Studies on Wheat Stem Rust Resistance Controlled at the Sr6 Locus. III. Ethylene and Disease Reaction

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ABSTRACT

Peroxidase activity can be induced in resistant lines of wheat by infection with race 56 of *Puccinia graminis tritici*. Since ethylene also can induce high peroxidase activity, resistant and susceptible infected lines carrying the respective *Sr6* alleles were treated with ethylene. Treatment caused infected susceptible leaves to increase in peroxidase activity to levels above the activity exhibited by infected, resistant untreated leaves, but treated leaves remained susceptible. In contrast, ethylene-treated resistant leaves at 20 C reverted to complete susceptibility, despite the fact of high peroxidase activity induced by ethylene and infection. There were no significant differences in pustule numbers among treated or untreated resistant and susceptible leaves. It is concluded that total peroxidase activity is not causally related to resistance, expressed either as infection type or as pustule density.

Susceptible infected leaves had much higher rates of ethylene production than leaves showing a resistant reaction, although the latter had rates significantly above healthy control leaves. The higher rates of ethylene production in susceptible reactions are consistent with the changes in infection type produced by ethylene, but are not consistent with the activation of peroxidase either by ethylene or by resistant reactions. The results raise the question as to whether it is resistance or susceptibility that is induced during the infection process in rust reaction controlled by *Sr6* alleles. Phytopathology 60:1648-1652.

Additional key words: Triticum, obligate parasitism.

In the second paper of this series we reported (26) that peroxidase activity increased in an infected near-isogenic line of wheat grown at 20 C and carrying the dominant *Sr6* allele for resistance to *Puccinia graminis* Pers. f. sp. *tritici* Eriks. & E. Henn., but that peroxidase did not increase significantly in the susceptible companion line carrying the recessive allele. The increase occurred at the time when resistance is first expressed and also when indoleacetic acid (IAA) decarboxylation increased (2). The *Sr6* allele is temp sensitive, and plants possessing the dominant allele are resistant at 20 C but susceptible at 26 C. Therefore, it was possible to "challenge" resistant plants (26) by placing them at higher temp after peroxidase increases were observed. Although susceptible infection types resulted from the temp treatment, there were no significant changes in peroxidase levels during the process of reversion from a low infection type (0;) to a high infection type (3+).

We did note (26), however, that the observable number of infected sites was less on plants so treated. The possibility existed that, although total peroxidase activity did not appear to correlate with resistance as measured by infection type, it might be related to a resistance mechanism controlling the initial establishment (hence numbers of colonies) of the pathogen. Consequently, it is necessary to try other approaches to ascertain the role of peroxidase in disease development.

In studies of plant disease resistance accompanied by metabolic changes, there are relatively few instances where biochemical changes in infected tissue clearly can be shown to be causally related to resistance. Correlations with final disease reaction can be obtained with a product or products of the host-parasite interaction, but the chronological sequence in the production of such materials and in the restriction of the pathogen development is not always clear. Furthermore, the role of the product in vivo is subject to uncertainty. The recent results of Chalutz et al. (6) illustrate the complications and also suggest some methods for further tests of any hypothesis involving enzyme activity or toxic chemicals in disease resistance.

Several papers have reported that ethylene treatment stimulates enzyme activity (24, 28), including enzymes possibly involved in aromatic biosynthesis such as peroxidase (7, 15, 28, 30, 32) and phenylalanine ammonia-lyase (21). Since these enzymes frequently are invoked (9, 20, 21, 28, 30, 32) in explaining disease resistance, we attempted to induce increased peroxidase production in wheat leaves by ethylene in order to determine if such induction would either change a susceptible reaction to one of resistance or to decrease pustule numbers in either resistant or susceptible reactions.

MATERIALS AND METHODS.—Temperature and light conditions for growth and inoculation of plants was as described previously (25) except that, in order to facilitate ethylene treatments, small growth chambers (ISCO) rather than controlled growth rooms were employed. The lines of wheat, near-isogenic for *Sr6* alleles, were the same, and race 56 of *Puccinia graminis tritici* was the pathogen. Peroxidase extractions and assays were identical to those carried out earlier (26).

Treatment of plants with ethylene was accomplished by sealing two pots each of healthy and three pots each of inoculated resistant or susceptible lines of wheat in

a single clear plexiglass chamber with a capacity of 125 liters. A measured volume of ethylene (Matheson reagent-grade) was injected through a port capped with a serum stopper.

Previous experience (11) with rusted plants kept in a close system has indicated that it is necessary to air periodically the chamber if normal sporulation is to occur. Furthermore, in contrast to storage tissues of plants (7, 15), carbon dioxide is required for infected leaves. Accordingly, after injection of ethylene, an amt of CO_2 equal to 0.06% of the chamber volume was generated from sodium bicarbonate with 3 N H_2SO_4. Both operations were carried out approximately 4 hr before the end of the light period. The chambers were kept closed for 21-22 hr, then opened for 2-3 hr during the light period before the next daily treatment with ethylene and CO_2. Although CO_2 does inhibit ethylene action, the max amt present at any time (approx 0.09%) was well below the values usually given for inhibitory effects (1, 5, 23).

Measurement of ethylene production was made by distributing 60-100 excised primary leaves equally among three 150-ml beakers, each containing 30 ml of water. Approximately 1 inch of the basal ends was immersed in the water. The beaker walls retained the leaves in an upright position.

The beakers containing excised leaves of healthy control plants or inoculated leaves of resistant and susceptible lines then were placed in separate cylindrical plexiglass chambers of 11.5 liters capacity. Each chamber contained three shallow dishes with 3 ml each of 0.25 M in 2 N perichloric acid (34). Paper wicks of approximately 1 × 5 inches were arranged in each dish so as to provide max absorptive area for trapping ethylene. After sealing the chambers for 12 or 24 hr, the wicks were removed and extracted thoroughly 3 times with fresh perchlorate solution. The washings and original perchlorate solutions were combined and made to a standard volume, usually 15 ml. A 5-ml aliquot was transferred to 15-ml Servall tubes, and sealed with a serum bottle stopper. Ethylene was regenerated with four equivalents of HCl (34), and the gas phase analyzed by the gas chromatographic procedure of Burg & Burg (4). An activated alumina column 6 ft × 0.25 inch was used.

The use of excised leaves circumvented the problem of extraneous ethylene production by noninoculated tissue of diseased plants and by soil, but raised problems associated with breakdown of resistance through excision (14) and bacterial contamination. For the latter reason, leaves were treated as above for either 12 or 24 hr, and fresh tissue used for each separate measurement. This introduces the possibility of variability in periodic measurements because of variations in infection intensity among leaves (26); however, the number of leaves in each sample was large enough to make this a minor effect. Because of limitations in the total amt of leaf material available, healthy control values were obtained by using equal numbers of resistant and susceptible noninoculated leaves in a single chamber. In cases when plants were enclosed in plexiglass chambers, the temp of a water reservoir in the chamber was monitored and no difference from the external environmental temp of 20 C was noted.

TABLE 1. Peroxidase activity in ethylene-treated healthy or inoculated lines of wheat resistant or susceptible to race 56 of *Puccinia graminis tritici* at 20 C

	Activity on day[a]					
	4	6	8	4	6	8
Tissue[b]	Control			75 ppm ethylene		
SH	13.8	13.3	15.0	19.0	29.3	28.3
SI	16.6	18.9	20.2	38.2	29.3	37.4
RH	15.5	16.3	15.5	24.0	29.3	28.5
RI	20.3	27.6	33.8	41.3	28.9	44.1

[a] △ OD/min per leaf.
[b] S = susceptible lines; R = resistant lines; H = healthy control plants; I = infected plants.

RESULTS AND DISCUSSION.—Although concn of ethylene from 0.1 to 10 ppm are sufficient to induce biochemical changes in many plant tissues, there is little information on the response of primary leaves of wheat. To ensure a response, wheat plants were treated with 75 ppm, starting immediately after inoculation. On day 4 (Table 1), all leaves showed increased peroxidase activity when compared to equivalent but untreated tissues. It is to be noted that, at all times tested, susceptible infected tissues treated with ethylene showed a higher peroxidase activity than untreated, resistant infected leaves, despite the fact that ethylene treatment did not alter the normal susceptible infection type. The data for day 6 in the ethylene treatments of infected plants were lower than for either day 4 or 8. The reason is unknown, but in more recent experiments, with different purposes, this decrease has not been observed. Most probably, the substrate solutions, freshly prepared for each assay, were limiting the reaction rate in extracts with high activity. The values were still higher, however, than any value for untreated leaves. The final reaction of ethylene-treated susceptible leaves was infection type 3 (Table 2).

In contrast, leaves normally showing a resistant reaction at 20 C reverted to a susceptible infection type (Table 2) at that temp when treated with ethylene, even though ethylene induced additional peroxidase activity above that of the same line showing normal resistance (Table 1). As is evident from Table 2, the

TABLE 2. Number of pustules, percentage sporulation, and infection type of rust-affected ethylene-treated or untreated wheat leaves at 20 C

Tissue[a]	Pustules/leaf[b]	% Sporulation on day[c]		Infection type
		7	9	
SIC	115	58	74	3
SIE	123	82	82	3
RIC	137	1	5	0; 1
RIE	118	27	70	3

[a] R = resistant lines; S = susceptible lines; I = infected tissue; E = ethylene treatment; C = control tissue not treated with ethylene.
[b] Average of 40 leaves.
[c] Average of 20 leaves for each day.

TABLE 3. Effect of 75 ppm of ethylene on peroxidase activity and disease reaction on a near-isogenic line of wheat resistant to race 56 of *Puccinia graminis tritici* at 20 C measured on day 14

Treatment[a]	Pustules/ leaf	% Sporulation	Peroxidase[b] activity	Infection type
RC			19.7	
RIC	75	5	30.8	0; 1
RI_2E	94	81	49.5	3
RI_3E	86	80	48.3	3
RI_6E	97	63	48.9	3

[a] R = resistant lines; S = susceptible lines; C = non-treated control plants; I = infected plants; E = ethylene treatment. The numerals refer to the day after inoculation when ethylene treatments were started.
[b] △ OD/min per leaf.

rate of development of susceptible reactions with ethylene treatment varied with the line of wheat. Leaves normally susceptible at 20 C appeared to sporulate earlier than untreated leaves of the same line. Ethylene-treated leaves of the line normally resistant at 20 C sporulated more slowly than either group of susceptible leaves. By day 9, however, the infections on all leaves, except those showing a resistant reaction, were essentially equivalent and could not be distinguished visually.

In Table 3, data are presented showing essentially the same effects of ethylene. In this experiment, resistant inoculated lines were treated with ethylene starting on day 2, 3, or 6 after inoculation. On day 14, peroxidase assays were made, and simultaneously pustule numbers and sporulation were estimated. The number of leaves available was not large enough to permit statistical evaluation. As in the previous experiment, the reversion from a resistant to susceptible infection type was evident within 2 or 3 days of initiation of ethylene treatments despite the very high levels of peroxidase.

The results are not in agreement with other disease situations (28, 30, 32) where resistance is apparently increased by ethylene treatment. The results do support a previous contention (26) that total peroxidase activity in vitro is not correlated with infection type. Unlike the uncertainties experienced with reversion of reaction through transfer of the resistant lines to higher temp (26), the reversion induced by ethylene was not accompanied by a reduction in observable infected sites. No statistically significant differences were found. It should be noted, however, that ethylene treatment was started immediately after inoculation. When temp transfers are made immediately after inoculation, reduction in number of infected sites does not occur (2, 26). Although a second type of resistance involving pustule number may exist (26), peroxidase does not appear to be involved. It is premature to suggest that high temp and ethylene treatments at 20 C are synonymous.

The effects of ethylene on enzyme activity and disease reaction may explain several aspects of the behavior of this host-parasite complex. In previous experiments where tissue was grown in large rooms at 20 C with adequate air circulation, most infections were type 0;, and only rarely were infections of type 1 observed on the resistant line. In smaller chambers at the same temp but filled with plants, as many as 50% of infected sites may be of type 1. In the experiment shown in Table 2, for example, there were 5% type-1 pustules, in contrast to previous papers of this series. Gases produced during disease development also may be responsible for the abnormal development of pustules when infected plants are kept continuously in a closed system for periods of 8 or 10 days (11). In terms of enzyme activity, it was shown previously (2) that very high rates of decarboxylation of IAA occurred with resistant infected leaves which could not be readily distinguished visually from healthy noninoculated leaves. In that case, either a relatively few invaded or disease-affected cells had extremely high rates of decarboxylation, or cells considerably removed from infected sites were being affected. The production of ethylene would explain such an effect and such considerations prompted experimentation with ethylene.

It appeared reasonable to assume that the high peroxidase or IAA decarboxylation activity in plants showing resistant reactions was a secondary consequence of ethylene production, perhaps because of cellular injury. In support of this idea, infected resistant lines at 20 C reverted to susceptibility when simply enclosed in sealed chambers without the addition of ethylene. The reversion was slower than when no extra ethylene was added, requiring 4 or 5 additional days to complete development.

The data of Table 4 suggest that such a simple model may not be sufficient to explain the relationship between ethylene and peroxidase activity. In two separate experiments, leaves with susceptible reactions showed much higher rates of ethylene production from the end of the 2nd day after inoculation than did leaves with resistant reactions. When sporulation occurred on susceptible tissue (96-100 hr), rates of ethylene production rose dramatically, perhaps due to the activity of the fungus. Although resistant infected tissue produces ethylene at higher rates than control tissues, the rates of ethylene production by susceptible or resistant infected tissue are inversely related to their peroxidase activities. This finding is not consistent with ethylene induction of peroxidase synthesis, or with a role for peroxidase in ethylene production (33).

It might be argued that the very high rates of ethylene production by infected susceptible tissue represses enzyme activity while more moderate rates induce enzyme activity. There are no readily available data that support such an idea, and it should be noted that 75 ppm of ethylene stimulated peroxidase in infected tissue of both resistant and susceptible plants (Table 1). With sweetpotato tissue, peroxidase levels increased successively with increments of ambient ethylene up to 100 ppm (15).

Our results cannot be easily reconciled with some current ideas concerning resistance, aromatic compounds, enzyme activity, and ethylene production. As was pointed out in a previous paper (25), the *Sr6* gene

TABLE 4. Ethylene production at 20 C by primary leaves of healthy or rust-affected resistant and susceptible lines of wheat

	Picoliters ethylene per leaf at stated hr after removal from inoculation chamber					
Tissue[a]	24-48	48-72	72-96	96-120	120-144	144-168
Experiment 1						
R + S	515	1,000	1,100	1,200		
RI	1,180	3,020	2,550	5,900		
SI	1,130	4,280	6,500	14,600		
Experiment 2						
R + S		900	1,220	1,100	1,200	1,350
RI		1,630	1,950	2,450	[b]	6,930
SI		1,920	4,680	18,000	21,000	37,500

[a] R + S = sample of equal numbers of resistant and susceptible noninoculated leaves; RI = infected resistant leaves; SI = infected susceptible leaves.
[b] Perchlorate solution lost.

for resistance may be unique with respect to the biochemical events it controls. With additional research, it may be possible subsequently to fit the data into an existing conceptual framework regarding resistance. It is conceivable that one of a group of peroxidase isozymes (27) (IAA oxidase?) may be produced in tissue exhibiting a resistant infection type, but that the peroxidase activity found during reversions of disease reaction by high temp or ethylene treatments involves different isozymes. Imaseki et al. (15), however, did not find any qualitative difference in peroxidase isozymes induced by ethylene or by infection. In addition, our data do not prove, even indirectly, that ethylene normally is in any way involved in either resistant or susceptible disease reactions. In the tests we have carried out so far, it is not possible to eliminate the accumulation of other volatile components, such as ethyl acetate (18), that might influence disease reaction. We have noted the presence, during chromatography, of a second gaseous component, but its identity is not known. Furthermore, it is not known whether ethylene per se or some metabolic derivative of ethylene acts to induce enzyme activity. Finally, the rate of production of any gaseous component does not permit a ready extrapolation to tissue concn of the gas. The level of ethylene within the tissue and the sensitivity of the cells may be crucial. These and similar problems are under current consideration.

Because of such uncertainties, the above data suggest caution while interpreting the effects of ethylene, enzymes, and aromatic compounds in this host-parasite combination as well as in others. The concept has been recognized for many years that resistance may be caused by induction in the host of synthetic events leading to pathogen inhibition through toxic chemicals. The concept has been popularized recently, largely through studies on storage or endocarp tissue and the coinage of unique terms (8). It is known that ethylene can activate at least some of these same events, either with no effect on disease reaction or pathogen growth (6) or, in the present instance, with induction of susceptibility. The catalogue of ethylene effects is lengthy (23) and hardly complete. With storage or endocarp tissues, the role of ethylene in wound healing versus chemical inhibition in explaining resistant reactions has not been explored adequately. This is especially true in challenge infections, where cell-wall thickening alone might be sufficient to prevent pathogen establishment. There is evidence that ethylene can cause other changes frequently observed in disease situations, such as changes in permeability (31).

The corollary concept that susceptibility is the inducible active property of the host, rather than (or as well as) resistance, has not been so widely emphasized. In cases of obligate parasitism, compatible relationships may require the gearing and entrainment, in successive steps, of host metabolism to provide a suitable metabolic environment for the pathogen. Resistance, even in "hypersensitive" reactions such as an immune response (29), may result when one or more of the necessary metabolic processes is not activated, thus leading to injury or death of the pathogen. Damage or irritation to host cells, accompanied by effects on enzyme activity and metabolism, may then follow.

It is of interest in this regard that three of four generally recognized plant hormones increase in compatible reactions involving obligate parasites. Interest normally has centered about their role in growth distortion, but growth distortion may be an effect of only secondary consequence for disease development (9). IAA accumulates at a very early stage of rust disease (10), and has been correlated with vegetative growth of the pathogen (12). Similarly, Bayliss & Wilson (3) found that growth alterations occurred very early in rust development of thistle and were correlated with increased gibberellin levels, as well as with IAA levels. IAA, kinins (13), and gibberellins each may be responsible for crucial events in the initial divergence (12) and in the maintenance of host metabolism as well as in the accumulation of necessary substrates (13, 16, 19). Our results indicate that a fourth hormone (23), ethylene, may be involved in reactions controlled by the *Sr6* locus. The interactions among hormones such as ethylene and IAA (4) or kinins and IAA (17) in regulation of normal plant development are complex, but the interactions also provide an opportunity for diverse regulatory action in disease situations where there is genetic specificity for each of several possible

infection types on a given variety. A pertinent example is the observation that kinetin maintains the normal rust reactions of excised wheat leaves (22). Along with control of disease reaction by regulatory enzymes and feed-back inhibition, the possibility of induced host chemical susceptibility should be considered as well as the recognized possibility of induced host chemical resistance.

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