Hypovirulence-Associated Suppression of Host Functions in *Cryphonectria parasitica* Can be Partially Relieved by High Light Intensity

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ABSTRACT

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Phenotypic expression by cultured isogenic virulent (EP155) and hypovirulent (EP713) strains of the chestnut blight fungus, *Cryphonectria parasitica*, was examined under different light regimens. Colony morphology and the levels of pigmentation, conidiation, oxalate accumulation, and phenol oxidase production were all influenced by light intensity, but to different degrees in the two strains. Significantly, exposure of strain EP713 to high light intensity partially relieved the hypovirulence-associated suppression of pigmentation, conidiation, and oxalate accumulation observed at low or intermediate light intensity. These results suggest that light and hypovirulence-associated double-stranded RNAs may influence *C. parasitica* gene expression by the same or converging regulatory pathways.

Additional keywords: biological control, mycovirus.

Transmissible hypovirulence in the chestnut blight fungus, *Cryphonectria parasitica* (Murrill) Barr, continues to receive attention because of its potential as a biocontrol strategy (2,9,32). Available evidence indicates that the genetic information responsible for hypovirulence resides on cytoplasmically replicating, double-stranded (ds) RNAs, presumably of viral origin (5,6,13,30, 33,34). Hypovirulence-associated dsRNAs, recently termed virulence inhibition factors (10), are transmitted to virulent strains by hyphal anastomosis (3). However, reduced virulence is only

one of a number of associated symptoms. These can include altered colony morphology (1,2,7), suppressed conidiation (1,8,9), and reduced oxalate accumulation (1,15), laccase production (26), pigmentation (1,2), and accumulation of specific fungal mRNAs and polypeptides (23,24). Moreover, these symptoms are reversible, i.e., loss of hypovirulence-associated dsRNAs results in complete reversion to the virulent phenotype (11, our unpublished results).

The mechanisms responsible for hypovirulence-associated suppression of fungal processes remain obscure. However, two recent advances have provided new experimental approaches to this problem. First, analyses at the RNA and cDNA levels have yielded insight into the genetic organization of the hypovirulence-associated dsRNAs (18,19,26,30) and have resulted in the identification of dsRNA-encoded polypeptides (26). Second, efficient DNA-mediated transformation has been achieved with *C. parasitica* (17, N. K. Van Alfen, *personal communication*). Thus, it may be possible to identify the precise genetic information responsible for hypovirulence-associated symptoms by examining the phenotypic consequences of introducing cDNA sequences corresponding to specific regions of the hypovirulence-associated dsRNAs into virulent strains. Events such as reduced pigmentation, conidiation, and metabolite accumulation provide convenient markers for such studies and need further characterization. In addition, a number of these processes also are influenced by environmental factors, e.g., pigmentation and conidiation are suppressed when virulent strains are grown in the absence of light (4,21,25). Examination of the combined effects of environmental factors and hypovirulence-associated dsRNAs on specific fungal processes may lead to an understanding of how each independently influences fungal gene expression. These considerations prompted us to initiate a systematic study of the influence of one environmental factor, light intensity, on phenotypic expression by isogenic virulent and hypovirulent strains of *C. parasitica*. The isogenic virulent and hypovirulent strains, EP155 and EP713, were chosen for this study, because they consistently exhibit clearly observable phenotypic differences and because the EP713 dsRNAs are the most thoroughly characterized of the hypovirulence-associated dsRNAs at the molecular level (26).

MATERIALS AND METHODS

Fungal strains and growth conditions. *Cryphonectria parasitica* strains EP155 (virulent) and EP713 (hypovirulent) were obtained from Dr. S. Anagnostakis (Conn. Agric. Exp. Stn.). Strain EP713 was originally produced by transfer of dsRNA from the French hypovirulent strain EP113 (3,12) to the North American virulent strain EP155 by anastomosis. EP155 and EP713 are therefore considered to be isogenic. In addition, results similar to those reported for EP155 were obtained using dsRNA-free, single conidial isolates derived from EP713. Such isolates have cultural and morphological characteristics that are identical to EP155. Stock cultures were maintained on potato-dextrose agar (PDA; Difco) on the laboratory bench: light <2,000 lx, temperature 22–24 C. Under these conditions, strain EP713 grew more slowly than EP155, was white, and produced no conidia or pynicidia within 10 days of culturing. Strain EP155 was light orange and produced abundant pycnidia and conidia. Experimental cultures were initiated by placing 3- × 3- × 3-mm agar cubes, excised from the margins of 7- to 10-day stock cultures, at the center of 85-mm-diameter petri dishes containing 28–30 ml of PDA with or without cellophane overlays. Variations in phenotypic expression will arise if the volume of medium is not maintained at a uniform level. Plates were incubated in an environmentally controlled growth chamber (Conviron) equipped with fluorescent and incandescent lights set for a 16-hr photoperiod at 24 C. One set of 10 plates received 10,000 lx (high intensity), one set was covered with 16 layers of cheesecloth so that mycelia received 2,500 lx (medium intensity), and one set was covered with a foil-wrapped box (dark). Other treatments directed at ensuring that the presence or absence of light was primary to the observed effects are described in the discussion section.

Phenotypic measurements. Radial growth of colonies plated on PDA or PDA/cellophane was determined by measuring the diameters of each colony at 90° angles. Mycelial pads were also stripped from colonies grown on PDA/cellophane and weighed. Total extractable pigment from colonies grown on PDA/cellophane was quantified as follows. Colonies were stripped from cellophane overlays after 7 days' growth, weighed, transferred to Corex tubes, and homogenized thoroughly with a Polytron tissue homogenizer in the presence of 10 volumes of absolute ethanol, in which all *Cryphonectria* pigments have been shown to be soluble (16). After 3 hr at room temperature, the homogenized fungal tissue was nearly uniformly cream-colored and had settled. Portions of the pigment-containing extracts were removed and further diluted fourfold with ethanol so that the final tissue/ethanol ratio was 1/40. The absorbances of the diluted extracts were measured with a Shimadzu UV2100 spectrophotometer at 454 nm, determined experimentally to be the maximum absorbance point for the most highly pigmented samples extracted under these conditions. Conidiation was measured by liberating conidia with a glass rod in 10 ml of 0.15% Tween 80 (Sigma). Solutions were strained through sterile Miracloth (Calbiochem) to remove mycelial pieces, and serial 10-fold dilutions were made from each filtrate. The number of conidia in each dilution was quantified by direct counting with the aid of a hemacytometer and by plating aliquots of the dilutions onto PDA plates supplemented with 100 mg of methionine and 1 mg of biotin per liter (PDAmb) and counting the resulting colonies. For determination of oxalate accumulation, 3- × 3- × 3-mm agar cubes were excised from stock cultures and placed in the center of empty 100-mm petri dishes. Potato-dextrose broth was added until even with the top of the agar plug (30–40 ml). When undisturbed, the mycelia formed pads on top of the broth. Aliquots of the culture fluid were removed at 3 or 7 days after inoculation, and the oxalate content was measured with the oxalic acid detection kit purchased from Boehringer Mannheim. Phenol oxidase activity was determined by measuring the conversion of L-dihydroxyphenylalanine (L-DOPA) to hallachrome as described by Leonard (20). Fluid from cultures grown as described for oxalate determination was collected by filtration at 4 C. Reaction mixtures (3 ml) containing 5 mM L-DOPA, 0.03 M sodium phosphate buffer, pH 6.5, and 1 ml of filtered culture fluid were incubated at 37 C and enzyme activity was measured spectrophotometrically. One unit of enzyme activity is defined as a 0.001 unit change in absorbance/min at 475 nm. Incubation of the culture fluid with syringaldazine indicated that laccase was responsible for more than 80% of the phenol oxidase activity (14).

Extraction, purification, and analysis of double-stranded RNA. We have found that rapid purification of dsRNA by cellulose column chromatography (22) gives excellent results as a preparative procedure, but is less reliable for accurate quantification of all dsRNA species in a given sample. We therefore relied on a scheme for dsRNA purification that allowed quantification of each nucleic acid species at various steps throughout the isolation procedure. Fungal tissue (0.25–2.0 g) was pulverized in liquid nitrogen with a mortar and pestle. Five volumes of extraction buffer (2× STE [1× = 100 mM NaCl, 50 mM Tris, pH 8.0, 2 mM EDTA], 2% sodium dodecyl sulfate [SDS], 2 mM dithiothreitol) were added to the mycelial powder to form a slurry, which was transferred to Corex tubes. One volume of neutralized phenol/chloroform/isoamyl alcohol (25/24/1) was added, and the mixture was homogenized vigorously with a Polytron tissue homogenizer. The emulsion was broken by centrifugation and the aqueous phase removed to clean tubes. One quarter volume of 10 M LiCl was added while vortexing, and the tubes were incubated on ice. After 2–3 hr, the samples were subjected to centrifugation at 10,000 *g* for 20 min. Supernatants were decanted into clean tubes, 2.5 volumes of cold ethanol were added and the tubes were placed at −80 C for 20 min. Nucleic acid was collected by centrifugation at 10,000 *g* for 20 min, pellets were washed with 70% ethanol, dried, and resuspended in water. At this point, samples contained predominantly DNA, dsRNA, tRNA, and minor amounts of single-stranded RNA. Samples were

adjusted to 20 mM Tris, pH 7.4, 20 mM $MgCl_2$, and 5 units of DNase (Promega) was added per gram of tissue. After incubation for 2 hr at 37 C, NaCl and RNase A were added to 0.3 M and 20 μg/ml, respectively, and samples were incubated for 30 min at 37 C followed by extraction once with phenol/chloroform/isoamyl alcohol and once with chloroform/isoamyl alcohol. Nucleic acid was precipitated from the aqueous phase with 2.5 volumes of ethanol, collected by centrifugation, washed with 70% ethanol, dried, and resuspended in 1× STE. At this point, preparations contained predominantly dsRNA and digestion products of other nucleic acids. Double-stranded RNA was analyzed by electrophoresis through agarose gels (0.6% to 1.0%) cast in TPE (35 mM Tris, pH 7.8, 30 mM NaH_2PO_4, 1 mM EDTA). Gels were stained with ethidium bromide and photographed. Total and individual dsRNA species from a given preparation were quantified by densitometer tracing of photographic negatives. A standard curve was generated by loading five twofold dilutions of purified dsRNA preparations of known concentration.

RESULTS

Effects of light intensity on phenotypic expression by isogenic *C. parasitica* strains EP155 and EP713. It was evident from visual inspection of colonies grown under different light regimens (examples shown in Fig. 1) that *C. parasitica* responds to light in a fairly dramatic and consistent fashion and that the presence of hypovirulence-associated dsRNAs altered that response. When grown in the absence of light, colonies of the virulent strain EP155 were white, occasionally with very light yellow-orange centers, contained considerable aerial hyphae, and grew completely across the 85-mm dish within 7 days. Colonies incubated in high light (10,000 lx) were deep orange, contained relatively fewer aerial hyphae, and covered only 65% of the agar surface. Colonies incubated in intermediate light intensity (2,500 lx) were generally intermediate in color and size. The response of the hypovirulent strain EP713 to light differed consistently from that of strain EP155. In the absence of light, EP713 produced small, irregularly shaped, white colonies. Incubation at 2,500 lx resulted in a significant increase in radial growth, but only a slight increase in pigmentation. However, incubation at 10,000 lx resulted in a significant increase in pigmentation with no increase in radial growth.

To obtain more precise information regarding the effect of light intensity on EP155 and EP713 phenotypic expression, sets of 10 cultures grown under three different light intensities were characterized with respect to colony morphology and levels of pigmentation, conidiation, oxalate accumulation, and laccase production. As indicated in Figure 2, the diameter and mass of EP155 colonies decreased as the light intensity increased, while the diameter and mass of EP713 colonies increased with increasing light intensity. However, there was not a strict relationship between colony diameter and mass. While the diameter of EP155 and EP713 colonies were comparable under intermediate and high light intensities, EP155 colonies were of considerably greater mass, even at 10,000 lx.

Increasing light intensity resulted in increased pigmentation in both strains. When grown in darkness, EP713 colonies were cream-colored and provided ethanol extracts with slightly higher absorbances at 454 nm than did similarly grown EP155 colonies, which were snow-white in appearance. However, the pigment produced by these cream-colored colonies was not the same as the well-characterized pigments associated with sporulation of *C. parasitica* (16,28,29). The pigments from sporulating colonies turn deep red when made alkaline (16). No color changes were observed in extracts from any of the dark-grown, nonsporulating, cream-colored colonies following the addition of NaOH to 0.01 N. Incubation at 2,500 lx resulted in a large increase in the absorbances at 454 nm for extracts of EP155 colonies but only a very slight increase for extracts of EP713 colonies. At 10,000

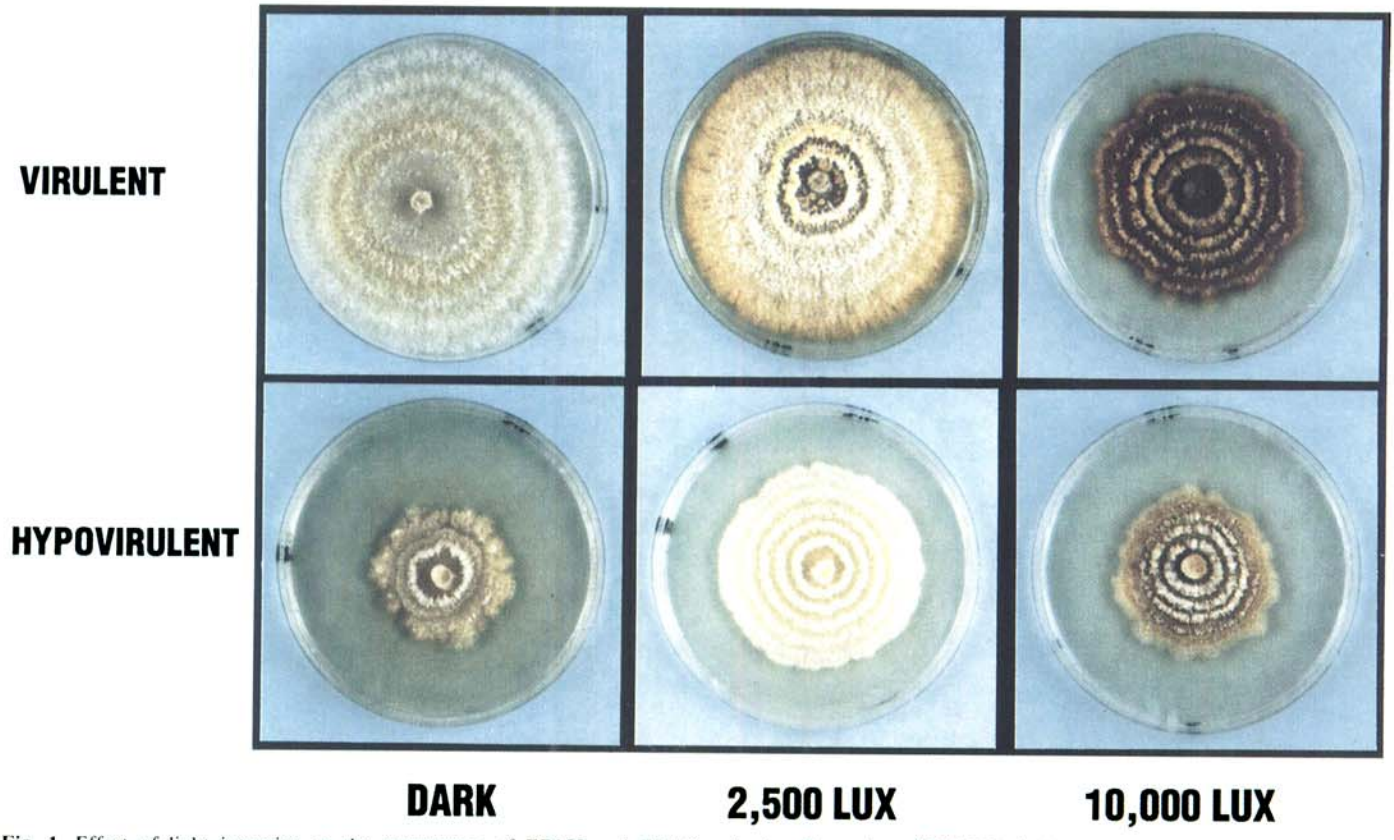

Fig. 1. Effect of light intensity on the appearance of EP155 and EP713 colonies. Examples of EP155 (virulent) colonies grown in the dark or at 2,500 and 10,000 lx for 7 days are shown in the top panels. Bottom panels show examples of EP713 (hypovirulent) colonies grown under the same three conditions. Photographic reproduction of colonies can slightly distort differences in the relative levels of pigmentation. Quantitative differences are more accurately expressed in Figure 3.

lx, values for EP713 rose dramatically, such that their values were equivalent to or slightly higher than the values for EP155 at 2,500 lx. Addition of NaOH to extracts of EP713 colonies grown at 2,500 lx resulted in a distinctly pink coloration, and led to a deep red coloration of all other extracts.

The trend in conidiation paralleled that of pigment production (Fig. 3B). Again, the differences between the two strains was much greater at intermediate light intensity than at high light. However, even at 10,000 lx, strain EP713 produced only 50% as many conidia as did strain EP155 at 2,500 lx.

Two biochemical events potentially involved in pathogenesis, oxalate accumulation, and phenol oxidase production, have been reported to be suppressed in hypovirulent strains of *C. parasitica* (15,27). As shown in Figure 3C, oxalate accumulation was significantly stimulated at high light intensity. In the absence of light, and at intermediate light intensity, strain EP155 produced 8.3- and 5.2-fold higher levels of oxalate, respectively, than did strain EP713. However, at high light intensity, EP713 produced significant levels of oxalate; 50% of the level produced by EP155 under the same conditions and approximately twice the level produced by EP155 incubated at intermediate light intensity. In this regard,

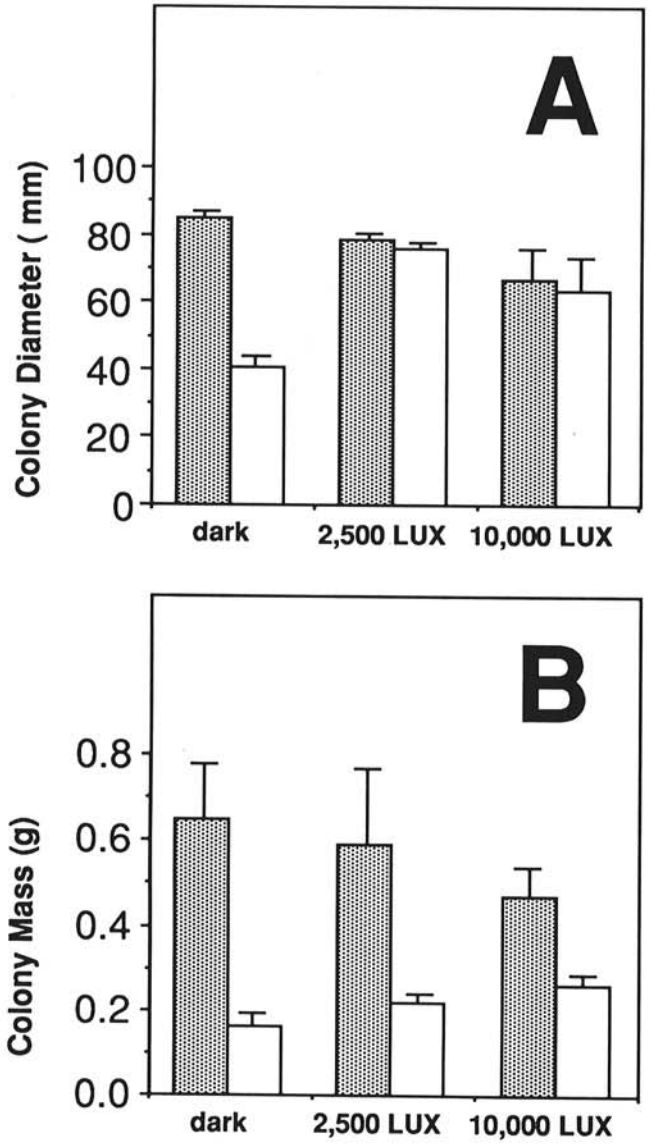

Fig. 2. Effect of light intensity on size and mass of EP155 and EP713 colonies. **A,** Average colony diameter (in mm) for EP155 (stippled bars) and EP713 (open bars) grown under different light conditions. Each value represents the average from 10 culture plates with standard deviations indicated. **B,** Average colony mass for the same set of colonies. Bars indicate standard deviations.

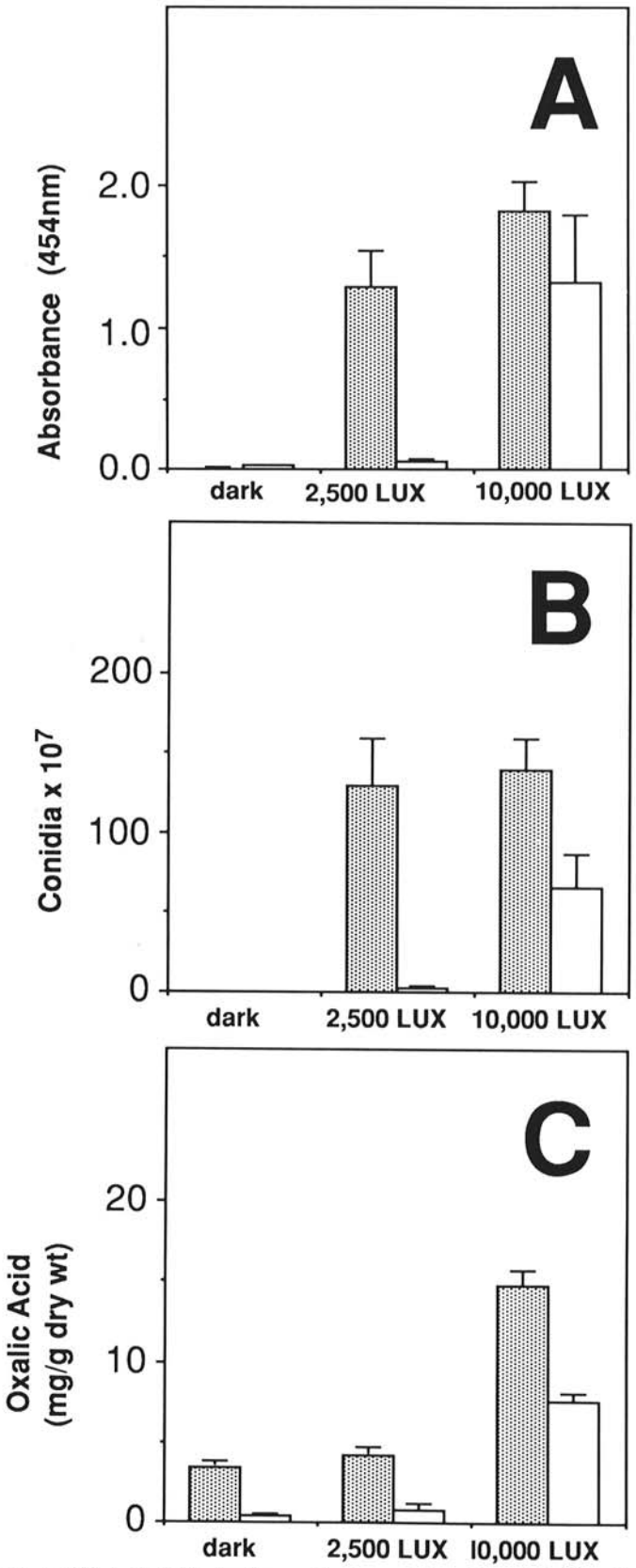

Fig. 3. Effect of light intensity on **A,** pigmentation, **B,** conidiation, **C,** and oxalate accumulation by *C. parasitica*. Values for EP155 and EP713 are indicated by stippled and open bars, respectively. The relative levels of pigmentation were determined spectrophotometrically as indicated in the methods section. The average numbers of conidia produced per colony at each light intensity are presented in Panel B. Forty percent of the EP155 plates grown in the dark produced conidia with an average value of $1.6 \times 10^7 \pm 1.8 \times 10^7$. Only one dark-grown EP713 plate produced conidia, at a level of less than 10^4. The levels of oxalic acid (mg/g dry wt) that accumulated after 7 days of culture are presented in Panel C.

the relationship between light intensity and oxalate production for the two strains paralleled that observed for pigmentation and conidiation. A different relationship, however, was observed between phenol oxidase production and light intensity (Table 1). Increasing light intensity resulted in a significant reduction in phenol oxidase production in both strains EP155 and EP713. Moreover, under each light condition tested, strain EP155 produced significantly higher levels of phenol oxidase activity than did strain EP713, in agreement with the recent report by Rigling et al (27) for other strains of *C. parasitica* incubated at an intermediate light intensity.

Effect of light intensity on hypovirulence-associated dsRNA accumulation. One possible explanation for the relief of hypovirulence-associated symptoms by high light is a concomitant reduction in the concentration of hypovirulence-associated dsRNAs. To test this possibility, total dsRNAs were extracted from EP713 colonies grown under a variety of light intensities and quantified by gel electrophoresis (Fig. 4) and densitometry. As indicated in Table 2, there was a reduction in the average concentration of dsRNAs as the light intensity was increased. On an overall per weight basis, 30% less dsRNA was recovered from mycelium incubated at 10,000 lx than from mycelium incubated in the dark. However, there were several individual cases in which as much, or more, dsRNA was isolated from a highly pigmented colony grown at 10,000 lx than from parallel, slightly pigmented colonies grown at a lower light intensity (e.g., in Figure 4, compare lane 2 of the 10,000-lx samples with lanes 2 and 3 of the 2,500-lx samples or compare lane 4 of the 2,500-lx samples with lanes 2 and 4 of the dark samples.). In addition, all dsRNA isolates had the same number and relative amounts of individual dsRNA species regardless of the light intensity under which the colonies were grown (Fig. 4). Thus, the relief of hypovirulence-associated symptoms by high light intensity cannot be considered to be a direct result of quantitative or qualitative changes in hypovirulence-associated dsRNAs.

TABLE 1. Effect of light on phenol oxidase accumulation in culture filtrates of *Cryphonectria parasitica* strains EP155 and EP713

	Day 3[a] Units/g dry wt[b]		Day 7[a] Units/g dry wt	
Strain	Dark	10,000 lx	Dark	10,000 lx
EP155	1.30 ± 0.2	0.50 ± 0.08	5.11 ± 0.31	2.52 ± 0.01
EP713	0.40 ± 0.03	ND[c]	1.98 ± 0.49	ND[c]

[a] Age of culture.
[b] One unit of phenol oxidase activity is defined as a 0.001 unit change in absorbance per minute at 475 nm with the assay described in the Materials and Methods section. The majority of phenol oxidase activity (>80%) was of the laccase type (see Materials and Methods) as previously described by Rigling et al (27).
[c] ND = not detectable.

DISCUSSION

Current interest in hypovirulence-associated dsRNAs stems primarily from their potential as biological control agents (2,9,32,33). Hypovirulence in *C. parasitica* also provides opportunities for examining the influence of viral factors on host functions. In addition, hypovirulence-associated symptoms can have a direct impact on the effectiveness of biological control, for example, suppression of conidiation may interfere with the effective dissemination of the hypovirulence phenotype (2). In an attempt to understand how hypovirulence-associated dsRNAs affect virulence and other fungal processes, we are applying a strategy that relies on molecular cloning of cDNA copies of the hypovirulence-associated dsRNAs coupled with DNA-mediated transformation of the fungai host (17). The isogenic virulent and hypovirulent *C. parasitica* strains EP155 and EP713 initially were selected for these studies because the hypovirulence-associated dsRNAs are present at a reasonably high concentration (17) and because pigmentation and conidiation were reported to be suppressed in strain EP713 relative to strain EP155, thus providing clearly observable phenotypic differences that correlate with the presence of hypovirulence-associated dsRNAs.

As early as 1914, Anderson (4) reported that light induced conidiation in *C. parasitica*. More recently, Puhalla and Anagnostakis reported that *C. parasitica* grown on either minimal media or PDA produced little or no pigment when cultured in the dark (25). Inspection of published experimental protocols

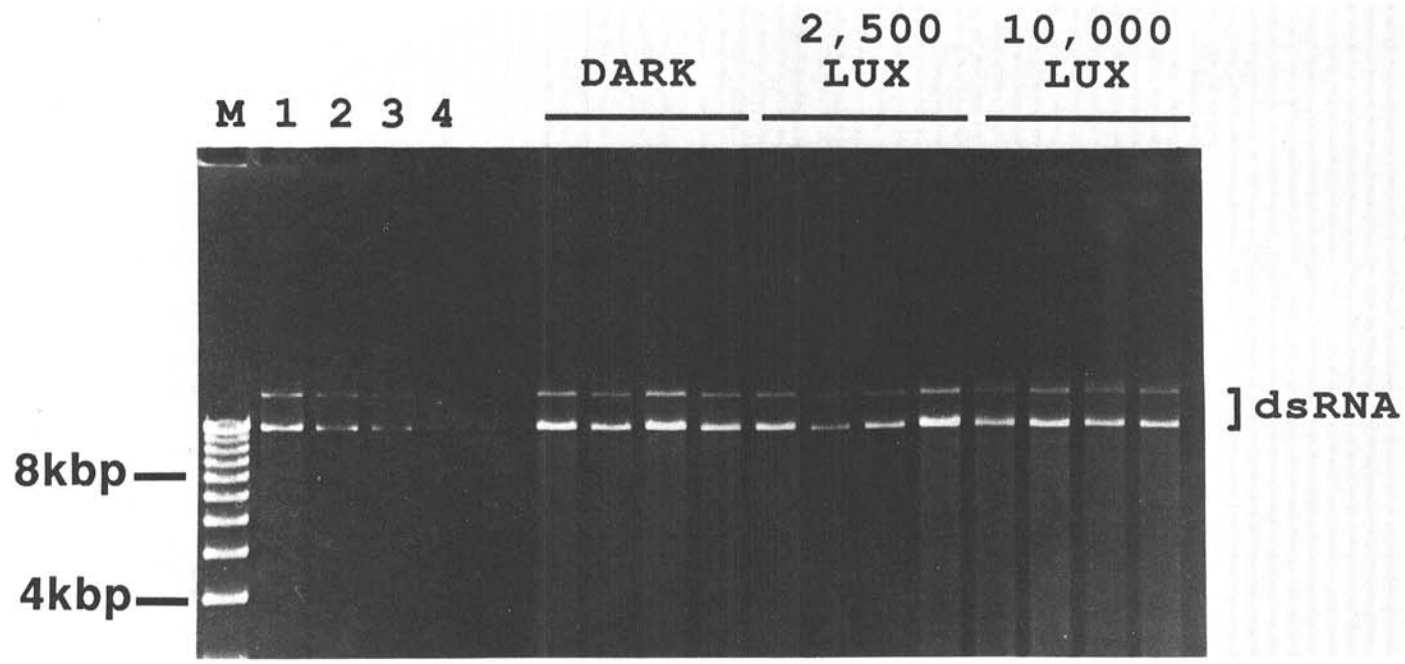

Fig. 4. Agarose gel electrophoretic analysis of dsRNAs extracted from strain EP713 grown under different light intensities. The migration positions of the EP713 large dsRNAs are indicated at the right. Lane M contains 200 ng of the BRL 1 kbp DNA ladder as size markers. Lanes 1–4 received 24, 12, 6, and 3 ng of purified EP713 large dsRNAs, respectively. In other gels, two- or fourfold higher concentrations of dsRNAs were used to generate a standard curve. The remainder of the lanes contain dsRNAs extracted from the equivalent of 10 mg of EP713 mycelium grown either in the dark, at 2,500 lx or 10,000 lx.

revealed that *C. parasitica* is frequently grown either in the dark or under unmeasured, low intensity light. Consequently, it was unclear whether a certain threshold of light was required for efficient pigmentation and conidiation or whether the magnitude of the response depended on the intensity of the light administered. In addition, since several of the processes influenced by light are also suppressed in hypovirvlent strains of *C. parasitica*, it was of interest to determine the combined effect of light intensity and hypovirulence-associated dsRNAs on phenotypic expression.

To ensure that light and not other environmental factors, e.g., difference in humidity, temperature, or gas (CO_2 or ethylene) accumulation were responsible for the observed effects, the following experiments were performed. Cultures of EP713 and EP155 were initiated at the centers of PDA-containing petri dishes with single 3- × 3- × 3-mm agar cubes taken from stock cultures. The plates were tightly wrapped with Parafilm and a piece of black construction paper was taped to the bottom to cover one-half of the plate, bisecting the agar inoculation cube. Plates were then placed inverted under high light conditions. In this way, an entire colony was uniformly exposed to the same level of humidity and gaseous environment while one-half of the colony was shaded and the other half was exposed to high light intensity. After 7 days of incubation, the resulting colonies were visually striking; portions of the colonies that grew on the lighted half of the plate were deep orange while the shaded portions were very light in color. A line corresponding to the position of the edge of the construction paper was clearly visible, bisecting the colony. Differences in temperature also appeared not to be a major factor in pigmentation or the induction of sporulation, consistent with results of Puhalla and Anagnostakis (25). Although we could not accurately record temperature at the agar surface, temperatures at the plate surface were 26 C for 0 lx, 27 C for 2,500 lx, and 29 C for 10,000 lx. Colonies grown in foil-covered plates that were incubated for 7 days at temperatures ranging from 20 to 35 C, the highest temperature at which *C. parasitica* grows (25), did not differ visually from each other in color nor did they sporulate.

A very similar light-response pattern was observed for pigmentation, conidiation, and oxalate production. In the absence of light, these processes proceeded at undetectable or very low levels during the 7-day culture period (Fig. 3). Illumination resulted in a significant stimulation of all three processes. However, the light-response was not dependent on exposure to a certain threshold of light intensity, but increased as light intensity increased over the light range tested. While all three processes were suppressed in the hypovirulent strain at each light intensity tested, the suppression was partially relieved at higher light intensity. For example, exposure to high intensity light resulted in a significant stimulation in pigmentation by the hypovirulent strain, to the extent that the hypovirulent strain grown in high light intensity often contained as much or more pigment than the virulent strain grown at 2,500 lx. The effect of high light intensity on conidiation by the hypovirulent strain was also significant. Although the virulent strain produced 50- to 60-fold more conidia than the hypovirulent strain at 2,500 lx, this value decreased to a factor of 2 at high light intensity. Our results regarding oxalate production were consistent with those of Havir and Anagnostakis (15) who reported that hypovirulent strains produced no detectable oxalate when grown on PDAmb under white fluorescent light with a 12-hr light-dark cycle, while isogenic virulent strains produced on the order of 7–10 mg of oxalate per gram dry weight by day seven of culturing under these conditions. The levels of oxalate produced by strains EP155 and EP713 grown at intermediate light intensity were in this range, except that the hypovirulent strain did produce detectable amounts of oxalate. Oxalate was produced by both strains in the absence of light, with strain EP155 producing 8.5-fold higher amounts than strain EP713 by day seven of culturing. Exposure to intermediate light intensity resulted in a moderate increase in oxalate production by both strains. However, exposure to high light intensity stimulated oxalate production 18.4-fold in strain EP713 and 4.3-fold in strain EP155, again resulting in a partial relief of the suppression observed in the hypovirulent strain at lower light intensity.

TABLE 2. Quantification of dsRNAs from individual cultures of *Cryphonectria parasitica* strain EP713 grown under different light regimes

Light intensity (lx)	dsRNA concentration[a] (ng/10 mg tissue)
0	37.5 ± 4.9
2,500	30.0 ± 7.7
10,000	23.0 ± 2.7

[a] Refers to major high molecular weight band as indicated in Figure 4. Quantification was performed as described in Materials and Methods.

Two noteworthy hypovirulence-associated symptoms were recently described: suppressed phenol oxidase activity of the laccase type (27) and reduced accumulation of specific fungal mRNAs and polypeptides (23,24). Since phenol oxidase activity could potentially participate in degradation of lignified zones produced by the infected host as a defense barrier, a reduction in phenol oxidase activity could contribute significantly to hypovirulence. Rigling et al also speculated that reduced sporulation by hypovirulent strains of *C. parasitica* could be linked to a reduction in phenol oxidase activity (27). This prediction is not consistent with the observed effect of light intensity on these two processes (Table 1; Fig. 3). Exposure to high light intensity resulted in a reduction in phenol oxidase activity and a concomitant stimulation, rather than a reduction, in sporulation. In this regard, phenol oxidase activity differed from the other parameters tested in that its level was reduced at the times of assay both by hypovirulence-associated dsRNA and by high light intensity. Additional studies with phenol oxidase deficient mutants should reveal the contribution of phenol oxidase activity to virulence in *C. parasitica*.

The observations by Powell and Van Alfen (23,24) that the accumulation of only a specific subset of fungal mRNAs and polypeptides is altered in hypovirulent strains suggest that hypovirulence-associated symptoms are not a consequence of a general debilitation of the fungal host. Rather, they suggest that the hypovirulence-associated dsRNAs, presumably through the action of encoded polypeptides, influence the expression of specific fungal genes at the level of mRNA transcription or stability. Based on analogies with other eukaryotic systems (e.g., 31), it is probable that the light-mediated stimulation of pigmentation, conidiation, and oxalate production in *C. parasitica* is also regulated at the level of mRNA transcription or stability. The observations that these processes are suppressed in the hypovirulent strain and that the suppression is partially relieved by exposure to high light suggests the possibility that both light and products of EP713-associated dsRNA influence fungal gene expression through the same or converging regulatory pathways. However, it is important to note that different hypovirulent strains of *C. parasitica* exhibit a range of associated symptoms. For example, in the North American strain, GH2, hypovirulence is not linked to suppressed pigmentation and conidiation (D. Fulbright, *personal communication*). The fact that hypovirulence-associated dsRNAs can have multiple unlinked effects on fungal gene expression provides additional support for the proposal that hypovirulence and associated symptoms are not related to a general response of the fungal host to the presence of dsRNA but may depend on the specific action of dsRNA-encoded gene products.

The means for determining the influence of individual dsRNA-encoded polypeptides on fungal gene expression are now available. Several polypeptides encoded by EP713 dsRNAs have been identified and expressed in vitro with the aid of partial cDNA clones of EP713 dsRNA (26, our unpublished results). Antisera to several EP713 dsRNA-encoded polypeptides are now available. In addition, conditions for efficient DNA-mediated transformation of *C. parasitica* have been established (17, N. K. Van Alfen, *personal communication*). Transformation experiments designed to express dsRNA-specific sequences in *C. parasitica* under the control of several different fungal promoters are currently in

progress. As indicated in this report, the hypovirulence-associated symptoms of suppressed pigmentation, conidiation, and oxalate accumulation provide convenient, well defined, and reproducible markers for transformation studies aimed at examining the influence of EP713 dsRNA-encoded polypeptides on fungal gene expression.

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