Effects of Benomyl and Ribavirin on the Lettuce Big Vein Agent and its Transmission

R. N. Campbell

Plant pathologist, Department of Plant Pathology, University of California, Davis 95616.
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ABSTRACT

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Treatment of lettuce plants with benomyl up to 72 hr after inoculation with lettuce big vein agent (BVA)-transmitting *Olpidium brassicae* killed the fungal vector and reduced the frequency of BVA transmission. Benomyl applied to inoculated plants during the symptom expression period had no demonstrable effect on the BVA or symptom expression. Ribavirin applied to inoculated plants during the symptom expression period reduced the severity of symptom expression and the titer of BVA in the *O. brassicae* population in the roots. The BVA was not eradicated from the tops of the plants by ribavirin. The sensitivity of the BVA to ribavirin is evidence of a viral nature of the BVA.

The lettuce big vein agent (BVA) is an infectious, graft-transmissible entity that causes a distinctive vein-banding symptom on lettuce leaves. It is harbored in the soil within the resting spores of the chytrid fungus, *Olpidium brassicae* (Wor.) Dang., and is transmitted to lettuce plants by zoospores of the fungus. The BVA is not mechanically transmissible and no morphological or chemical entity has been associated with it (1).

O. brassicae zoospores mixed with the fungicide benomyl at 100 μg/ml remained motile and infective (2), but thalli in the roots were killed if the roots were treated with benomyl (100–500 μg/ml) applied either before or after inoculation (2,14). Fewer of these plants developed big-vein symptoms (2,14) and others had abnormal or mild symptoms (14) when benomyl treatments were applied within 72 hr of inoculation with BVA-transmitting *O. brassicae*. Symptom expression by lettuce or tobacco plants infected by beet western yellows virus or tobacco mosaic virus (TMV), respectively, was surpressed when the plants were treated with methyl benzimidazole-2-yl carbamate (MBC) (13). Benomyl is converted to MBC and this derivative accounts for the in vivo fungicidal activity observed in treated plants (4).

Antiviral agents, originally developed for animal viruses (8), have found no practical application for plant virus diseases (7). One of these chemicals, ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) (11) has been tested against some plant viruses. It inhibited local lesion formation by potato virus X in *Capsicum* sp. (6) and promoted the production of virus-free tobacco plants from infected, protoplast-derived calluses (10). Ribavirin also caused remission of symptoms of the rose ring pattern agent (9) and prevented multiplication of apple chlorotic leafspot virus in *Chenopodium quinoa* (5).

The present study was done to determine whether benomyl and ribavirin affected the BVA and symptom expression in lettuce plants or the transmission of BVA to lettuce plants.

MATERIALS AND METHODS

***O. brassicae* cultures and BVA assays.** *O. brassicae* was maintained in the roots of Climax lettuce in sand culture in 10-cm-diameter pots at 16–18 C and irrigated with weak nutrient solution as needed (14). The experimental pots consisted of about 50 lettuce seeds sown in plastic pots (100-ml capacity) with drainage holes. These pots contained about 50 ml of white quartz sand with a void volume of about 20 ml. The preparation of zoospore suspensions was done as previously described (14). The standard inoculum for each experimental pot was 10 ml of a zoospore suspension with

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$\geqslant 10^6$ zoospores per milliliter; this was pipetted into the pots 4–7 days after seeding. Plants used in experiments were transplanted singly to 100-ml pots and treatments were applied during the transplanting period or later. If the plants were inoculated for a BVA assay, they were left in the pot and observed for symptoms 3–8 wk after inoculation. The assay was positive if any of the plants showed symptoms and usually most of the plants had symptoms.

Recovery of BVA. Plants that did not have symptoms or had abnormal symptoms, as well as control plants, were tested for the BVA by the in vivo acquisition method. The shoots were excised and rooted (14) to produce plants free from *O. brassicae*. The surviving rooted plants were verified to be free from *O. brassicae* about 8 wk after excision by saturating the sand in which each plant was growing with tap water. Ten minutes later additional tap water was added and the drainage water was collected and examined for *O. brassicae* zoospores. Then the plants were inoculated with an isolate of *O. brassicae* free from BVA. After incubation for 3 wk, *O. brassicae* zoospores were harvested from each plant and assayed for BVA.

Benomyl treatment. Two methods were used to treat lettuce plants with benomyl. In the first, seedlings were removed from sand in pots from 4–72 hr after inoculation with *O. brassicae*. Their roots and part of the hypocotyl were immersed in a solution of 500 μg a.i./ml of distilled water for 24 hr. After treatment they were rinsed, planted individually, and observed for 6–8 wk for symptoms of big vein. In the second method, seedlings transplanted 1 wk after inoculation were drenched with benomyl starting 17–19 days after inoculation. Each plant was drenched with 2.5 mg a.i. benomyl in 10 ml of solution. The excess benomyl was flushed from the pot 8 or 24 hr after each drenching with 40 ml of weak nutrient solution. Controls received distilled water in place of the benomyl and were flushed with nutrient solution.

Ribavirin treatment. Results from preliminary trials indicated that ribavirin (1–4 mg per plant) was severely phytotoxic to young seedlings. The method finally adopted was to drench each transplanted seedling with 1 mg ribavirin per plant in 20 ml of distilled water twice a week for 3 wk starting 19 days after inoculation with *O. brassicae*. One day after each drenching, each pot was flushed with 40 ml of weak nutrient solution. Controls received distilled water, instead of ribavirin, and the weak nutrient solution flush.

RESULTS

Effect of benomyl on symptom expression. *O. brassicae* was usually killed during the first generation by immersing lettuce seedlings in benomyl 4–72 hr after inoculation with BVA-*Olpidium*. In four trials that involved a total of 119 treated plants, *O. brassicae* survived in only five plants that were not tested further. Of the remaining 114 plants, only 17 had normal symptom development. Eight of those were tested and the BVA was recovered by *O. brassicae* from seven plants. Among the 97 remaining plants, 69 were symptomless and 28 had possible abnormal symptoms. The abnormal symptoms consisted of a transient, fine vein-clearing as seen in early stages of normal symptom expression or small patches of vein banding about 1 cm long along a few veins of a leaf as illustrated previously (14). BVA was not recovered from any of the 74 plants tested. By contrast, BV symptoms developed in 75 of 79 inoculated plants that were not treated with benomyl. BVA was recovered from 17 of 21 plants with symptoms and from one of four plants without symptoms. Twenty-two uninoculated plants remained free from BV symptoms and *O. brassicae* contamination. BVA was not recovered from any of them.

When benomyl was drenched onto lettuce seedlings at intervals during the period when symptoms were expressed, there was no reduction in the total number of plants with symptoms (Table 1), the rate at which the plants developed symptoms, or symptom severity. *O. brassicae* was not found in any of the benomyl-treated plants at the end of the experiments. Although excess benomyl was flushed from the pots, there was some phytotoxicity as shown by a reduction in plant weight (Table 1) and a lighter green color of the leaves. Infection by *O. brassicae* and the BVA also reduced the plant weight below that of the controls.

Effect of ribavirin on BVA. Ribavirin ameliorated symptom expression when it was applied for 3 wk during the symptom expression period (Table 2). Treated plants had less-distinct, light-green vein banding than the controls and sometimes the vein banding was restricted to a few veins on one or two leaves. In the second trial the BVA titer apparently was higher and normal symptoms developed on leaves that expanded during the early part of the treatment period. The leaves which formed later showed partial or complete remission of symptoms; thus, five plants were classified as having mild symptoms in Table 2 and four as having no symptoms, although normal symptoms had been observed earlier. Seven to 8 wk after inoculation the treated plants were chlorotic and smaller than the controls due to the phytotoxicity of ribavirin. In the second trial the average weight of tops of treated plants was 13.5 ± 2.1 gm, whereas the nontreated plants weighed 27.8 ± 3.0 gm. The tops of the plants in both trials still contained BVA that was recovered from most treated plants. After excision of the tops, the roots of plants in both trials were assayed for zoospores. Many zoospores were recovered from treated plants but most replicates

TABLE 1. Effect of benomyl on development of big vein symptoms in lettuce and on plant weight when applied during the period of symptom expression

	Symptom expression[a]				Plant weight (g)[b]		
	Trial no.				Trial no.		
Treatment	1	2	3	total	1	2	3
O. brassicae[c] plus benomyl[d]	7/10	6/10	8/10	21/30	17.0 x	10.6 x	15.1 x
O. brassicae[c]	8/10	7/10	6/10	21/30	21.6 y	14.9 y	20.5 y
Uninoculated	0/10	0/10	0/10	0/30	37.6 z	23.7 z	28.9 z

[a] Number of plants with big vein symptoms in 8 wk divided by number of plants tested.

[b] Average fresh weight of plant tops. Values in the same column that are followed by different letters are significantly different according to Duncan's multiple range test ($P = 0.05$).

[c] *Olpidium brassicae*, the fungal vector of lettuce big vein agent (BVA).

[d] Benomyl (2.5 mg/plant) was applied as a drench beginning 17-19 days after inoculation with BVA-*O. brassicae*. The treatment was done once a week for 3 wk in trial 1 and twice a wk for 4 wk in trials 2 and 3. Excess benomyl was flushed from the pots 24 hr after treatment in trial 1 and 8 hr after treatment in trials 2 and 3.

TABLE 2. Effect of ribavirin on the lettuce big vein agent (BVA)[a] and on big vein symptom expression in lettuce

	Ribavirin treated[b]		Untreated control	
Effects	Trial 1	Trial 2	Trial 1	Trial 2
Symptom severity[c]				
Mild	18	13	0	0
Normal	2	0	18	19
None	0	7	2	1
Recovery of BVA[d]	13/17	18/20	5/5	20/20
BVA in zoospores[e]	6[f]/20	3[g]/20	9/9	20/20

[a] BVA is transmitted by a fungal vector, *Olpidium brassicae*.

[b] Ribavirin was applied at 1 mg per plant twice a week for 3 wk starting 19 days after inoculation. Each pot was flushed with nutrient solution 24 hr after each treatment.

[c] Number of plants in each category, 20 plants per treatment in each trial were observed for 7–8 wk from date of inoculation. See text for description of mild symptoms.

[d] Tops of plants were excised, rooted, and tested for presence of BVA by *O. brassicae* recovery; results given as number of plants from which BVA was recovered divided by the number of plants tested.

[e] Number of plants from which zoospores transmitted BVA divided by the number of plants tested.

[f] In five of six cases, there were only one or two positive seedlings among 50 in an assay pot.

[g] In two of three cases, there were only one or two positive seedlings among 50 in an assay pot.

were free from, or had a very low titer of, BVA.

In another trial, zoospores of BVA-*Olpidium* in 0.05 M glycine-NaOH buffer, pH 7.6, were mixed with ribavirin to give 0, 50, or 100 mg ribavirin per milliliter. After incubation for 1 hr the zoospores were sedimented by centrifugation at 10,000 rpm for 10 min in Sorvall SS-34 rotor, resuspended in buffer without ribavirin, and inoculated to two replicate pots of lettuce seedlings. Ten seedlings were transplanted from each replicate 6 days after inoculation and observed for symptoms of big vein. Normal development of big vein symptoms occurred in all 60 inoculated plants regardless of the treatment the zoospores had received. Twenty uninoculated controls were symptomless.

DISCUSSION

This study confirms that benomyl or MBC, its degradation product (4), kills *O. brassicae* within lettuce root cells (2,14). The fungus is killed whether it is inoculated to plants previously treated with benomyl (2) or benomyl is applied during the first vegetative generation after *O. brassicae* is introduced (2,14). In either case, fewer treated plants developed big vein symptoms than those of the nontreated controls. The BVA in plants with symptoms was not affected by exposure to benomyl. The symptoms were normal and BVA was recovered by BVA-free *O. brassicae*. Although some plants treated with benomyl exhibited atypical symptoms, the BVA was not recovered from them and it must be concluded that they had not been infected by the BVA. These atypical symptoms are regarded as transient abnormalities that were noted only because the plants were scrutinized intensively. Furthermore, benomyl applied during the symptom expression period did not ameliorate symptom expression. Thus, neither BVA nor its multiplication in the lettuce host are directly affected by benomyl. The BVA-lettuce system differs from the TMV-tobacco system in which MBC ameliorates symptom expression by inhibition of RNA synthesis by maintaining leaves in a condition unsuitable for virus multiplication (3). Benomyl probably affects the *Olpidium*-BVA-lettuce system at the initial transfer of BVA from fungus to host cytoplasm or during establishment of BVA in the host. It is not clear, however, why the process is affected by benomyl treatments made as late as 72 hr after inoculation. It might be expected that the transfer of BVA to the host would have been completed by this time. Transfer of BVA would seem to be easier during the first 36 hr after infection when the thalli are limited only by an ectoplast rather than later when a sporangium wall has formed (12).

Ribavirin reduces the severity of foliar symptoms and the titer of BVA in *O. brassicae* in the roots, but does not kill the fungus. Presumably, ribavirin interferes with synthesis of the BVA, as it has been reported to do with plant viruses (5,6,9,10). Assuming that ribavirin is specific for plant viruses and not for other types of causal agents, the sensitivity of BVA to ribavirin provides evidence for a viral nature of BVA. Although the mild symptoms of big vein during and after ribavirin treatment probably resulted from reduced synthesis and therefore a lower BVA titer, BVA was not eradicted from the plants. There was adequate time for resumption of synthesis during the 8–10 wk period between the last ribavirin treatment and the *O. brassicae* recovery tests. This is supported by the observation that many plants developed normal big vein symptoms in this interval.

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