportional to the amount of N available to the fungus (Table 1), and for any level of N tested there was no significant difference between urea and nitrate in ability to support pseudothecia formation. The number of fertile pseudothecia did, however, differ between urea and nitrate treatments at 900 ppm added N. At this level, urea supported formation of many fertile pseudothecia and a high number of fertile asci per fertile pseudothecium, resulting in a greater estimated spore production than in any other N treatment. Nitrate at this level, however, was inhibitory to spore production. Spore production at 90 and 9 ppm added N (urea or nitrate) was progressively less than that at 900 ppm urea-N, such that over the 100-fold range of N levels, ascospore production also ranged over two orders of magnitude. Statistical analysis showed that spore production was strongly correlated with urea-N level ($r = 0.97$, 14 df). As N level decreased, both the numbers and the fertility of ascocarps declined; N form did not affect these results. Most of the pseudothecia produced at low levels of added N (9 ppm and check) were small (<450 μm in diameter), whereas large ascocarps (500–1,100 μm) accounted for 88–98% of those produced at 90 and 900 ppm. The pH of the medium remained near neutrality during the entire experimental period (3 mo) for all treatments except 900 ppm nitrate-N, in which the final pH reached 8.7.

In preliminary experiments, various urea-containing solutions were prepared, each lacking one of the following nutrients: P, K, S, Mg, Ca, Mn, or Fe. When applied to the cellulose plates, only those treatments lacking P or K significantly reduced the number of pseudothecia formed, in comparison with the complete medium. Thus, the requirements of *P. tritici-repentis* for S, Mg, Ca, Mn, Fe, and other micronutrients were met by background levels in the medium.

In contrast, P and K were required in amounts larger than the background levels. P and K were both required for the production of fertile pseudothecia at 900 ppm N (urea) plus the salts included in the complete medium (Table 2). In the absence of either P or K, a small number of pseudothecia were produced, almost all smaller than 450 μm. When P and K were the only nutrients added to the cellulose-urea medium, large (mostly >450 μm) fertile ascocarps were produced. The concentrations of P and K in this nutritional environment were important to the number and fertility of ascocarps produced. At a very low concentration of added K_2HPO_4 (2 ppm P and 5 ppm K), the number of ascocarps was significantly less than the number produced at all higher K_2HPO_4 levels tested. Approximately 50% of the ascocarps produced at these low P and K levels were small (<450 μm), producing relatively few fertile asci per fertile ascocarp. When the K_2HPO_4 concentration was increased either 10- or 100-fold, more ascocarps were produced; these were mostly large, containing significantly more fertile asci than did the fertile ascocarps at the low K_2HPO_4 level (Table 2). At the highest concentration (2,000 ppm of P and 5,000 ppm of K) the ascocarps were large but mostly infertile. Thus, high levels of P + K inhibit ascospore—but not pseudothecium—formation. The presence of other salts appears to ameliorate the inhibitory effects of high K_2HPO_4 levels, although total sporulation remains less than with the intermediate levels of K_2HPO_4. The mechanism of this amelioration is unknown.

In the absence of other salts, P alone supports a limited number of ascocarps (Table 2). At 200 ppm P and below, most of the ascocarps are large and a small number are fertile, but total sporulation is very limited. K alone does not support appreciable numbers of pseudothecia at any concentration, and no spores were observed in the absence of P.

The pH of all tests shown in Table 2 remained near neutrality throughout the experimental period. However, since K_2HPO_4 has buffering capacity, its beneficial influence on sporulation may have been due to buffering rather than to nutritional factors. To test this possibility, two amendment solutions containing 0.05 M HEPES

TABLE 1. Influence of nitrogen on sexual reproduction by *Pyrenophora tritici-repentis* [a]

Added N		Pseudothecia >450 μm			Asci per	Total	Final
Form	ppm [b]	Total	diameter	Fertile	ascocarp [c]	ascospores [d]	pH ± 0.2
Urea	900	116 x [e]	115 x	70 x	38 x	20,400 x	7.2
	90	52 y	46 y	36 y	15 y	4,400 y	7.4
	9	7 z	0 z	3 z	4 z	300 z	7.3
Nitrate	900	126 x	119 x	6 z	1 z	90 z	8.7
	90	39 y	38 y	31 y	15 y	4,000 y	7.5
	9	12 z	4 z	9 z	8 yz	700 z	7.3
	0	5 z	2 z	1 z	1 z	13 z	7.2

[a] Data (means of two experiments with two replications per experiment) represent observations per test plate (20 cm^2 area). All treatments contained complete salts, including P and K.

[b] Micrograms of N per gram of dry cellulose; background level of N in cellulose was 25 ppm.

[c] Average number of fertile asci per fertile pseudothecium.

[d] Per plate, estimated by: 8 × (average number of fertile asci per fertile pseudothecium) × (number of fertile pseudothecia).

[e] Values within a column followed by the same letter do not differ significantly ($P = 0.05$), as determined by Duncan's new multiple range test.

buffer (pH 7.0) were prepared, one containing the complete nutrient complement and the other lacking K_2HPO_4. There were two replicates per treatment, and the experiment was repeated once. In the treatment lacking K_2HPO_4, no fertile ascocarps formed, i.e., the same result observed in an unbuffered medium lacking P or K. The HEPES itself was not inhibitory to pseudothecia formation, as shown by the large number of fertile ascocarps (average 52 per plate) formed in the HEPES-containing complete nutrient treatment.

DISCUSSION

The cellulose-based medium described here was useful for determining the influence of the macronutrients N, P, and K on ascospore production by *P. tritici-repentis*. Ascocarps were produced on this medium under the proper nutritional and environmental conditions, were readily visible, and could be easily removed for examination of contents. Maintenance of adequate moisture in the cellulose plates was essential for success of the method.

As has been reported for various other ascomycetes, sexual reproduction by *P. tritici-repentis* is influenced by the level and, to some extent, the form of available N. Over the 100-fold range of N concentrations we tested, ascospore production was proportional to the level of N supplied as urea. The same was true for nitrate-N in the low range of concentrations used. At the highest concentration, nitrate was inhibitory to ascospore production, although abundant pseudothecia still formed. The mechanism of this inhibition was not investigated. The inhibition could result directly from the nitrate ion itself or perhaps be associated with the moderate rise in pH that occurred in the high-nitrate treatment. Inhibition of ascospore production by nitrate has also been observed in *Venturia inaequalis* Cooke (Wint.) (14); fertile perithecia formed at 30 or 60 ppm nitrate-N, but perithecia without ascospores developed at 125 ppm N.

As N level was decreased, *P. tritici-repentis* produced progressively fewer, and eventually smaller, pseudothecia, with progressively fewer fertile asci. Ascospore production was directly related to N level. Hall (3) found that perithecial production by *Sordaria fimicola* (Roberge) Ces. & de Not. was proportional to N concentration but that for a given level of N the maximum number of perithecia was produced when the initial C:N ratio was between 5:1 and 10:1. We did not test for the optimum C:N ratio but held C constant as N was lowered. We were interested in C:N ratios similar to those in straw, which start in the 100:1 range and increase as saprophytic microorganisms remove the available N from the substrate. The relationship of straw C:N ratios to nutritional requirements of fungal colonists is not straightforward, however, since some of the C and N included in the analysis used to calculate the C:N ratio is not actually available to a fungus colonizing the substrate. Most fungi are unable to obtain the C contained in lignin or lignin-complexed cellulose, and total N content of a substrate may include N already incorporated into microbial biomass, which, again, may be largely unavailable to a new colonist of the substrate. In any case, a large proportion of the plant N in straw is thought to be readily available to microorganisms (9), and it is therefore reasonable to expect that competition among microorganisms could result in reduced N availability to the less able competitors.

Our test medium, cellulose moistened with nutrient solution, is a simplified nutritional model. The form and availability of the various nutrients are clearly different from those in straw. Therefore, the nutrient response observed in our study cannot necessarily be extrapolated directly to *P. tritici-repentis* on its natural substrate. However, certain generalizations concerning the effects of nutrient availability and competition on *P. tritici-repentis* inoculum production in straw can be made. Our results suggest that it may be possible to inhibit or reduce ascospore production by *P. tritici-repentis* through N competition by other organisms. It is significant that sporulation is proportional to N level over a fairly broad range of concentrations. Thus, some reduction in sporulation could be expected even if N were not reduced below the threshold level for spore production. However, an unresolved but important question remains: At what stage(s) during the process of growth and sporulation by *P. tritici-repentis* could N competition be effective in reducing eventual ascospore production?

Both P and K are clearly needed at substantial levels for sexual reproduction in *P. tritici-repentis*. At the highest levels we tested, these nutrients were somewhat inhibitory to ascospore production. High levels of available phosphate cause major changes in biochemical processes of fungal cells, including inhibition of

TABLE 2. Influence of phosphorus and potassium on sexual reproduction by *Pyrenophora tritici-repentis* [a]

Nutrients supplied[b]			Pseudothecia					
P (ppm)	K (ppm)	Micronutrients	Total	>450 μm diameter	Fertile	Asci per ascocarp[c]	Total ascospores[d]	Final pH
2,000	5,000	+	133 x[e]	129 y	81 y	30 y	21,100 y	7.1 ± 0.1
2,000	5,000	0	181 w	181 x	15 z	9 z	1,100 z	7.4 ± 0.1
200	500	0	146 wx	146 y	122 x	38 x	37,700 x	6.5 ± 0.1
20	50	0	157 wx	141 y	121 x	32 xy	32,400 x	6.7 ± 0.5
2	5	0	52 y	24 z	5 z	4 z	300 z	6.6 ± 0.4
2,000	0	+	0 z	0 z	0 z	0 z	0 z	7.6 ± 0.1
2,000	0	0	24 yz	0 z	0 z	0 z	0 z	6.9 ± 0.5
200	0	0	26 yz	22 z	1 z	1 z	10 z	7.2 ± 0.1
20	0	0	30 yz	28 z	1 z	1 z	10 z	7.1 ± 0.1
2	0	0	38 yz	20 z	4 z	1 z	30 z	6.8 ± 0.2
0	5,000	+	11 yz	0 z	0 z	0 z	0 z	7.4 ± 0.3
0	5,000	0	4 z	1 z	0 z	0 z	0 z	6.4 ± 0.5
0	500	0	0 z	0 z	0 z	0 z	0 z	5.8 ± 0.1
0	50	0	0 z	0 z	0 z	0 z	0 z	6.4 ± 0.5
0	5	0	0 z	0 z	0 z	0 z	0 z	6.4 ± 0.5
0	0	+	23 yz	1 z	0 z	0 z	0 z	7.7 ± 0.1
0	0	0	17 yz	0 z	0 z	0 z	0 z	6.8 ± 0.3

[a] Data (means of two experiments with two replications per experiment) represent observations per test plate (20 cm^2 area).
[b] All treatments contained urea at 900 ppm N (dry weight basis); P and K levels are expressed as fraction of cellulose dry weight; micronutrients = S, Mg, Ca, Mn, and Fe.
[c] Average number of fertile asci per fertile pseudothecium.
[d] Per plate, estimated by: 8 × (average number of fertile asci per fertile pseudothecium) × (number of fertile pseudothecia).
[e] Values within a column followed by the same letter do not differ significantly ($P = 0.05$), as determined by Duncan's new multiple range test.

uptake of some monovalent cations (1) and inhibition of conidial formation (10). Although the levels we found to be inhibitory were comparable, on a dry weight basis, to those in wheat straw (0.2% P and 0.5% K [16]), they represent concentrations of 28 mM P and 56 mM K in the solution saturating the cellulose of the medium. Their greater availability in this form than in the straw undoubtedly accounts for their inhibitory influence at these concentrations. When P and K concentrations were reduced through three orders of magnitude, a 100-fold range of concentrations that supported abundant sporulation was followed by levels too low to permit significant ascospore production. At these low levels of P and K, ascocarps were fewer and smaller and contained fewer fertile asci per fertile ascocarp than those produced at optimal P and K levels. These effects are similar to those observed when N is limiting. Although neither P nor K alone supported spore production by *P. tritici-repentis*, P alone did permit production of a few small ascocarps.

These data suggest that inhibition of sporulation through competition for P and/or K may be more difficult to impose than inhibition through N competition. In our experiments, sporulation was proportional to P and K concentration over only a small range (less than one order of magnitude). We saw a threshold level below which essentially no sporulation occurred and above which sporulation was abundant. Thus, P and K levels would have to be reduced below the threshold level before an effect would be expected; this contrasts with the proportional relationship we observed for sporulation over a broader range of N levels.

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