Research Reports

Detection of Bispyribac-sodium Residues in Walnut Leaves after Simulated Drift

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SUMMARY. Walnut (*Juglans regia*) and rice (*Oryza sativa*) are among the most important crops grown in the Sacramento Valley of California. Because rice herbicides are often applied by air, there are occasional allegations of rice herbicide drift onto walnut trees. This study was established to investigate bispyribac-sodium residues on walnut leaves after simulated drift treatments. The objectives were to determine whether bispyribac-sodium can generate visual symptoms on walnut trees without leaving detectable residues in leaf tissues and to evaluate the subsequent impacts on walnut yield. Two experiments were conducted in a 3-year-old walnut orchard. In the first experiment bispyribac-sodium was applied to walnut trees at 0.125%, 0.25%, 0.5%, and 1% of the normal use rate in rice (45 g·ha⁻¹). In the second experiment, rates were 1%, 3%, 10%, and 100% of the normal use rate in rice. Bispyribac-sodium caused general leaf chlorosis and discrete yellow spotting on walnut leaves even at very low concentrations; symptoms were recorded on trees exposed to rates as low as 0.125% of the normal use rate in rice. However, based on high-performance liquid chromatography analysis, the lowest simulated drift treatment from which bispyribac-sodium could be detected 10 d after treatment was 1% of the rice use rate. In general, visual injury symptoms remained constant over time, or even worsened, whereas bispyribac-sodium residues decreased or became not detectable. There was no measurable impact on walnut yield from any of the simulated drift treatments in these experiments.

The Sacramento Valley of California is a diverse cropping region where rotational field crops

and fruit and nut trees are grown in close proximity. Walnut is one of the most important crops with a total of ≈143,000 acres for a gross dollar value

of ≈\$800 million reported in 2015 in the region [California Department of Food and Agriculture (CDFA), 2016; U.S. Department of Agriculture (USDA), 2016]. In addition, nearly all of the California rice production area is based in the Sacramento Valley, with 410,000 acres harvested in 2015 for a gross dollar value of about \$732 million (CDFA, 2016).

Weeds can greatly reduce rice yields (Hill et al., 2006); in California, weed management programs almost entirely rely on the use of herbicides (Fischer et al., 2010). Generally, California rice growers apply herbicides at planting and follow up with one or two additional postemergence applications later in the season. The majority of rice herbicide applications are made by airplane between May and early July (California Department of Pesticide Regulation, 2016). During this time of the year, walnut trees are actively growing, initiating and differentiating buds that will produce vegetative shoots and flowers in the subsequent year (Sabatier et al., 2003).

In recent years, complaints of damage from rice herbicides allegedly drifting into young walnut orchards have been reported in the Sacramento Valley (J.W. Beauchamp, personal communication). Symptoms observed are consistent with acetolactate synthase (ALS) inhibitor herbicide damage: leaf chlorosis, chlorotic spots, and internode shortening (Al-Khatib, 2015).

The effects of ALS inhibitor herbicides on off-target crops have been widely studied in annual and perennial crops (Al-Khatib and Tamhane, 1999; Al-Khatib et al., 1992, 1993; Boutin et al., 2000; Fletcher et al., 1996; Hensley et al., 2012; Rana et al., 2014). For example, high concentrations of chlorsulfuron during

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Units			
To convert U.S. to SI, multiply by	U.S. unit	SI unit	To convert SI to U.S., multiply by
0.4047	acre(s)	ha	2.4711
29,574	fl oz	μL	3.3814 × 10 ⁻⁵
29.5735	fl oz	mL	0.0338
73.0778	fl oz/acre	mL·ha ⁻¹	0.0137
9.3540	gal/acre	L·ha ⁻¹	0.1069
2.54	inch(es)	cm	0.3937
1.6093	mph	km·h ⁻¹	0.6214
28.3495	oz	g	0.0353
70.0532	oz/acre	g·ha ⁻¹	0.0143
1	ppb	μg·kg ⁻¹	1
6.8948	psi	kPa	0.1450
(°F - 32) ÷ 1.8	°F	°C	(°C × 1.8) + 32

reproductive growth phase reduced sweet cherry (*Prunus avium*) fruit production (Bhatti et al., 1995).

Bispyribac-sodium is widely used in California rice fields for the control of grass species such as early watergrass (*Echinochloa oryzoides*), late watergrass (*Echinochloa oryzicola*), barnyardgrass (*Echinochloa crus-galli*), and broadleaf species such as arrowhead (*Sagittaria montevidensis*), red-stem (*Ammannia* sp.), monochoria (*Monochoria vaginalis*), and ducksalad (*Heteranthera limosa*) (Fischer et al., 2004). Although many rice herbicides in California are applied in a granular form to minimize drift, bispyribac-sodium is applied in liquid spray mixtures. Evaluating simulated drift rates of three of the most commonly used rice herbicides in the Sacramento Valley (bispyribac-sodium, bensulfuron-methyl, and propanil), Galla et al. (2018a) identified bispyribac-sodium, an ALS inhibitor herbicide, as the herbicide with higher impact potential on walnut trees. Bispyribac-sodium simulated drift rates slowed walnut shoot growth and were negatively correlated with walnut kernel quality (Galla et al., 2018a). These results were confirmed in a subsequent study on the effect of multiple exposure of simulated drift rates of bispyribac-sodium on walnut (Galla et al., 2018b).

Laboratory analysis of walnut leaf samples displaying ALS-inhibitor symptoms usually does not detect bispyribac-sodium residues (J.W. Beauchamp, personal communication). Lack of detection could simply mean that the observed symptoms are not caused by bispyribac-sodium. However, it could also be related to the analytical limits of detection and the low exposure levels because typical downwind drift ranges from 1% to 8% or even lower (Al-Khatib and Peterson, 1999). It is also possible that bispyribac-sodium residues are metabolized by walnut leaves before symptoms develop sufficiently for a grower or consultant to notice and collect samples for analysis.

Previous research on bispyribac-sodium drift on walnut trees was based on visible injury symptoms (Galla et al., 2018a, 2018b), but to our knowledge, there are no data available on the detection of bispyribac-sodium on walnut leaves after a drift event occurs. Therefore, the objectives of this research were 1) to estimate the level of drift

exposure necessary to generate detectable bispyribac-sodium residues in walnut leaves and 2) to determine whether there is a correlation between symptoms, yield, and bispyribac-sodium residues on leaf tissue.

Materials and methods

The study was conducted in an experimental orchard at the University of California (UC) Davis Plant Science Field Station, near Davis, CA. Walnut trees (cultivar Chandler) were 3 years old and grafted on Paradox (*Juglans hindsii* × *Juglans regia*) rootstock. The soil was classified as Yolo silt loam and Reiff very fine sandy loam (USDA, 2015). Trees were spaced 6 m within and 6 m between rows. Trees were monitored for insects and diseases and were treated according to the UC Cooperative Extension Walnut Pest Management Guidelines (Strand, 2003). Weeds in the experimental area were managed with a combination of preemergent and postemergent herbicides. No in-season ALS inhibitors or glyphosate were used to avoid the confounding effects of other amino acid inhibiting herbicides. Trees were irrigated and fertilized with microsprinklers through the growing season.

Two experiments were conducted to correlate the levels of observed symptoms with bispyribac-sodium concentrations in walnut leaves. The experiments were established as a randomized complete block with three replicates with single trees as experimental units. In the first experiment, bispyribac-sodium (Regiment®; Valent USA, Walnut Creek, CA) was applied on 9 May 2017 at four plausible simulated drift rates (Al-Khatib and Peterson, 1999): 0.125%, 0.25%, 0.5%, and 1% of the normal use rate in rice (45 g·ha⁻¹). In the second experiment, bispyribac-sodium was applied on 19 June 2017 at 1%, 3%, 10%, and 100% of the normal use rate in rice, to simulate drift, sprayer contamination, and accidental overspray scenarios. Nonionic surfactant (Broadspread®; Custom Ag Formulators, Fresno, CA) was added at 1.6 fluid oz/acre to all treatments. Although not fully mimicking droplet and concentration dynamics of herbicide drift, treatments were applied at constant volume with low doses of herbicide to ensure reproducibility and accuracy of the treatments (Marple et al., 2008;

Rana et al., 2014). A nontreated control was also included. The two experiments were conducted in different sites to avoid possible drift contamination between single trees.

Both sites were sprayed during the time window in which rice growers generally apply their herbicide treatments (California Department of Pesticide Regulation, 2016). In both experiments, trees were treated at the same growing stage: internode elongation following pistillate flowering. Bispyribac-sodium treatments were applied to one side of the canopy, by a researcher using a hand-held carbon dioxide (CO₂)-pressurized boom held vertically. The sprayer was equipped with three 8001 flat fan tips (Teejet Technologies, Wheaton, IL) spaced 20 inches apart and was calibrated to deliver 10 gal/acre at 20 psi. Environmental conditions were as follows: 21 °C, 57% relative humidity (RH), and 1 mph wind speed on 9 May 2017 and 31 °C, 4% RH, and 1 mph wind speed on 19 June 2017.

On the basis of previous experience, injury was estimated visually 10, 20, and 40 d after treatment (DAT). Visual injury estimates were based on a scale from 0 (no injury) to 100 (tree canopy completely necrotic and/or defoliated). At the same time, six leaflets were collected by hand from each plot from the side of the tree directly exposed to the treatment. To simulate conventional practice in field investigations, we intended to sample only leaflets with visible symptoms. However, because symptoms developed slowly, particularly at very low rates, no symptoms were observed 10 DAT. If no symptoms were visible, leaflets were randomly sampled from the side of the canopy directly exposed to the drift-simulation spray. To avoid contamination during sampling, plots were sampled in the order from the lowest to the highest rate, and researchers used a new pair of gloves for each treatment. Samples were double-bagged, kept on ice, and frozen at -20 °C until further processing.

Walnuts were harvested from both experiments on 20 Oct. 2017. Nuts were shaken from the trees using rubber mallets and poles, handpicked, and separated from the hulls. Nuts were subsequently dried at 26 °C for 72 h and total in-shell nut weight and number of nuts per tree were recorded.

ANALYTICAL METHODS. Walnut leaf samples were rinsed with distilled water and ground in a mortar with liquid nitrogen. Subsequently, the ground tissue was stored at -80°C in a cryofreezer.

Samples were prepared for analysis as follows: 1.0 g of homogenized sample was added to a test tube and soaked for 1 h in 10 mL of a solution consisting of 4 μmol ammonium acetate in high-performance liquid chromatography (HPLC)-grade methanol fortified with 0.1% formic acid. The test tube was vortexed for 1 min and centrifuged at 2322 g_n for 5 min.

Analytical methods were developed and conducted by a commercial laboratory (Environmental Micro Analysis Laboratory, Woodland, CA). Reverse-phase HPLC analyses were conducted with a HPLC system equipped with an autosampler (1290; Agilent, Santa Clara, CA) using a kinetic column [50 $\times$ 4.60 mm, 2.6 μm (C18) (Phenomenex, Torrance, CA)]. The mobile phase used was a gradient of A (4 mmol ammonium formate and 0.1% formic acid in water) and B (4 mmol ammonium formate and 0.1% formic acid in methanol). The initial composition of the mobile phase was 95% A:5% B, which ramped up to 100% B over 10 min, held at 100% B for 14 min, tapered back to 95% A over 0.1 min, and held at 95% A for the final 10 min. Injection volume was 3.0 μL , flow rate 0.50 $\text{mL}\cdot\text{min}^{-1}$ and the column temperature was maintained at 25°C .

Mass spectrometric detection was conducted using a liquid chromatography triple quadrupole mass spectrometry system with atmospheric pressure chemical ionization (API 4000; AB SCIEX, Framingham, MA) in electrospray positive-ion multiple reaction mode. Source-dependent parameters were as follows: 5500 V ion spray voltage, 40 psi atomization air pressure (GS1), 60 psi auxiliary gas (GS2), 30 psi curtain gas (CUR), 500°C ion source temperature (TEM), 5 V collision-activated dissociation (CAD). Limit of detection was 10 ppb.

STATISTICAL ANALYSIS. Visual injury estimates were subjected to analysis of variance (ANOVA), using lmerTest package in R (Kuznetsova et al., 2016), considering block as random effects. ANOVA statistical assumptions were tested before performing the analysis. Means were separated according to Tukey's test at 5% level

of probability, using multcomp package in R (Hothorn et al., 2008). For 1% and higher rates, the relationships between visual injury estimates and bispyribac-sodium residues and between yield and bispyribac-sodium residues were evaluated using Pearson's correlation test using R (R Core Team, 2017).

Results and Discussion

VISUAL SYMPTOMS. All bispyribac-sodium rates tested caused injury on walnut trees similar to symptoms previously reported and included leaf chlorosis, stunted growth and internode shortening in young shoots (Al-Khatib, 2015). In addition, veins of leaves exposed to the 100% rate started turning purple ≈ 20 DAT. Generally, trees at the site treated on 9 May 2017 had more severe symptoms than the experiment initiated 1 month later. The walnut leaves treated on 9 May were at a younger stage and thus may have been more sensitive to ALS herbicide damage. In addition, average air temperatures at the time of the second experiment treatment were significantly higher than long-term averages (California Irrigation Management Information System, 2017), which may also have

influenced walnut response to the herbicide.

Bispyribac-sodium caused injury on walnut leaves at very low concentrations, with symptoms observed on trees exposed to rates as low as 0.125% of the full rice use rate (Table 1). In the second experiment, where higher doses were tested, symptoms appeared ≈ 1 week after application and peaked 20 DAT (Table 2). By 40 DAT, all trees exposed to bispyribac-sodium rates 3% or lower were recovering and had less visual injury compared with the earlier assessments. Conversely, the 100% rate took longer to cause the peak of symptom severity. At 10 DAT, no difference in injury ratings was observed among treatments, however, by 20 DAT, the full bispyribac-sodium use rate caused the highest level of injury and symptoms continued to worsen with 55% visual injury recorded 40 DAT.

LEAF RESIDUES. No residues were detected in walnut leaf tissues sampled from trees exposed to rates lower than 1% (data not shown), suggesting that walnut trees are so sensitive to bispyribac-sodium that levels below the analytical detection limit are still sufficient to cause symptoms.

Table 1. Visual injury ratings of walnut trees treated with simulated drift rates of bispyribac-sodium applied on 9 May 2017 at the University of California Davis Plant Sciences Field Station near Davis, CA. Means are averages of three replicates.

Bispyribac-sodium rate (%) ^a	Mean visual injury (%)	
	10 DAT	20 DAT
0.125	1 a ^b	1 a
0.25	2 a	2 a
0.5	2 a	3 a
1.0	12 b	15 b

^aRate expressed as percentage of the full rice use rate: 45 $\text{g}\cdot\text{ha}^{-1}$ (0.64 oz/acre). Nonionic surfactant at 1.6 fl oz/acre (116.9 $\text{mL}\cdot\text{ha}^{-1}$) was added to all treatments.

^bMeans followed by same letter within a column are not statistically different according to Tukey's test ($P < 0.05$). DAT = days after treatment.

Table 2. Visual injury ratings of walnut trees treated with simulated drift rates of bispyribac-sodium applied on 19 June 2017 at the University of California Davis Plant Sciences Field station near Davis, CA. Means are average of three replicates.

Bispyribac-sodium rate (%) ^a	Mean visual injury (%)		
	10 DAT	20 DAT	40 DAT
1	6 a ^b	2 a	1 a
3	12 a	13 a	10 a
10	21 a	22 a	22 b
100	15 a	44 b	55 c

^aRate expressed as percentage of the full rice use rate: 45 $\text{g}\cdot\text{ha}^{-1}$ (0.64 oz/acre). Nonionic surfactant at 11.6 fl oz/acre (116.9 $\text{mL}\cdot\text{ha}^{-1}$) was added to all treatments.

^bMeans followed by same letter within a column are not statistically different according to Tukey's test ($P < 0.05$). DAT = days after treatment.

Table 3. Bispyribac-sodium residues in walnut leaf tissue following simulated drift treatments applied on 19 June 2017 at the University of California Davis Plant Sciences Field Station near Davis, CA.

Bispyribac-sodium rate (%) ^z	DAT	Residues (ppb)		
		Rep 1	Rep 2	Rep 3
1	10	10	ND	ND
	20	ND	ND	ND
	40	ND	ND	ND
3	10	ND	10	70
	20	ND	20	ND
	40	ND	ND	10
10	10	90	60	60
	20	60	20	40
	40	40	20	30
100	10	100	570	230
	20	360	170	420
	40	40	120	330

^zRate expressed as percentage of the full rice use rate: 45 g·ha⁻¹ (0.64 oz/acre). Nonionic surfactant at 1.6 fl oz/acre (116.9 mL·ha⁻¹) was added to all treatments.

DAT = days after treatment; Rep = replicate; ND = not detectable at the analytical limit of detection of 10 ppb; 1 ppb = 1 µg·kg⁻¹.

Fig. 1. Walnut leaves presenting typical symptoms caused by 0.5% of bispyribac-sodium rice use rate, 45 g·ha⁻¹ (0.64 oz/acre), 10 d after treatment. It was not possible to detect bispyribac-sodium residues from walnut trees exposed to this rate at any sampling time.

Alternatively, it is possible that the process leading to visible injury occurs relatively quickly but that the herbicide is subsequently metabolized by the plant to levels below the limit of detection (LOD).

The 1% rate was the lowest at which bispyribac-sodium residues were detected 10 DAT (Table 3). By 20 DAT, no residues were detected from samples taken from trees exposed to the 1% rate. Higher levels of bispyribac-sodium residues were detected in samples from the 3% rate at 10 DAT with a median value of 10 ppb and maximum residue amount of 70 ppb (Table 3). By 20 DAT,

bispyribac-sodium median residue value was below the LOD, with a maximum residue of 20 ppb found. Finally, at 40 DAT, the maximum bispyribac-sodium residue detected was 10 ppb but the median value was less than the LOD.

Bispyribac-sodium residues were detected at all sampling timings in leaves taken from trees treated with 10% of the rice use rate. Again, as observed for the 3% rate, residue levels detected declined between sampling intervals. In particular, between 10 and 20 DAT, the median value decreased from 60 to 40 ppb and the maximum value decreased from 90 to 60 ppb. As expected, walnut leaves exposed to the full rice use rate also had the highest level of bispyribac-sodium residues over the duration of the study (Table 3). The level of residues detected in these trees decreased throughout the study, but at 40 DAT, the median residue level was still 40 ppb, and the maximum was 330 ppb.

WALNUT YIELD. Because of the young age of the trees, there was considerable variability in nut yield among the single-tree plots in both experiments. Trees from the second experiment, sprayed on 19 June 2017, appeared to have lower yield compared with the trees used in the first experiment sprayed on 9 May 2017. However, none of the rates tested caused a statistical difference in yield or average nut weight (data not shown) compared with the non-treated control. Although a positive

correlation between bispyribac-sodium residues and visual symptoms 20 DAT (Pearson's $r = 0.67$, $P = 0.02$) and 40 DAT (Pearson's $r = 0.72$, $P = 0.01$) was found, no significant correlation was found between yield and bispyribac-sodium residues or visual injury ratings (data not shown).

This study shows that bispyribac-sodium can cause visual symptoms on walnut leaves even if the herbicide cannot be analytically quantified from symptomatic leaves using methods with a 10 ppb LOD (Fig. 1). The 1% rate was the lowest tested rate that left detectable residues in walnut leaf tissues 10 d after exposure. Over time, symptoms remained constant or even worsened while bispyribac-sodium residues decreased and finally dropped below the analytical limit of detection. In particular, considering the 3% rate, a relatively high but still plausible drift rate (Al-Khatib and Peterson 1999) visual injury remained about constant between 10 and 20 DAT (12% and 13%, respectively), but bispyribac-sodium residues ranged from not detectable to 70 ppb at 10 DAT and decreased to a range of not detectable to 20 ppb at 20 DAT. The same trend was also observed for the 10% and 100% rate, although in the latter case, residues did not decrease substantially until 40 DAT.

Although symptoms generally start to appear within 1 or 2 weeks of exposure, they reached their peak of severity 2 to 3 weeks after treatment. It is plausible, therefore, that in case of suspected drift, a grower or crop consultant may not notice or recognize symptoms for 2 weeks or more after the drift event occurred. By that time, although symptoms could be severe, it might be possible to detect bispyribac-sodium only in leaf tissue exposed to 10% or more of the rice use rate, which is an uncommonly high level of drift (Al-Khatib and Peterson 1999).

In conclusion, drift rates of bispyribac-sodium can cause leaf symptoms on walnut, but the results of this study and other associated field trials (Galla 2018a, 2018b) suggest that low levels of injury may not result in direct effects on gross walnut yield or nut weight. Importantly, in suspected cases of bispyribac-sodium drift, analytical sampling results for residues in leaf tissues may

be inconclusive because of the high levels of biological activity on walnut and the relatively high analytical detection limits and short time-window of detection. Field investigations of suspected bispyribac-sodium drift on walnut should not depend exclusively on detecting this herbicide in symptomatic tissues in determining the causes of walnut disorders in the field.

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