Verticillium Wilt of *Ailanthus altissima*: Susceptibility of Associated Tree Species

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ABSTRACT

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Verticillium albo-atrum causes unprecedented wilt and mortality of the invasive tree-of-heaven (*Ailanthus altissima*) within forests of south-central Pennsylvania. Stem inoculation of potted *Ailanthus* seedlings in the greenhouse and canopy *Ailanthus* trees in the field with *V. albo-atrum* resulted in 100% mortality. Stem inoculation of understory striped maple saplings in the field also resulted in 100% mortality. However, the high susceptibility of striped maple was not observed in naturally infected stands, where only 1% of striped maple saplings exhibited Verticillium wilt. Inoculation of chestnut oak, northern red oak, red maple, sugar maple, white ash, and yellow-poplar seedlings or canopy trees with *V. albo-atrum* did not induce wilt symptoms. Non-*Ailanthus* tree species growing adjacent to dead and dying *Ailanthus* trees in the field were asymptomatic. Pending further studies, *V. albo-atrum* should be considered as a potential biocontrol agent for invasive *Ailanthus*.

Invasive plants cost \$20 billion in losses annually in the United States, which includes cost of damage and control, and comprise 8 to 47% of total plant species within some states (9). Introduced species such as tree-of-heaven (*Ailanthus altissima* (Mill.) Swingle), designated hereafter as *Ailanthus*, often have the ability to replace indigenous plants and disrupt native ecosystems (9,18). The ability of exotic plants to become naturalized and thrive in new geographic regions may be related to escape from pathogens in their native area. In contrast, plants introduced to a new geographic region may encounter indigenous pathogens that are foreign to the introduced host species. Such indigenous pathogens have potential to serve as biological control agents for introduced invasive plant species (20).

Control of *Ailanthus* in nonforested areas traditionally has been accomplished by using chemical herbicides. However, it is often difficult to apply chemical herbicides within a forest stand without causing adverse effects to nontarget species. Biological controls such as mycoherbicides may provide an efficient, inexpensive, and sustainable control of invasive plants in forests (9,27,28). In support of this concept, Barton (2) listed 26 species of fungi that have been used as biocontrol agents against weeds in seven countries. *Verticillium albo-atrum* Reinke & Berthold has

killed thousands of *Ailanthus* trees within forests in south-central Pennsylvania and has potential as such a biocontrol agent for this invasive tree species (24). However, host specificity and susceptibility of nontarget organisms must be determined prior to biocontrol applications (30), especially when conducting risk analyses (7). Rigorous host range testing combined with efficacious application methods can ensure that plant pathogens used as biocontrol agents can be a safe and environmentally benign method of weed control (2).

Verticillium wilts occur on more than 200 different dicotyledonous species, including diverse crops such as nuts, vegetables, legumes, herbs, root crops, and oilseed crops, as well as soft, pome, and stone fruit (10,25). More than 60 genera of landscape trees are susceptible to *Verticillium* spp. including *Acer* (maple), *Amelanchier* (serviceberry), *Fraxinus* (ash), *Liriodendron* (yellow-poplar), *Populus* (aspens), *Prunus* (cherry), *Quercus* (oak), *Rhus* (sumac), *Robinia* (black locust), *Sassafras* (sassafras), *Tilia* (basswood), and *Ulmus* (elm) (25), all of which are indigenous tree genera within Pennsylvania forests. *Verticillium* subspecies or host-adapted isolates may attack only certain host species (15,22). Striped maple (*Acer pennsylvanicum* L.), a common understory shrub in Pennsylvania forests and woody species of interest in this study, was reported to be susceptible to *V. albo-atrum* in a landscape setting (13). Verticillium wilt may also occur on common weeds (22) that occur within forest openings or along forest edges. Weeds such as common blackberry (*Rubus allegheniensis* Porter ex L. H. Bailey), nettle (*Urtica* spp.), and Pennsylvania smartweed (*Polygonum pennsylvanicum* L.) occur within forests of

our study area and may serve as hosts, or symptomless carriers, of *Verticillium* spp. (22,29).

Although Verticillium wilts are common in landscape trees (12,25), they are seldom reported from forest settings in North America (11). To the best of our knowledge, our recent article (24) dealing with Verticillium wilt of *Ailanthus* in forests of south-central Pennsylvania is the only article reporting *V. dahliae* (Kleb.) being capable of causing serious tree wilt and mortality in a forest setting. There are only three reports of *V. albo-atrum* causing wilt or mortality within a forest: wilting yellow-poplar seedlings (*Liriodendron tulipifera* L.) in Delaware (21); wilting and dying deer brush (*Ceanothus integrifolius* Hook & Arn.) in California (11); and *Ailanthus* in Pennsylvania (24).

Due to its high pathogenicity to *Ailanthus* (24) and ability to survive for years in the soil (1), *V. albo-atrum* has potential to be an effective, perennial bioherbicide (31). However, *V. albo-atrum* should not be considered as a biocontrol agent until additional, nontarget hosts are investigated. The main objective of this study was to determine if tree species commonly associated with *Ailanthus* in Pennsylvania forests are susceptible to *V. albo-atrum*. Studies were conducted at three levels: (i) inoculation of potted seedlings in the greenhouse, (ii) inoculation of canopy trees in the forest, and (iii) evaluation of associated tree species growing intermingled with dead and dying *Ailanthus* in naturally infected forest stands.

MATERIALS AND METHODS

Inoculation of potted seedlings (four tree species). In March 2006, 3-year-old *Ailanthus*, northern red oak (*Quercus rubra* L.), sugar maple (*A. saccharum* Marsh.), and white ash (*Fraxinus americana* L.) seedlings were placed in 15.2-cm pots containing a blend of 20% Hagerstown silt loam topsoil and 80% Metro Mix 200 (The Scotts Company, Marysville, OH) and maintained in a greenhouse. Potted seedlings were fertilized once with 12:12:12 (N:P₂O₅:K₂O) Osmocote (Scotts Miracle-Gro, Marysville, OH) and watered daily. Seedling species selected were those economically important cohorts of *Ailanthus* that could be purchased locally.

V. albo-atrum isolate PSU140 (GenBank accession no. FJ424082), originally isolated from dying *Ailanthus* trees in the study area (24), was used in all inoculations. An *Ailanthus* isolate was used be-

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cause *Verticillium* isolates from a given host generally cause the most severe symptoms on the host species from which they were obtained (4), perhaps due to host adaptation (4).

Inoculum consisted of conidia harvested from 6-week-old cultures growing on plum extract agar (PEA; 900 ml of distilled water, 20 g of agar, 100 ml of concentrated plum extract, 1 g of yeast, 5 g of lactose, pH 5.6 to 6.0), a semiselective medium for *Verticillium* spp. (3), amended with streptomycin and neomycin. Spore concentrations were adjusted to 10^7 conidia ml⁻¹. Inoculum viability was confirmed by evaluating spore germination (approximately 80%) immediately prior to inoculation.

On 1 June 2006, 20 seedlings of each of the four species ($n = 80$), in two replicates of 10, were inoculated into the xylem at two points approximately 1 cm apart with 0.1 ml of 1×10^7 conidia ml⁻¹ using a 1-ml syringe (6) that penetrated approximately 1 mm into the xylem. Stems of five control seedlings of each species ($n = 20$) were injected at two points with sterile distilled water. Following inoculation, symptoms were rated weekly for 8 weeks using a 0-to-4 scale, where 0 = healthy leaves, 1 = chlorotic leaves, 2 = necrotic leaf margins, 3 = wilting leaves, and 4 = dead or defoliated seedlings (3,24). After 8 weeks, tissue samples from symptomatic leaves were removed and cultured onto PEA. *V. albo-atrum* was identified in resultant cultures, here and throughout this article, using morphological characteristics that had been confirmed using polymerase chain reaction (PCR)/DNA molecular techniques (24).

Inoculation of forest trees (eight tree species). In early May 2007, forest stands that contained healthy *Ailanthus* trees were selected for inoculation studies. In these stands, *Ailanthus* trees were growing intermingled with healthy chestnut oak (*Q. montana* Willd.), northern red oak, red maple (*A. rubrum* L.), striped maple saplings, sugar maple, white ash, and yellow-poplar. Mean diameters of inoculated tree species were *Ailanthus*, 14.0 cm; red maple, 24.7 cm; sugar maple, 28.2 cm; striped maple, 6.5 cm; chestnut oak, 35.5 cm; northern red oak, 47.0 cm; white ash, 46.1 cm; and yellow-poplar, 44.0 cm. Groups of six healthy trees of each species were randomly selected at two sites (replicates) separated by 1 to 3 km. Due to the abundance of red and striped maple trees in the selected stands, one additional replicate of six trees was added for each of these species. Treatment trees were tagged and GPS coordinates recorded.

Inoculum from *V. albo-atrum* isolate PSU140 was produced as described for seedling inoculations. Inoculum viability was confirmed by evaluating spore germination (approximately 80%) immediately prior to inoculation. At each replicated site,

five trees of each of the eight species were inoculated three times at the base of each trunk using a Hypo-Hatchet (OEM Fabricators Inc, Woodville, WI), which delivered 1 ml of 1×10^7 conidia ml⁻¹ each time the trunk was struck (24). One control tree/species/replicate was wounded with a sterile ax at three locations at the trunk base and treated with sterile distilled water. Disease severity on the 108 treated trees ([8 species $\times$ 6 trees/species $\times$ 2 replications] + [6 red maples and 6 striped maples]) was rated monthly for 5 months, using the 0-to-4 scale described for seedling evaluations.

Evaluation of trees on naturally infected forest plots (17 tree species). During July and August 2007, 12 circular forest plots (0.08 ha, 16.1-m radius) were established within a 20-ha stand. The stand contained various levels of dead and dying *Ailanthus* trees from which *V. albo-atrum* had been isolated (24). Locations of plot centers were recorded using GPS. All trees ≥ 2.5 cm diameter at breast height (dbh, 1.37 m) on each plot were evaluated, resulting in rating 853 individual trees within the following 17 species: American basswood (*Tilia americana* L.), American elm (*Ulmus americana* L.), black ash (*F. nigra* Marsh.), black gum (*Nyssa sylvatica* Marsh.), black locust (*Robinia pseudoacacia* L.), butternut (*Juglans cinerea* L.), chestnut oak, northern red oak, red maple, sassafras (*Sassafras albidum* (Nutt.) Nees), shellbark hickory (*Carya laciniata* (F. Michx.) L.), striped maple, sweet birch (*Betula lenta* L.), tree-of-heaven (*A. altissima*), white ash, witch-hazel (*Hamelis virginiana* L.), and yellow-poplar. Most individuals were co-dominant canopy trees, except striped maple and witch-hazel, which were understory saplings.

Plot trees and saplings ≥ 2.5 cm dbh were rated using a 0-to-2 scale, where 0 = healthy, 1 = wilting, and 2 = dead. Symptomatic leaves were removed from trees and saplings with leaves within reach, and wood chips removed from trunks of taller symptomatic trees. Leaf and wood-chip samples were cultured onto amended PEA and grown at 22°C in the dark. Resultant *V. albo-atrum* colonies were identified using morphological characteristics (24).

Data analyses. Analysis of variance (ANOVA) revealed disease severity ratings between replications for seedlings, as well as canopy trees, that were not significantly different and were pooled for analyses. Differences in disease severity values among species were evaluated at the end of the seedling or tree study using area under the disease progress curve (AUDPC; 16):

$$[(y_i + y_{i+1})/2](t_{i+1} - t_i)$$

where y_i is the disease severity rating, t_i is the time of the i th rating, and $i = 1, 2, 3, \dots, n - 1$. ANOVA and Tukey's mean comparisons (19) were used to detect significant ($P < 0.05$) differences among final AUDPC

ratings at the end of each study for seedlings and canopy trees. Temporal trends in weekly disease severity ratings for inoculated seedlings and monthly ratings for canopy trees were illustrated graphically (Figs. 1 and 2).

On the naturally infected forest plots, data from species that composed $\leq 3\%$ total trees per hectare were combined into a miscellaneous (MISC) category. Species in the MISC category were American elm, basswood, black ash, black gum, butternut, chestnut oak, hickory, northern red oak, sassafras, white ash, witch-hazel, and yellow-poplar. ANOVA and Tukey's mean comparisons were used to detect significant ($P < 0.05$) mean disease severity ratings among species, including the MISC group (19).

RESULTS AND DISCUSSION

Inoculation of seedlings. Inoculated *Ailanthus* seedlings developed visible symptoms 4 weeks after inoculation (Fig. 1). Disease severity progressed rapidly thereafter and symptoms on *Ailanthus* remained greater than on other species until the end of the experiment at week 8, when most inoculated *Ailanthus* seedlings were dead (rating of 40 = death). Foliage of inoculated sugar maple and white ash seedlings developed slight discoloration by 3 to 5 weeks (Fig. 1). However, similar symptoms were observed on noninoculated control seedlings and were related to greenhouse stress rather than *Verticillium* wilt. Inoculated northern red oak, sugar maple, and white ash seedlings, as well as controls injected with sterile distilled water, did not develop *Verticillium* wilt symptoms. Final AUDPC values for inoculated seedlings were significantly greater for inoculated *Ailanthus* than for other species, whose AUDPC values were near zero and not statistically different from each other (Table 1).

V. albo-atrum was reisolated from all inoculated *Ailanthus* seedlings but not from other inoculated species (or non-inoculated controls), indicating that *V. albo-atrum* likely did not persist within the aboveground vascular elements of inoculated northern red oak, sugar maple, and white ash seedlings. Because conditions used in this host range study were conducive to infection and disease of *Ailanthus* (2), we consider white ash, sugar maple, and northern red oak to be tolerant (23) to *V. albo-atrum* isolate PSU140 under conditions of this greenhouse study.

Inoculation of forest trees. Disease severity on inoculated *Ailanthus* trees developed rapidly within 4 weeks after inoculation and remained high until the end of the study at 20 weeks (Fig. 2). Severity ratings for inoculated *Ailanthus* trees and striped maple saplings were greater than the other six species from week 8 through the end of the study. Inoculated striped maple saplings did not develop significant symptoms

until 8 weeks after inoculation, a month later than *Ailanthus* trees. However, by the end of the study, disease levels on striped maple were similar to those of *Ailanthus* (Fig. 2). All inoculated *Ailanthus* trees and inoculated striped maple saplings were dead after 20 weeks.

AUDPC values of inoculated *Ailanthus* trees were significantly greater than for all other species, followed closely by striped maple (Table 1). The slightly lower AUDPC values for inoculated striped maple saplings were likely due to the early, 4-week lag in the disease progress curve (Fig. 2). AUDPC values of inoculated striped maple saplings were significantly

greater than all remaining species, which were not significantly different from each other. Inoculated chestnut oak, northern red oak, red maple, sugar maple, white ash, and yellow-poplar trees did not exhibit wilt symptoms and are considered tolerant (23) to *V. albo-atrum* isolate PSU140 under conditions of this field study.

The high susceptibility of canopy *Ailanthus* trees to *V. albo-atrum* isolate PSU140 confirmed findings with *Ailanthus* seedlings (Fig. 1; Table 1) and our recent article (24). Inoculation of canopy trees also confirmed the tolerance of northern red oak, sugar maple, and white ash to *V. albo-atrum*, findings similar to our results from

oak, maple, and ash seedling inoculations. Although we observed yellow-poplar trees to be tolerant to *V. albo-atrum*, Morehart et al. (21) reported yellow-poplar seedlings to be susceptible to *V. albo-atrum*. Differences between their findings and ours may relate to host age, variability in susceptibility among host populations, variability in pathogenicity among *V. albo-atrum* strains, or other unknown factors. In addition, our isolate of *Verticillium* was originally from *Ailanthus*; isolates from a given host usually cause most severe symptoms in host species from which they were obtained (4).

The high susceptibility of inoculated striped maple saplings was surprising, because we had not observed wilting and dying of striped maples in stands naturally infected with *V. albo-atrum*. The small size of striped maple saplings compared with other inoculated species, which were all canopy trees, may have influenced susceptibility of striped maple to artificial inoculations, as discussed below. Hibben (13) reported that *V. albo-atrum* caused annual wilting of a striped maple landscape tree in Ithaca, NY, over a 3-year period. However, the tree did not die during the 3-year period, indicating some degree of tolerance (23) or misidentification of the *Verticillium* sp. responsible for the decline.

Inoculated 3-year-old *Ailanthus* seedlings died more quickly than older inoculated *Ailanthus* canopy trees (Fig. 1 versus Fig. 2), although both succumbed by the end of the growing season in which they were inoculated. The faster demise of seedlings may have been related to various factors. The small *Ailanthus* seedlings received more inoculum per unit of biomass compared with the larger *Ailanthus* trees. In addition, *V. albo-atrum* likely colonized seedlings' small xylem volume much more quickly than the greater xylem volume of large canopy trees. Also, an older tree might have had time to develop partial defense mechanisms in attempts to slow *Verticillium* spp. colonization compared with a 3-year-old seedling. Finally,

Fig. 1. Mean disease severity ratings of potted *Ailanthus*, northern red oak, sugar maple, and white ash seedlings inoculated in the greenhouse at two points on the stem with *Verticillium albo-atrum* conidia at 0.1×10^7 spores ml^{-1} in sterile distilled water. Disease severity was rated using a 0-to-4 scale, where 0 = healthy leaves, 1 = chlorotic leaves, 2 = necrotic leaf margins, 3 = wilting leaves, and 4 = dead or defoliated seedlings. Inoculated controls did not develop symptoms.

Fig. 2. Mean disease severity ratings of canopy *Ailanthus*, chestnut oak, red maple, red oak, sugar maple, striped maple, white ash, and yellow-poplar trees in the field inoculated at three locations on the trunk base with *Verticillium albo-atrum* conidia at 3×10^7 spores ml^{-1} in sterile distilled water. Disease severity was rated using a 0-to-4 scale, where 0 = healthy leaves, 1 = chlorotic leaves, 2 = necrotic leaf margins, 3 = wilting leaves, and 4 = dead or defoliated seedlings. Inoculated chestnut oak, red maple, red oak, sugar maple, yellow-poplar trees, and controls did not develop characteristic symptoms.

Table 1. Area under the disease progress curve (AUDPC) for seedlings evaluated weekly from time of inoculation (0 weeks) until end of study (8 weeks), AUDPC for trees evaluated at 4-week intervals from time of inoculation (0 weeks) until end of study (20 weeks)²

Species	AUDPC	
	Trees	Seedlings
<i>Ailanthus</i>	63.64 a	39.98 a
Striped maple	45.87 b	...
White ash	3.20 c	0.85 b
Red maple	0.00 c	...
Sugar maple	0.00 c	1.05 b
Yellow-poplar	0.00 c	...
Northern red oak	0.00 c	0.00 b
Chestnut oak	0.00 c	0.00 b

² Disease rating values followed by the same letter within a column are not significantly different according to Tukey's test ($P = 0.05$); ... indicates no data.

the greenhouse environment may have predisposed potted seedlings to more rapid colonization than *Ailanthus* trees growing in native forest soils.

V. albo-atrum was reisolated from all inoculated *Ailanthus* trees and inoculated striped maple saplings but not from other inoculated species (or noninoculated controls). As with the seedling study, conditions used in this host-range testing were deliberately conducive to *Verticillium* spp. infection and disease (2). Because *V. albo-atrum* could not be isolated from the vascular system of inoculated but asymptomatic chestnut oak, northern red oak, red maple, sugar maple, white ash, and yellow-poplar trees, we consider these tree species to be tolerant (23) to *V. albo-atrum* isolate PSU140 under conditions of this field inoculation study.

Permanent forest plots. Disease rating data from trees on the forest plots were normalized using a square-root transformation. ANOVA revealed significant differences in mean disease severity ratings among species. Tukey's mean comparison test showed that mean disease ratings on *Ailanthus* and black locust were not statistically different but were significantly greater than values for striped maple, sweet birch, red maple, and the MISC group of species (Table 1). The mean severity value for striped maple was significantly greater than for sweet birch, red maple, and the MISC group. Mean disease severity values for red maple, sweet birch, and the MISC group were not significantly different from each other.

Of the 184 *Ailanthus* trees evaluated on the natural forest plots, 122 (66.3%) were dead or wilted from natural *V. albo-atrum* infections (Table 1). Isolations were not made from dead trees due to the high incidence of saprobes but *V. albo-atrum* was isolated from all wilting *Ailanthus* trees. Of the 185 black locust trees examined, 108 (58.4%) were dead but none of the 77 living locust trees on the forest plots were wilted (Table 1). Similar levels of black locust mortality (*data not shown*) were observed off the plots, in stands that did not have *Verticillium* wilt of *Ailanthus*. Bark had fallen from the dead locust trees

and the outer wood surface had numerous sapwood scars caused by the locust borer (*Megacyllene robiniae* Forster; 17). The high incidence of locust borer damage in the stand, coupled with the relatively short life span of early successional black locust trees (5), appeared responsible for the black locust mortality throughout the local forest. Dead locust trees were heavily colonized by fungal saprophytes, precluding attempts to isolate and culture *V. albo-atrum*. However, it is unlikely that *V. albo-atrum* was involved in the black locust mortality.

Of the 193 striped maple saplings evaluated on the natural forest plots, 42 (21.8%) were dead. However, all dead striped maples had severe stem cankering. Isolation and culturing from the cankers followed by molecular PCR/DNA identification consistently yielded *Botryosphaeria dothidea* (Moug.) Ces. & De Not. *B. dothidea* is a ubiquitous fungus that causes cankers and mortality on various hardwood species (25). Only 2 (1.3%) of 151 living striped maple saplings exhibited foliar wilt (Table 2). One of the two wilted saplings had vascular discoloration from which *V. albo-atrum* was isolated. Remaining living striped maples were not wilted. We attribute death of the understory striped maples primarily to *B. dothidea* rather than *V. albo-atrum*.

Results of this study and our previous research (24) confirm the high susceptibility of *Ailanthus* seedlings and trees to *V. albo-atrum*, based on inoculations and natural infections in a forest setting. Understory striped maple saplings were also susceptible to *V. albo-atrum* based on field stem inoculations but were generally tolerant based on natural infections within the forest. This apparent discrepancy may be related to the high levels of conidia in the inoculum or inoculation method. Inoculation with $3 \text{ ml} \times 10^7$ conidia per small striped maple sapling may have overwhelmed any inherent tolerance to *V. albo-atrum*. Stem inoculation directly into the xylem may have bypassed extravascular root defenses (26) occurring in a natural setting. Extravascular root defenses are important means of excluding wilt fungi,

because the root cortex plays a vital role in delaying ingress of such fungi into the root stele and subsequent systemic infection (14).

Chestnut oak, northern red oak, red maple, sugar maple, white ash, and yellow-poplar were tolerant to *V. albo-atrum* isolate PSU140. In the forest plots, associated tree species tolerant to natural infections by *V. albo-atrum* included American basswood, American elm, black ash, black gum, black locust, butternut, chestnut oak, northern red oak, red maple, sassafras, shellbark hickory, sweet birch, white ash, witch-hazel, and yellow-poplar. Host range data presented in this article may be used in risk analysis (2,7) if *V. albo-atrum* isolate PSU140 is considered to be a potential biocontrol agent for invasive *Ailanthus* (8).

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Table 2. Number of trees examined and number followed by percentage of healthy, wilted, and dead trees, as well as mean disease severity ratings, recorded during summer 2007 on twelve 0.08-ha permanent forest plots in the field exposed to natural *Verticillium albo-atrum* inoculum

Species	No. of trees examined	No. healthy (%)	No. wilted (%)	No. dead (%)	Mean disease rating ^z
Black locust	185	77 (41.6)	0 (0.0)	108 (58.4)	1.123 a
<i>Ailanthus</i>	184	62 (33.7)	36 (19.6)	86 (46.7)	1.005 a
Striped maple	193	149 (77.2)	2 (1.0)	42 (21.8)	0.437 b
Sweet birch	82	79 (96.3)	1 (1.2)	2 (2.4)	0.086 c
Red maple	116	114 (98.3)	0 (0.0)	2 (1.7)	0.036 c
Misc. group	93	92 (98.9)	0 (0.0)	1 (1.1)	0.021 c

^z Mean disease severity rating in the last column is the average rating (0-to-2 scale) of all trees ≥ 2.5 cm in diameter for a given species across all 12 plots. Mean disease rating values followed by the same letter in the last column are not significantly different ($P < 0.05$) according to Tukey's mean comparisons.

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