Isolation and Evaluation of a Plant-Virus-Inhibiting Quinone from Sporophores of *Agaricus bisporus*

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ABSTRACT

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A pink quinone, tentatively identified as γ-L-glutaminyl-3,4-benzoquinone, present in the sporophores of *Agaricus bisporus* is a potent systemic inhibitor of plant virus infection. A high level of resistance (98.5 or 81.5% reduction in numbers of lesions) is observed when cowpea or pinto bean plants are mechanically inoculated with tobacco ringspot or tobacco mosaic virus, respectively, 8 days after treatment.

A previous study (6) showed that the viruslike particles (VLPs) that cause La France disease of the cultivated mushroom, *Agaricus bisporus* (Lang.) Imbach, occur at a higher concentration in spawn than in sporophores derived from the same spawn. It was thought that the presence of a compound (or compounds) in the sporophore but not in the spawn might be responsible either for inhibition of virus replication in the sporophore or virus loss during extraction. Many basidiomycetes have been found to contain plant virus inhibitors (4). Before this hypothesis could be tested, we had to determine whether virus inhibitors were present in mushroom sporophores. The sequence of structural and biochemical events in the zygote of *A. bisporus* prior to sporulation indicates suppression of energy, protein, and nucleic acid synthesis (1,8,10). When protoplasm is transformed from a vegetative to a dormant state, as during sporulation of *A. bisporus* and other basidiomycetes, there is a total, yet reversible, suppression of metabolic activity (7,10). Evidence indicates that dormancy is initiated in the spores of *A. bisporus* by two quinones that appear in the zygote prior to sporulation (8,10). A colorless phenol, γ-L-glutaminyl-4-hydroxybenzene, is a precursor of the two quinoids. It is not effective as a metabolic inhibitor (8,10–13). An oxidation product of this phenol is a pink quinone tentatively identified as γ-L-glutaminyl-3,4-benzoquinone (11–13). Further oxidation converts this compound to a brown quinone and changes the color of the gill tissue from pink to brown (11–13). The pink compound is designated as inhibitor 490 and the brown as inhibitor 360 (8).

Inhibitor 490 has a pink color, absorption maximum at 490 nm, and molar extinction coefficient of 10,000 (14). It passes freely across mitochondrial and cellular membranes, inhibits mitochondrial oxidative enzymes, and participates in the induction of dormancy by inhibiting energy production (14). Inhibitor 360 has a brown color, absorption maximum at 360 nm, and molar extinction coefficient of 800–1,000 (D. G. Graham, *personal communication*). It passes freely across mitochondrial and cellular membranes, and inhibits protein and ribonucleic acid synthesis (7). Both compounds are known to have bacteriostatic, bactericidal, antiviral, and antitumorigenic properties both in vitro and in vivo (when introduced into animal tissue) (8–10).

In this communication, we report the effects of these two quinones on tobacco mosaic virus (TMV) infection of pinto bean and tobacco ringspot virus (TRSV) infection of cowpea. These virus-host systems were used in the absence of a reliable bioassay for mushroom viruses.

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MATERIALS AND METHODS

Isolation of inhibitors. Mushrooms were grown at the Mushroom Research Center of The Pennsylvania State University at 15 C. Sporophores were picked shortly after rupture of the velum while the gills were pink. The two quinoids were isolated according to the methods of Vogel et al (10). The gills were scraped from the pileus (cap) and homogenized at 4 C in distilled water (1:10, w/v) in a Waring Blendor. Particulate material was removed by centrifugation (30,000 *g* for 30 min, then 300,000 *g* for 1 hr). Aliquots of the supernatant were layered on G-25 fine Sephadex columns (2 × 100 cm) at 4 C and eluted with distilled water. After the protein peak, three major colored bands were eluted: the brown quinone with an absorption maximum at 360 nm; an as yet unidentified dark-orange band; and a pink band with an absorption maximum of 490 nm. The pink quinone was oxidized to brown following a 10-fold concentration at 50–60 C in a Buchler Flash Evaporator. The oxidized brown compound possessed all the physical and biological properties of inhibitor 360 (F. S. Vogel, *personal communication,* and *unpublished data* from our study). Although the chemical structures of the two compounds were not determined in our experiments, their physical properties were identical to those of inhibitors 360 and 490 as previously determined (11). Five to 10 milliliters of 150–200-mM inhibitor 360

TABLE 1. Effect of concentration of inhibitor 360 from mushrooms on infection of cowpea by tobacco ringspot virus

Concentration of inhibitor (mM)[a]	Local lesions[b] (no. per half leaf)	Inhibition (%)
Control	80.1(68–93)	0.0
10	57.9(51–66)	27.7
20	19.5(12–30)	75.7
30	17.1(8–24)	78.7
40	9.3(4–17)	88.4
50	8.1(4–13)	89.9
60	6.3(2–11)	92.1
70	6.6(1–14)	91.8
80	3.9(0–8)	95.1
90	2.1(0–5)	97.4
100	1.5(0–6)	98.1

[a] Test plants were sprayed with distilled water (control) or the corresponding solution of inhibitor 48 hr prior to inoculation.

[b] Means of three replications, 12 plants per treatment. The range of numbers of lesions for each concentration of inhibitor 360 is given in parentheses. Purified TRSV (0.1 mg/ml) was used as inoculum.

TABLE 2. Effect of inhibitor 360 from mushrooms on the infection of pinto bean by tobacco mosaic virus (TMV) and of cowpea by tobacco ringspot virus (TRSV)

	Local lesions (no. per half leaf)[b]					
	Control		Inhibitor		Inhibition (%)	
Time of application[a]	TMV	TRSV	TMV	TRSV	TMV	TRSV
24 hr preinoculation	81.2(64–91)	72.1(56–83)	6.5(4–9)	1.2(0–3)	92.0	98.3
48 hr preinoculation	78.3(67–85)	70.4(59–78)	0.9(0–2)	0.7(0–3)	98.9	99.0
3 days preinoculation	73.8(67–77)	64.7(56–72)	2.7(0–4)	0.8(0–2)	96.3	98.8
4 days preinoculation	73.6(59–78)	61.7(51–74)	0.8(0–3)	0.4(0–2)	99.0	99.3
8 days preinoculation	66.5(52–78)	45.3(40–56)	13.6(4–21)	0.7(0–3)	81.5	98.5
24 hr postinoculation	89.4(74–102)	73.7(62–82)	59.6(43–68)	47.8(38–54)	33.3	35.1

[a] Test plants were sprayed with a solution of inhibitor 50 μm or distilled water.
[b] Means of three replications, 12 plants per treatment. The range of numbers of lesions for each treatment is given in parentheses. Inoculum consisted of TMV (0.1 mg/ml) or TRSV (0.1 mg/ml) purified just prior to the beginning of each experiment.

TABLE 3. Effect of inhibitor 360 from mushrooms on infectivity of tobacco mosaic virus (TMV) in bean and tobacco ringspot virus (TRSV) in cowpea

	Local lesions (no. per half leaf)[b]			
	Distilled H_2O		Inhibitor	
Fraction[a]	TMV	TRSV	TMV	TRSV
Pellet	63.6(55–78)	90.8(83–102)	61.8(51–68)	91.5(80–99)
Supernatant	0	0	0	0

[a] Purified TMV (0.1 mg/ml) or TRSV (0.1 mg/ml) were incubated with equal volumes of distilled water or a solution of inhibitor (50 mM) at 22 C for 30 min. The virus was precipitated (see Materials and Methods) and the pellets were resuspended in 0.05 M phosphate buffer pH 7.2 to the original volume. Virus infectivity of the supernatant and resuspended pellet solutions was tested using mechanical inoculation.
[b] Means of three replications, 12 plants per treatment. The range of numbers of lesions for each treatment is given in parentheses.

TABLE 4. Systemic protection against infection of bean by tobacco mosaic virus (TMV) and of cowpea tobacco ringspot virus (TRSV) induced by inhibitor 360 from mushrooms

		Local lesion[a] (no. per half leaf)		Inhibition (%)	
Virus	Control	Unsprayed	Sprayed[b]	Unsprayed	Sprayed
TMV	88.7(82–97)	29.5(23–37)	3.1(1–7)	66.7	96.5
TRSV	80.5(67–92)	24.9(17–31)	4.1(3–6)	69.1	94.9

[a] Means of three replications, 12 plants per treatment. The range of numbers of lesions for each treatment is given in parentheses. Inoculum consisted of TMV (0.1 mg/ml) or TRSV (0.1 mg/ml) purified just prior to the beginning of each treatment.
[b] One of the primary leaves of test plants was sprayed with a solution of inhibitor (50 mM) 48 hr prior to inoculation of both leaves.

in distilled water was routinely obtained from 1 g (fresh weight) of gill tissue.

Effect of the inhibitors on plant susceptibility to virus infection. Pinto bean (*Phaseolus vulgaris* L.) and cowpea (*Vigna unguiculata* (L.) Walp.) served as the local-lesion hosts for TMV and TRSV, respectively. Greenhouse temperature and relative humidity were maintained at approximately 21 C and 40%, respectively. Depending upon the experiment, 12 plants were sprayed with distilled water or a 50-mM inhibitor solution (0.5 ml per leaf) 5–12 days after emergence. Following inhibitor application onto the leaves of the test plants, the droplets were allowed to dry, and the next morning treated and untreated leaves were sprayed with distilled water to wash off excess inhibitor. Test plants were then inoculated at the indicated times. This procedure was followed to ensure that excess inhibitor did not enter (or kill) the wounded cells (potential infection sites) at the time of inoculation. The effects of the inhibitor concentration and the time of inhibitor application on virus infection were studied. The potential of systemic protection against virus infection was tested by spraying one of the primary leaves of test plants with a solution of inhibitor 48 hr prior to inoculation of both primary leaves.

Effect of the inhibitors on virus infectivity. Purified virus solution (0.1 mg/ml) was incubated with an equal volume of 50-mM inhibitor solution or distilled water at 22 C for 30 min. The virus was precipitated by adjusting the mixture to 4% polyethylene glycol (PEG 6000) in 0.3 M NaCl (TMV) or 8% PEG in 0.3 M NaCl (TRSV), slow stirring for 1 hr and low-speed centrifugation (10,000 *g* for 10 min). The pellets were resuspended in 0.05 M sodium phosphate buffer pH 7.2 to the original volume. Virus infectivity of the supernatant and resuspended pellet solutions was tested by using mechanical inoculation.

Tobacco mosaic and tobacco ringspot viruses were purified according to the methods of Hariharasubramanian et al (2) and Stace-Smith et al (5), respectively. A Latin-square design was used for the local-lesion assays. Twelve test plants per treatment were utilized. Each experiment was replicated three times.

RESULTS AND DISCUSSION

Preliminary experiments showed that the inhibitor 360, the brown quinone, was more effective against virus infection than inhibitor 490 or a mixture of the two. Thus, unless otherwise indicated, inhibitor 360 was utilized in our studies. The two quinoids were not detected in spawn extracts.

At concentrations as low as 20 mM compound 360 caused more than 75% inhibition of infection (Table 1). Similar results were obtained with TMV infection of inhibitor-treated pinto bean. Percent inhibition of infection of plants sprayed with a 20-mM inhibitor solution did not change with virus concentration (0.01–1.0 mg/ml).

The effects of inhibitor 360 upon virus infection of inhibitor-sprayed plants prior to or postinoculation are shown in Table 2. Test plants were highly resistant when they were mechanically inoculated up to 8 days after spraying, the longest time interval between spraying and inoculation tested in this study. In addition, lesions developed approximately 24 hr later on leaves of plants sprayed with inhibitor solution compared to those on leaves of control plants sprayed with distilled water. Inhibition of infection was significantly lower in plants sprayed after inoculation.

Our results showed that inhibitor 360 does not act on the virus per se (Table 3). Inhibitor- or distilled-water-treated virus solutions showed equal levels of infectivity for both tobacco mosaic and tobacco ringspot viruses used in this study.

When one primary leaf of each test plant was sprayed with a 50-mM inhibitor 360 solution, and both leaves, sprayed and unsprayed, were inoculated 48 hr later, there was approximately a 70% inhibition of virus infection on the unsprayed leaves compared to approximately 95% inhibition on the sprayed leaves (Table 4). These results, in conjunction with the results of the experiment described previously, suggest that inhibitor 360 affects the host rather than the virus. The resistance to virus infection induced by compound 360 is systemic and of long duration. This study provides additional evidence that supports the hypothesis of elicitation of defense reaction in plants and protection against subsequent infection (3). Perhaps further attention should be given

to utilizing elicitors of defense mechanisms for controlling diseases under field conditions.

Preliminary experiments showed that aliquots of inhibitor-treated, virus-infected spawn of *A. bisporus* contained lower virus concentrations than the distilled-water-treated aliquots as determined by the degree of serological reactions. Precipitation lines corresponding to extracts of inhibitor-treated, virus-infected spawn were more diffuse, appeared approximately 12 hr later, and were closer to antigen-containing wells than those of water-treated, virus-infected spawn extracts. We have suggested (6) that single-spore cultures may be a good way of obtaining mushroom isolates free of these viruses. A combination of chemotherapy with inhibitor 360, heat treatment, and hyphal-tip culture might be effective in achieving this objective. In other preliminary experiments with the cowpea mosaic virus (CPMV)-cowpea system, compound 360 reduced virus multiplication and symptom severity when applied 24 hr postinoculation. Leaf disks, 1 cm in diameter, taken from primary leaves of CPMV-infected cowpea 5 days postinoculation were used for mechanical inoculation of *Chenopodium amaranticolor* plants that served as the local lesion host. The number of local lesions, formed on *C. amaranticolor* leaves infected with inoculum derived from CPMV-infected, inhibitor-treated cowpeas, was 54% lower than that of CPMV-infected, water-treated cowpeas. Systemic symptoms appeared later and were less severe in CPMV-infected cowpeas sprayed with a 50-mM inhibitor solution as compared to CPMV-infected, distilled water-treated cowpeas.

The studies of Vogel et al (8–10) underscore the likelihood that natural agents such as inhibitor 360, concerned with the induction of cryptobiosis might have useful therapeutic properties against the growth of tumor cells. Our studies show that inhibitor 360 is highly effective against plant virus infection. Phytotoxic side-effects were not observed under the conditions of our experiments. Further research may show that inhibitor 360 can be useful in control of diseases caused by plant viruses as well as in research concerning molecular aspects of virus replication.

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