INSECTICIDE RESISTANCE AND RESISTANCE MANAGEMENT

Pyrethroid Resistance Mediated by Enzyme Detoxification in *Listronotus maculicollis* (Coleoptera: Curculionidae) From Connecticut

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ABSTRACT In 2009, pyrethroid resistance was confirmed for seven “annual bluegrass weevil” *Listronotus maculicollis* Kirby (Coleoptera: Curculionidae) adult populations from southern New England. The mechanisms responsible for conferring this resistance were unknown. In this study, topical application bioassays with bifenthrin and bifenthrin combined with synergists affecting three detoxification systems were conducted on four field-collected adult populations of *L. maculicollis* from Connecticut to determine whether cytochrome P450 monooxgenases (P450s), glutathione S-transferases (GSTs), and/or carboxyl-esterases (COEs) mediated metabolic detoxification. Because a susceptible *L. maculicollis* laboratory strain does not exist, the most susceptible field-collected population (New Haven) provided a baseline against which all other populations were compared. In the population with the lowest resistance (Norwich), only detoxification by P450s was significant. Detoxification in the population with the second highest level of resistance (Stamford) involved both P450s and GSTs. Detoxification in the population with the highest level of resistance (Hartford) involved P450s, GSTs, and COEs. This study suggests that enzyme-mediated metabolic detoxification plays an important role in annual bluegrass weevil pyrethroid resistance.

KEY WORDS cytochrome P450, carboxyl-esterases, glutathione S-transferase, synergists

The “annual bluegrass weevil” *Listronotus maculicollis* Kirby (Coleoptera: Curculionidae), is a pest of highly maintained annual bluegrass *Poa annua* L. (Poaceae) found on many golf courses in the northeastern United States (Potter 1998, Vittum et al. 1999, Vargas and Turgeon 2004). Chlorinated hydrocarbons were used to manage weevil populations until around 1969, and throughout the 1970s and 1980s, less persistent organophosphates were deployed (Schread 1970, Cameron and Johnson 1971, Tashiro 1976, Vittum 1999). Since the early 1990s, when pyrethroids became widely available for use on turf, they have been the most effective option for control of *L. maculicollis*. The strategy has been to intercept adult weevils with pyrethroid residues as they emerge from overwintering sites (Cowles et al. 2008). Although pyrethroids are preferred by many golf course superintendents for managing this species, in 2009, Ramoutar et al. (2009) confirmed pyrethroid (bifenthrin and *H*9261-cyhalothrin) resistance in adults of seven *L. maculicollis* populations from Connecticut, Rhode Island, and Massachusetts. The mechanisms responsible for conferring this resistance were unknown at the time.

In most cases, pyrethroid resistance can be linked to insecticide metabolic detoxification through interaction with endogenous enzymes (Casida 1970, Brattsten et al. 1986). The best known of these enzymes, the cytochrome P450 monooxgenases (P450s or mixed function oxidases), the glutathione S-transferases (GSTs), and the carboxyl-esterases (COEs), sequester and/or render insecticides water soluble. These enzymes bind and metabolize lipophilic substrates, for which their original function may include the synthesis and degradation of steroids and molting and juvenile hormones and in the metabolism of pheromones (Bergé et al. 1998). According to Gould (1984), P450s, GSTs, and COEs evolved additional functions in insects detoxifying secondary plant substances such as terpenes and alkaloids, many of which are lipophilic. Pyrethroids are also lipophilic and are thus prone to increased metabolic detoxification (Dauterman and Hodgson 1978, Wilkinson 1983, Bergé et al. 1998).

The P450s and COEs inactivate pyrethroids by hydrolyzing the chemical’s central ester bond (Soderlund et al. 1983, Heidari et al. 2005). GSTs play an important role in detoxifying pyrethroid-induced lipid peroxides and by sequestering pyrethroids or their metabolites (Kostaropoulos et al. 2001, Vontas et al. 2001). The P450s have been implicated as major factors in detoxifying several classes of insecticides, including pyrethroids (Scott 1999, Ranson et al. 2002).

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and P450s are more commonly linked to pyrethroid resistance than GSTs and COEs (Soderlund and Bloomquist 1991).

The involvement of the P450s, GSTs, and COEs in insecticide resistance can be shown with inhibitors of these enzymes, also known as insecticide synergists. The most widely used P450, GST, and COE inhibitors are piperonyl butoxide (PBO) (Hodgson and Kulkarni 1983, Bergé et al. 1998), diethyl maleate (DEM) (Zhu et al. 2007), and S,S,S-tributyl phosphorothioate (DEF) (Soderlund and Bloomquist 1991), respectively. PBO may also block COE (Young et al. 2005), and DEF may partially block P450 enzymes (Pridgeon et al. 2002). If the application of a synergist with an insecticide results in increased insecticide toxicity, the enzyme blocked by that synergist is implicated in resistance to that insecticide.

In this study, topical application bioassays with bifenthrin and bifenthrin combined with the synergists PBO, DEM, and DEF were conducted on four field-collected populations of adult annual bluegrass weevils from Connecticut to determine if P450s, GSTs, and COEs mediated metabolic detoxification. For each population, LD₅₀ values were determined with and without synergist exposure and compared. Because pyrethroid resistance has emerged as a serious hurdle to adequately managing insecticide resistance can be shown with inhibitors of these enzymes, also known as insecticide synergists.

Materials and Methods

Insects. Adult annual bluegrass weevils were collected from four golf courses in Connecticut (Hartford, second generation; New Haven, first generation; Norwich, first generation; Stamford, overwintered generation). Because we did not have a susceptible laboratory strain, the field-collected population that showed the lowest LD₅₀ to bifenthrin (New Haven) provided a baseline against which all other populations were compared. Weevils were collected during the summer of 2008 by hand from golf course fairways or greens and kept at 21–23°C in P. annua plugs until bioassays were performed. Insect bioassays were conducted within 24 h of collection.

Chemicals. The technical grade insecticide used in this study was bifenthrin (>95% purity; Sigma-Aldrich, St. Louis, MO). The technical grade synergists used in this study were PBO (90% purity; TCI America, Portland, OR), DEM (97% purity; Sigma-Aldrich), and DEF (98% purity; Chem Services, West Chester, PA). Insecticides and synergists were dissolved in reagent quality acetone (>95% purity; Sigma-Aldrich). Tween 20 (ICI Americas, Wilmington, DE) was used as an emulsifier.

LD₅₀ Bioassays With Bifenthrin. Dose–mortality lines for each treatment were estimated from 6 to 13 doses (0.0005- to 2.0-ng/ml concentrations expressed as ng/insect) using 10–15 unsexed adults per dose. Bifenthrin/acetone solutions were topically applied in 1 µl per insect dorsally to the intersegmental membrane between the prothorax and the elytra. Applications were made using a Burkhard microapplicator (Burkhard Mfg., Rickmansworth, United Kingdom) equipped with a 1-ml tuberculin syringe. Control insects were treated with acetone. After dosing, adults were placed in 100 by 15-mm petri dishes with 9-cm-diameter filter paper and held at 20 ± 2°C for 24 h, after which mortality was recorded. Insects were counted as dead if they displayed no movement when probed. The LD₅₀ for each treatment and population was determined and compared. Bioassays were replicated twice and results were pooled across replicates. Control mortality was ≤20%, and mortality at the highest dose was ≥85%.

LD₅₀ Bioassays With Bifenthrin and Synergists. In addition to populations being tested with bifenthrin alone, they were also tested against bifenthrin in combination with PBO, DEM, or DEF. Ninety microliters of each synergist was mixed with 100 ml of distilled water (900 parts per million [ppm]) and 50 µl of Tween 20, which acted as an emulsifier. Nine hundred parts per million of each synergist was used because it is analogous to the rate of PBO found in several commercially available products. One milliliter of each synergist/water/Tween 20 solution was added to 9-cm-diameter filter paper that was placed in a 100 by 15-mm petri dish. Insects were put on the synergist-treated filter paper while it was still wet and kept there at 20 ± 2°C for 12 h with food (blades of P. annua). After 12 h, insects were treated with bifenthrin as previously described and returned to the synergist-treated filter paper and kept at 20 ± 2°C for 24 h without food, after which they were scored for mortality as previously described. Bioassays were replicated twice, and results were pooled across replicates. Control mortality was ≤20%, and mortality at the highest dose was ≥85%.

Statistical Analysis. Mortality data were subjected to probit analysis using the Statistical Analysis System Version 9.1 program PROC PROBIT (SAS Institute 2003). When comparing LD₅₀ values, a failure of 95% CL to overlap determined significant differences between populations or between comparisons of bioassays with and without synergists (Robertson and Preiser 1992). Because we did not have a standard susceptible laboratory strain, the most sensitive field population was used for calculating resistance ratios (resistance ratio [RR] = LD₅₀-resistant population/LD₅₀-most susceptible population) (Perez-Mendoza 1999). In all cases, the likelihood ratio (LR) χ² goodness-of-fit values indicated that the data adequately conformed to the probit model (Robertson and Preiser 1992).

Results

The New Haven population, which was the most susceptible to bifenthrin, exhibited no evidence of metabolic detoxification. Reduction in the LD₅₀ of bifenthrin alone (from 3.1 to 2.2 ng/insect, yielding a synergist ratio [SR] of 1.4) was seen when this population was exposed to DEF but not from exposure to PBO or DEM (LD₅₀ of 3.9 and 5.7 ng/insect, respec-
duced sensitivity to bifenthrin and the most susceptible population that was found in New Haven, CT, exhibit low to high levels of pyrethroid resistance compared with the Seekonk, MA, and Rumford, RI, exhibited low to high levels of pyrethroid resistance. The Stamford, West Hartford, and Willimantic, CT, populations, the precise role of insecticide use in resistance development is unknown. In this study, the New Haven population was compared with the Norwich, Stamford, and West Hartford populations because the levels of pyrethroid susceptibility for these groups had been previously determined. In this case, the pattern of resistance among this group was similar to the findings of Ramoutar et al. (2009), as measured the previous year. Because biochemical detoxification by enzymes such as the P450s, COEs, and GSTs are major causes of insecticide resistance in several species (Wilkinson 1983, Brattsten et al. 1986), it was probable that these systems could have a role in L. maculicollis pyrethroid resistance.

The biosynthesis of the P450s, GSTs, and COEs can either be induced in insects by the presence of substrates such as insecticides or are constitutively expressed at various levels (Brattsten et al. 1986, Berenbaum et al. 1990). In either case, they can metabolize toxins before they reach their target site (Bergé et al. 1998). Selective pressure may maintain lower levels of detoxifying enzymes than are found in resistant populations because increased enzyme activity may adversely affect metabolism of important hormonal molecules (Hodgson and Kulkarni 1983, Cuany et al. 1998). Selective pressure may maintain lower levels of pyrethroid resistance among these populations, the precise role of insecticide use in resistance development is unknown. In this study, the New Haven population was compared with the Norwich, Stamford, and West Hartford populations because the levels of pyrethroid susceptibility for these groups had been previously determined. In this case, the pattern of resistance among this group was similar to the findings of Ramoutar et al. (2009), as measured the previous year. Because biochemical detoxification by enzymes such as the P450s, COEs, and GSTs are major causes of insecticide resistance in several species (Wilkinson 1983, Brattsten et al. 1986), it was probable that these systems could have a role in L. maculicollis pyrethroid resistance.

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**Table 1.** LD<sub>50</sub> for adult L. maculicollis populations for bifenthrin and bifenthrin/synergist combinations

<table>
<thead>
<tr>
<th>Population</th>
<th>Treatment</th>
<th>N</th>
<th>Slope (SE)</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</th>
<th>χ&lt;sup&gt;2&lt;/sup&gt; (df)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>95% FL</th>
<th>RR&lt;sup&gt;c&lt;/sup&gt;</th>
<th>SR&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>New Haven</td>
<td>Bifenthrin</td>
<td>180</td>
<td>3.3 (0.6)</td>
<td>3.1a</td>
<td>4.7 (3)</td>
<td>2.4–3.8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Bifenthrin + PBO</td>
<td>140</td>
<td>2.4 (0.5)</td>
<td>3.9</td>
<td>1.2 (4)</td>
<td>2.7–5.6</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Bifenthrin + DEM</td>
<td>140</td>
<td>1.7 (0.3)</td>
<td>5.7</td>
<td>3.7 (4)</td>
<td>3.7–9.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Bifenthrin + DEF</td>
<td>140</td>
<td>1.7 (0.3)</td>
<td>2.2</td>
<td>1.9 (4)</td>
<td>1.4–3.3</td>
<td>1.4</td>
<td>—</td>
</tr>
<tr>
<td>Norwich</td>
<td>Bifenthrin</td>
<td>140</td>
<td>1.7 (0.3)</td>
<td>25.3b</td>
<td>5.7 (4)</td>
<td>14.9–48.5</td>
<td>8.2</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Bifenthrin + PBO</td>
<td>140</td>
<td>1.1 (0.3)</td>
<td>4.9</td>
<td>2.5 (4)</td>
<td>1.7–8.8</td>
<td>5.2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Bifenthrin + DEM</td>
<td>140</td>
<td>0.9 (0.2)</td>
<td>8.7</td>
<td>5.1 (4)</td>
<td>3.1–16.8</td>
<td>2.8</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Bifenthrin + DEF</td>
<td>210</td>
<td>1.4 (0.2)</td>
<td>26.9</td>
<td>4.3 (4)</td>
<td>18.6–40.5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Stamford</td>
<td>Bifenthrin</td>
<td>216</td>
<td>3.5 (0.9)</td>
<td>57.1e</td>
<td>3.2 (6)</td>
<td>66.3–126</td>
<td>28.1</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Bifenthrin + PBO</td>
<td>216</td>
<td>2.3 (0.3)</td>
<td>14.6</td>
<td>8.3 (5)</td>
<td>10.6–19.4</td>
<td>5.9&lt;sup&gt;f&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Bifenthrin + DEM</td>
<td>210</td>
<td>2.1 (0.4)</td>
<td>27.4</td>
<td>3.7 (5)</td>
<td>18.8–37.9</td>
<td>3.2&lt;sup&gt;g&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Bifenthrin + DEF</td>
<td>216</td>
<td>1.7 (0.3)</td>
<td>56.6</td>
<td>10.5 (5)</td>
<td>38.9–85.3</td>
<td>1.5</td>
<td>—</td>
</tr>
<tr>
<td>Hartford</td>
<td>Bifenthrin</td>
<td>220</td>
<td>1.9 (0.3)</td>
<td>638d</td>
<td>9.7 (13)</td>
<td>468–824</td>
<td>206</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Bifenthrin + PBO</td>
<td>220</td>
<td>1.4 (0.2)</td>
<td>143</td>
<td>4.4 (8)</td>
<td>84.6–224</td>
<td>4.5&lt;sup&gt;g&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Bifenthrin + DEM</td>
<td>220</td>
<td>1.0 (0.2)</td>
<td>260</td>
<td>6.7 (8)</td>
<td>144–457</td>
<td>2.5&lt;sup&gt;g&lt;/sup&gt;</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Bifenthrin + DEF</td>
<td>220</td>
<td>1.5 (0.3)</td>
<td>199</td>
<td>6.1 (8)</td>
<td>109–309</td>
<td>3.2&lt;sup&gt;g&lt;/sup&gt;</td>
<td>—</td>
</tr>
</tbody>
</table>

<sup>a</sup> LD<sub>50</sub> (ng/insect) followed by different letters are significantly different (P = 0.05) for unsynergized bifenthrin treatment compared among the four populations; significant difference is based on a failure of 95% fiducial limit overlap.

<sup>b</sup> LR χ<sup>2</sup> goodness-of-fit values. Tabular values at P = 0.05: 3 df, 7.82; 4 df, 9.49; 5 df, 11.07; 6 df, 12.59; 8 df, 15.51; and 13 df, 22.36.

<sup>c</sup> Resistance ratio: LD<sub>50</sub> resistant population/LD<sub>50</sub> most susceptible population.

<sup>d</sup> Synergism ratio: LD<sub>50</sub> bifenthrin alone/LD<sub>50</sub> bifenthrin + synergist, either PBO, DEM, or DEF.

<sup>e</sup> Synergism ratio is significant, P = 0.05 (LD<sub>50</sub> within a population for bifenthrin + synergist is significantly lower than the LD<sub>50</sub> for bifenthrin; significant difference is based on lack of 95% fiducial limit overlap).

Discussion

In 2009, Ramoutar et al. (2009) found that seven L. maculicollis adult populations from Bloomfield, Norwich, Stamford, West Hartford, and Willimantic, CT, Seekonk, MA, and Rumford, RI, exhibited low to high levels of pyrethroid resistance compared with the most susceptible population that was found in New Haven, CT. The resistant populations exhibited reduced sensitivity to bifenthrin and λ-cyhalothrin, type 1 and type 2 pyrethroids, respectively, and shared a history of high chemical exposure from several insecticide classes. Although there may be a correlation between insecticide exposure and the development of L. maculicollis pyrethroid resistance among these populations, the precise role of insecticide use in resistance development is unknown. In this study, the New Haven population was compared with the Norwich, Stamford, and West Hartford populations because the levels of pyrethroid susceptibility for these groups had been previously determined. In this case, the pattern of resistance among this group was similar to the findings of Ramoutar et al. (2009), as measured the previous year. Because biochemical detoxification by enzymes such as the P450s, COEs, and GSTs are major causes of insecticide resistance in several species (Wilkinson 1983, Brattsten et al. 1986), it was probable that these systems could have a role in L. maculicollis pyrethroid resistance.

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tible populations (Fragoso et al. 2007) and were at an overall fitness disadvantage (Oliveira et al. 2007).

In this study, the New Haven population exhibited the greatest susceptibility to bifenthrin and did not exhibit significantly increased susceptibility when exposed to any of the synergists. This outcome may have been because of the low levels of detoxifying enzymes expected in such a susceptible population. However, not all forms of P450s are equally susceptible to inhibition by PBO, so lack of synergism may not always reflect a lack of metabolic detoxification (Soderlund and Bloomquist 1991).

We anticipated a significant increase in bifenthrin toxicity in the resistant populations after exposure to PBO, a P450 inhibitor. The P450s comprise several groups of ubiquitous enzymes with nearly 100 forms identified in insects and are commonly associated with pyrethroid resistance (Scott 1999). As expected, all three bifenthrin-resistant populations studied here, Norwich, Stamford, and Hartford, showed a significant increase in bifenthrin toxicity when exposed to PBO, implicating the cytochrome P450 system as a major factor in conferring bifenthrin resistance to L. maculicollis adults.

Recent research has implicated GST content in pyrethroid resistance in the Curculionidae (Fragoso et al. 2007). Transferases may play an important role in pyrethroid resistance by detoxifying lipid peroxidation products that are induced by the pyrethroids and can lead to oxidative damage and stress (Vontas et al. 2001) or in the sequestration of the insecticide (Kostaropoulos et al. 2001). Additionally, GSTs are capable of catalyzing the secondary metabolism of substrates after P450 oxidation (Wilce and Parker 1994) and higher GST activity parallels higher P450 activity in many resistant insect strains (Dauterman and Hodgson 1978). In this study, the Stamford and Hartford populations exhibited a significant increase in bifenthrin toxicity when exposed to DEM, a GST inhibitor, and PBO, a P450 inhibitor. Although the Norwich population showed a significant increase in bifenthrin toxicity when exposed to PBO, it did exhibit a nonsignificant increase in bifenthrin toxicity when exposed to DEM (SR = 2.8). In the resistant populations we studied, both the P450s and GSTs seem to have roles in bifenthrin metabolism.

Carboxylesterases are major factors in malathion (organophosphate) resistance but are less common in pyrethroid resistance. Enhanced esterase activity has rarely been linked to pyrethroid resistance (Soderlund and Bloomquist 1991), even though esterases-dependent cross-resistance between organophosphates and pyrethroids has been recorded in the green peach aphid, Myzus persicae (Devonshire and Moores 1982). B-type Bemisia tabaci (Young et al. 2006). Chinese populations of the common cutworm, Spodoptera litura (Huang and Han 2007), and both West African and Australian populations of the cotton bollworm Helicoverpa armigera (Gunning et al. 1999, Martin et al. 2002) exhibit evidence of increased esterase activity conferring pyrethroid resistance. This phenomenon remains uncommon among weevil species other than pests of stored grain products. In the Curculionidae, Brazilian populations of the maize weevil exhibit enhanced esterase activity that may be linked to pyrethroid resistance (Ribeiro et al. 2003).

In this study, significant esterase activity was detected in the Hartford population, which also showed the highest level of resistance (resistance ratio of 206; Table 1). Esterase activity may be involved in conferring bifenthrin resistance to the Norwich and Stamford populations, but at lower levels than for the Hartford population. Based on our data, we suggest that highly resistant annual bluegrass weevils, such as those found at Hartford, use all available enzyme detoxifying systems discussed, including the esterases.

The role of enzyme-mediated insecticide metabolic detoxification resulting in pyrethroid resistance has been widely documented from several insect orders. For the L. maculicollis populations studied here, enzyme mediated detoxification plays a role in resistance to bifenthrin. As resistance level increases, the involvement of enzyme-mediated detoxification systems increases as well. Because enhanced cytochrome P450 enzyme activity is implicated among all the pyrethroid-resistant L. maculicollis populations, and other detoxification enzymes do not seem to have significantly enhanced activity in the absence of elevated P450 activity, selection for greater oxidative metabolism may be a critical step for pyrethroid resistance evolution in L. maculicollis.

Intriguingly, all of the superintendents of the golf courses represented in this study have relied on pyrethroids to manage L. maculicollis, starting with fluvinate in 1987. Variations in mixing pyrethroids with other products could have influenced the degree to which pyrethroid resistance has evolved at each golf course. In the late 1980s, fluvinate often was used in combination with chlorpyrifos. Chlorpyrifos toxicity is enhanced by its activation to chlorpyrifos-oxon by P450 enzymes, which would counteract selection for greater P450 activity by pyrethroids. In house flies, the CYP6D1 form of the P450 enzyme is implicated both in pyrethroid metabolism and in activation of chlorpyrifos (Scott et al. 1998); therefore, through competitive inhibition by chlorpyrifos or its oxon, blocking this P450 could prevent pyrethroid metabolism. More recently, superintendents may have combined pyrethroids (typically bifenthrin or λ-cyhalothrin) with propiconazole, a fungicide that, along with many other substituted imidazoles, is a potent insecticide synergist because it blocks the P450 enzymes (Wilkinson et al. 1974; R.S.C., unpublished data).

The shown link between the role of multiple enzyme systems in the evolution of pyrethroid resistance in L. maculicollis is shared by other insects for which the range of selected traits has been studied (David et al. 2005). The potential role for nonenzymatic modes for resistance, such as altered cuticular penetration and target site insensitivity traits, remains to be studied in L. maculicollis. It is apparent that the intense selection for resistance with pyrethroids poses special challenges for future chemical-based management, because enhancement of general purpose detoxifica-
tion enzyme systems could influence the performance of other, chemically unrelated compounds.

Acknowledgments

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