Estimating the combined toxicity of flufenacet and imazaquin to sorghum with pore water herbicide concentration

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ABSTRACT

Combined toxicity of herbicides to non-target crops is usually resulted from their successive application. The present study was conducted to assess the combined toxicity of flufenacet (FLU) and imazaquin (IMA) to sorghum with their concentration in soil pore water. The concentrations that inhibited growth by 50% (IC50) of FLU and IMA individually and their combination estimated from the herbicide concentrations in soil pore water notably differed from those based on the amended concentrations, due to the decline in bioavailability resulting from adsorption of the herbicides onto soil. According to the amended concentrations, the combined effect of FLU and IMA in soil on sorghum growth was identified as additive action. Based on the concentration in soil pore water, however, it was determined to be antagonism, which was identical to that observed in a test using culture solution. The results revealed that pore water herbicide concentration might be an effective tool to assess the combined toxicity of herbicides in soil to rotational crops.

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Introduction

Herbicides are widely used for the control of weeds in agricultural systems. However, injury by residual herbicides to rotational crops has been frequently observed (Greenland, 2003; Wibawa et al., 2009; Fast et al., 2011) and is becoming an increasing concern in agriculture in China (Wang et al., 2003). For instance, imazethapyr applied at recommended rates to soybean for the control of annual broadleaf and grass weeds can persist in soil and cause injury to following crops wheat and corn (Li et al., 2008). More importantly, the herbicides are present in agricultural fields as a mixture of two or more of them rather than as individuals, which results from their successive applications over a growing season (Beckie and Reboud, 2009). There is, therefore, an increasing need to evaluate the combined toxicity of residual herbicides in soil to rotational crops for decision-making on succession crops (Lydy et al., 2004).

Plant assays based on amended concentrations are usually used for assessment of the toxicity of residual herbicides in soil to non-target crops (Katayama et al., 2010) to obtain the concentration that inhibits growth by 50% (IC50). However, the obtained value of IC50 varies with soil type (Szmigielski et al., 2009) and thus cannot be a predictive reference for different soils, because the amended concentration tends to overestimate the bioavailable fraction of an herbicide in soil (Reid et al., 2000). The concentration of a chemical in soil pore water (PW) has been regarded as the exposure level or the bioavailable portion to organisms (Sijm et al., 2000). Pore water chemical concentrations might be more accurate for assessing the toxicity of residual herbicides in soils to plants,
especially for combinations of herbicides, because the combined toxicity relies upon the interactions among the target plant, the herbicide, and soil (Clark et al., 2004; Leistra and Matser, 2004; Lu et al., 2012).

In the present study, the pore water herbicide concentration was adopted for estimating the toxicity of flufenacet (FLU) and imazaquin (IMA), individually and in combination, to sorghum. FLU is an oxyacetamide herbicide and has been recommended for control of a wide range of monocot weeds in crops like corn, cereals, rice, and soybean when applied post- or pre-emergence or is preplant-incorporated. It inhibits the synthesis of long-chain fatty acids in plants and causes injury to sensitive crops, with symptoms of twisting and/or curling of leaves, and consequently restrains plant growth (Geier et al., 2009). IMA is an imidazolinone herbicide recommended for use as a pre-plant incorporated, pre-emergence and post-emergence herbicide in soybean and other legume crops to control broadleaf weeds. It inhibits acetolactate synthase (ALS), and therefore would inhibit the growth of susceptible following crops (Smith et al., 2005).

Additionally, the long residual time of herbicides (Gupta and Gajbhiye, 2002; Smith et al., 2005) makes them coexist in soil and subsequently cause combined toxicity to sensitive crops such as wheat, sugar beet and sorghum (Wang et al., 2003). The objective was to find an effective approach for the estimation of the combined toxicity of these two herbicides in soil.

1. Materials and methods

1.1. Chemicals

FLU (99.00%) and IMA (98.50%) were purchased from Dr. Ehrenstorfer GmbH, Augsburg, Germany. Physicochemical properties of the herbicides (Tomlin, 2009) are shown in Table 1. All other chemicals and solvents used in this study were of analytical grade.

1.2. Soil and treatment

The soil used in this study was collected from the 0–20 cm layer of the field at Huajiachi campus, Zhejiang University, Hangzhou, China. The soil was air-dried and passed through a 2 mm sieve to remove stones and debris. Physical and chemical properties of the soil were measured by the Institute of Soil Science, Zhejiang Academy of Agricultural Sciences, China and listed as follows: sand, 21.5%; silt, 71.1%; clay, 7.4%; organic matter (OM) content, 3.05%; cationic exchange capacity (CEC), 10.6 cmol/kg; water holding capacity (WHC), 39.4%; pH, 6.8.

The soil sample (200 g dry wt equivalent) was weighed into a pot and mixed thoroughly with the desired aqueous herbicide solution to achieve the concentrations of 0.55, 1.10, 2.20, 3.85, and 5.51 μmol/L for FLU, and 0.64, 1.61, 3.21, 6.43, and 12.85 μmol/L for IMA, respectively. The moisture of the herbicide-amended soil was adjusted to 70% of its WHC, and then passed through a 2-mm sieve twice to distribute the herbicide evenly. Soil receiving the same amount of water without the herbicide served as a control. Each treatment was replicated for six times. The treated soil was transferred to a nutrition bowl (diameter 9 cm, height 10 cm) and covered with an aluminum foil to equilibrate for 24 h at 25°C in the dark, and was then ready for the plant assay and the determination of herbicide concentration in soil pore water.

1.3. Adsorption experiment

Adsorption experiments were carried out with the batch equilibration method according to Organization for Economic Co-operation Development guideline 106 for the testing of chemicals (OECD, 2000). Each soil sample (5.0 g) was mixed with 10 mL of 0.01 mol/L CaCl₂ containing one of the herbicides and 100 mg/L NaN₃. The herbicide concentration was set to 13.76–68.81 μmol/L for FLU and 3.21–128.5 μmol/L for IMA, respectively. The mixture was shaken on a shaker (HZ-9310 K, Taicang Kejiao, China) at 150 r/min and 25°C in the dark for 24 hr, and subsequently centrifuged at 3000 g for 10 min. The resulting supernatant was passed through a 0.45 μm filter, and was then ready for the determination of the herbicides by high performance liquid chromatography (HPLC). Each treatment was performed in triplicate. The control experiment was conducted with the same procedures as above. No loss of the herbicides was observed due to degradation, volatilization, or sorption onto the wall of the tubes.

<table>
<thead>
<tr>
<th>Table 1 – Molecular structure and properties of the herbicides.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Herbicides</strong></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Flufenacet (FLU)</td>
</tr>
<tr>
<td>Imazaquin (IMA)</td>
</tr>
</tbody>
</table>

ND: no dissociation.
Competitive adsorption of FLU and IMA onto the soil was evaluated with the same procedures described above. The herbicides were combined as follows: (1) a series of concentrations of FLU (13.76–68.81 μmol/L) mixed with IMA at levels of 3.21, 32.1, and 128.5 μmol/L, separately; (2) a series of concentrations of IMA (3.21–128.5 μmol/L) mixed with FLU at levels of 13.76, 41.28, and 68.81 μmol/L, separately.

1.4. Toxicity of the herbicides in soil to sorghum

Sorghum (Sorghum bicolor (L.) Moench. cv. Gadambalia) was selected for the growth assay based on its sensitivity to both of the herbicides. Sorghum seeds were soaked in water for 6 h, and then germinated for 10 h in an incubator at 25°C. Four germinated seeds with extended radicles were sowed evenly into the treated soil at a depth of about 1 cm. The planted bowls were placed randomly in an artificial climate incubator (RXA-380, Ningbo Jiangnan Instrument Factory, China) at 25°C under 16:8 h light:dark cycles (lights at 350 μE/(m² · sec)) with a humidity of 70%. The plants were harvested on day 14 for the measurement of height.

To assess the combined toxicity of FLU and IMA in soil to sorghum, diverse concentration compositions shown in Table 2 were selected based on the equitoxic concentration estimated from their individual toxicity tests.

1.5. Separation of soil pore water

The treated soil sample (20 g · wt) was weighed into the inner layer of a centrifugal filter (Amicon Ultra-15, 30,000 molecular weight cut-off; Millipore, Ireland) after removing the original filter membrane, and centrifuged at 8000 g for 20 min with a Beckman J2-21 centrifuge (Beckman TA-14 rotor, Germany). The collected pore water was filtered through a 0.45-μm filter and then injected into an HPLC to determine the concentration of the herbicides.

1.6. Toxicity of the herbicides in culture solution to sorghum

Six germinated sorghum seeds were fixed on a foam board with degreasing cotton and then transferred into a plastic container filled with 1 L of one-quarter strength Hoagland hydroponic culture consisting of (in μmol/L): 250 Ca(NO₃)₂, 197.50 K₂SO₄, 2.50 KH₂PO₄, 50 MgSO₄, 100 NH₄NO₃, 0.03 (NH₄)₆Mo₇O₂₄, 2.5 MnSO₄, 0.19 ZnSO₄, 0.08 CuSO₄, 8 H₃BO₃, and 5 Fe-EDTA (Hoagland and Arnon, 1950), and one of the herbicides at a level of 0.014, 0.028, 0.055, 0.14, and 0.28 μmol/L for FLU, and 0.48, 0.89, 0.92, 3.82, and 7.28 μmol/L for IMA, respectively. The planted plastic containers were placed randomly and incubated in an artificial climate incubator at 25°C under 16.8 hr light-dark cycles (lights at 350 μE/(m² · sec) with a humidity of 70%. Each treatment was replicated three times. The plants were harvested on day 14 for the measurement of the height.

For the test of the combined toxicity of the binary mixture in culture solution to sorghum, the herbicide concentrations based on the equitoxic concentration of individual toxicity test in culture solution are shown in Table 2. Other procedures were carried out as described above.

1.7. Analysis of the herbicides

The herbicides were analyzed by an HPLC equipped with a diode array detector (1200 series, Agilent Technologies, USA). A Hewlett-Packard stainless steel analytical column (ZORBAX SB-C18, 25 cm × 4.6 mm, 5 mm) was used for chromatographic separation, with a mobile phase of acetonitrile and water (80:20, V/V) containing 0.1% H₃PO₄ (V/V) at a flow rate of 1.0 mL/min. Injection of 10 μL samples was performed in HPLC for quantitative analysis. The peak of FLU and IMA was recorded at 210 and 240 nm with a retention time of approximately 4.50 and 3.50 min, respectively.

1.9. Data analysis

Toxic unit (TU) has been regarded as a useful approach for the assessment of combined toxicity (Sprague and Ramsay, 1965; Anderson and Weber, 1975; Schuler et al., 2009) and was employed to estimate the combined toxicity of the herbicides:

\[
TU_i = \frac{C_i}{IC_{50i}}
\]

\[
TU = \sum TUi
\]

\[
TU_b = \frac{TU}{\max(TUi)}
\]

where, \(C_i (\mu mol/kg)\) is the concentration of herbicide i at the IC₅₀ of the mixture; IC₅₀ is the concentration of herbicide i corresponding to 50% inhibition. If \(TU = 1\), the effect of two toxicants is additive; If \(TU > TU_0\), the effect of two toxicants is antagonism; If \(TU < 1\), the effect of two toxicants is synergism.

Estimation of IC₅₀ values and analysis of variance (ANOVA) were carried out with SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Differences between the means were compared by means of the LSD (Least significant digit) test at \(p < 0.05\).
2. Results and discussion

2.1. Adsorption of FLU and IMA in soil

The adsorption isotherms of the herbicides onto the soil are shown in Fig. 1. The adsorption coefficients ($K_f$) and $1/n$ together with determination coefficients ($r^2$) of FLU and IMA, calculated from the Freundlich equation, are shown in Table 3. The higher adsorption was mainly due to the higher OM content in the tested soil (Gupta and Gajbhiye, 2002). The $K_f$ value of FLU was 0.65, which is lower than most of the values (0.6–2.4) observed by Weber et al. (2003), indicating its weak adsorption onto the tested soil. The data revealed that the sorption affinity of FLU onto the tested soil was much higher than that of IMA.

The adsorption of FLU and IMA declined in the presence of the co-existing substance in all cases (Fig. 1). The adsorption isotherm of FLU was shifted down notably in the presence of IMA. However, CIMA-PW was similar to CIMA-a as a result of its lower affinity to the soil, with a $K_f$ value of 0.65. The data revealed that the difference between the concentration of the herbicides in soil pore water and their amended concentrations is mainly governed by their affinity to the soil. A similar observation was made by Liu et al. (2012).

$C_{FLU-PW}$ and $C_{IMA-PW}$ in soil amended with the binary mixture of these two herbicides were found to be much greater than when they were present individually (Fig. 2). This was resulted from the lesser adsorption caused by competition of the herbicides co-existing in soil.

The portion of a chemical dissolved in soil water is usually regarded as the fraction available to organisms (Van der Wal et al., 2004). Consequently, FLU and IMA in soil pore water might be more closely related to their bioavailability than the amended concentration.

2.2. FLU and IMA in soil pore water

The variation in the concentration of FLU and IMA in soil pore water ($C_{FLU-PW}$ and $C_{IMA-PW}$, respectively) with their amended concentration ($C_{FLU-a}$ and $C_{IMA-a}$, respectively) is shown in Fig. 2. Although $C_{FLU-PW}$ increased almost linearly with $C_{FLU-a}$, the value of $C_{IMA-PW}$ was much smaller than that of $C_{FLU-a}$ due to its high affinity to soil. However, $C_{IMA-PW}$ was similar to $C_{IMA-a}$ as a result of its lower affinity to the soil, with a $K_f$ value of 0.65. The data revealed that the difference between the concentration of the herbicides in soil pore water and their amended concentrations is mainly governed by their affinity to the soil. A similar observation was made by Liu et al. (2012).

$C_{FLU-PW}$ and $C_{IMA-PW}$ in soil amended with the binary mixture of these two herbicides were found to be much greater than when they were present individually (Fig. 2). This was resulted from the lesser adsorption caused by competition of the herbicides co-existing in soil.

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2.3. Toxicity of FLU, IMA, and their binary mixture to sorghum estimated with amended concentration

The effect of FLU and IMA individually on sorghum growth in soil is shown in Fig. 3. The inhibitory effect of FLU and IMA on sorghum growth was enhanced with the increase in their amended concentrations. The corresponding dose-response curves are shown in Fig. 4a. The $IC_{50a}$ value of FLU estimated with the Probit regression model was 2.34 μmol/L in the tested soil (Table 4). The $IC_{50a}$ value for IMA in the tested soil toward sorghum was 3.43 μM, which is accordant with that (2–12 μmol/L) reported by Zhou et al. (2007). The data demonstrated that FLU was more toxic to sorghum than IMA.

Table 3 – Freundlich coefficients measured for flufenacet (FLU) and imazaquin (IMA).

<table>
<thead>
<tr>
<th>Herbicide</th>
<th>$K_f$ (μmol/kg)</th>
<th>$1/n$</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLU</td>
<td>6.39 ± 0.92</td>
<td>0.75 ± 0.056</td>
<td>0.984</td>
</tr>
<tr>
<td>FLU (3.21 μmol/L IMA)</td>
<td>3.23 ± 0.073</td>
<td>0.92 ± 0.010</td>
<td>0.999</td>
</tr>
<tr>
<td>FLU (32.1 μmol/L IMA)</td>
<td>3.11 ± 0.12</td>
<td>0.93 ± 0.016</td>
<td>0.998</td>
</tr>
<tr>
<td>FLU (128.5 μmol/L IMA)</td>
<td>3.10 ± 0.46</td>
<td>0.90 ± 0.054</td>
<td>0.988</td>
</tr>
<tr>
<td>IMA</td>
<td>0.65 ± 0.006</td>
<td>0.86 ± 0.003</td>
<td>0.999</td>
</tr>
<tr>
<td>IMA (13.76 μmol/L FLU)</td>
<td>0.55 ± 0.016</td>
<td>0.86 ± 0.008</td>
<td>0.999</td>
</tr>
<tr>
<td>IMA (41.28 μmol/L FLU)</td>
<td>0.52 ± 0.002</td>
<td>0.88 ± 0.002</td>
<td>0.999</td>
</tr>
<tr>
<td>IMA (68.81 μmol/L FLU)</td>
<td>0.48 ± 0.011</td>
<td>0.89 ± 0.007</td>
<td>0.999</td>
</tr>
</tbody>
</table>

Fig. 1 – Adsorption isotherms of flufenacet (FLU) (a) and imazaquin (IMA) (b) in soil alone and coexisting. $C_s$: the concentration of herbicides adsorbed on the soil at adsorption equilibrium; $C_{aq}$: the concentration of herbicides in the aqueous phase at adsorption equilibrium.
In the binary mixture experiment, the growth of sorghum was also impaired by the combination of FLU and IMA (Fig. 3c) with the dose–response curve shown in Fig. 4c. The corresponding IC50a value was 2.70 μmol/L (Table 4). Dose-response curves based on the concentration of the single herbicides included in the mixture are also shown in Fig. 4c, with IC50a values of 1.17 and 1.53 μmol/L, respectively, suggesting that the mixture containing 1.17 μmol/L FLU and 1.53 μmol/L IMA would inhibit sorghum height by 50% compared with the control. Based on Eqs. (1) and (2), the value of TU was estimated to be 0.95, suggesting that there was an additive action in the binary mixture to impair the growth of sorghum.

2.4. Toxicity of FLU, IMA, and their binary mixture to sorghum estimated with the concentration in soil pore water

The single-component dose-response curves of FLU and IMA based on their concentrations in pore water are shown in Fig. 4b, respectively. The IC50PW value of FLU individually calculated from CFLU-PW was 0.66 μmol/L (Table 4). The value was significantly lower than that (2.34 μmol/L) based on the amended concentration. On the other hand, the IC50PW value of IMA individually was 3.83 μmol/L (Table 4) which is similar to that (3.43 μmol/L) based on the amended concentration. The data indicated that there exists a difference between IC50PW and IC50a caused by the difference between C_PW and C_a resulting from the adsorption of the herbicides onto the tested soils. The herbicides adsorbed by soil particles could not be taken up by sorghum and thus have no toxic effect on the plant growth (Yu et al., 2009). Accordingly, using the IC50PW value might be a more effective approach for the estimation of the toxicity of residual herbicide in soil to non-target plants, since the herbicide in soil pore water reflects the bioavailable fraction (Folberth et al., 2009).

The IC50_PW value for the binary mixture was 3.50 μmol/L based on the obtained dose-response curve shown in Fig. 4d. The value of TU was consequently calculated to be 2.18 with a TU0 of 1.42, demonstrating an antagonistic action in the toxic influence of FLU and IMA on the growth of sorghum. This diametrically opposes to the additive action between FLU and IMA concluded from the amended concentration, implying that estimating the impact of a mixture of herbicides is complicated since interactions may occur between the individual herbicides as well as with the soil (Amorim et al., 2005; Perez et al., 2011). The overall effect of a mixture in soil usually depends upon the dose–response relationships (Loureiro et al., 2009) and composition ratios (Liu et al., 2014), as well as interactions between the herbicides and soil (Jonker et al., 2004). In the present study, the difference between the individual effects and those of the binary mixture only
involved the interactions occurring between the herbicides and the soil, since the herbicides, sorghum species and composition ratios of the herbicides were fixed.

To determine the reliability of $C_{PW}$ or $C_{sa}$ for the evaluation of the combined toxicity, similar assays were conducted in culture solution as a reference. The response of sorghum growth to FLU, IMA and their mixture in culture solution is shown in Fig. 5, with the dose–response curves given in Fig. 6, respectively. The IC50 values of FLU and IMA in culture solution toward sorghum were 0.29 and 1.31 $\mu$mol/L (Table 4), which are significantly lower than those based on the concentration in soil pore water. This difference could be interpreted as arising from the reduction in bioavailability of the herbicides induced by the adsorption of the chemicals onto the dissolved organic matter (DOM) present in soil pore water (Lee et al., 2003). This is accordant with the observation by Gigliotti et al. (2005), who indicated that the toxicity of the herbicide triflusulfuron methyl in culture solution toward oilseed rape was decreased by a factor of 4.8 by the presence of DOM extracted from municipal waste compost.

The IC50 value of the mixture in culture solution was 2.20 $\mu$mol/L (Table 4), which included 0.27 $\mu$mol/L FLU and $C_{sa}$: the amended concentration; $C_{PW}$: the concentration in pore water; $C_{CS}$: the concentration in culture solution.

### Table 4 – IC50 values with 95% confidence intervals (CI) of flufenacet (FLU), imazaquin (IMA), and their binary mixture (MIX).

<table>
<thead>
<tr>
<th>Concentration Component</th>
<th>IC50 (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individual toxicity</td>
<td></td>
</tr>
<tr>
<td>$C_{sa}$</td>
<td>FLU 2.34 (2.09–2.63)</td>
</tr>
<tr>
<td></td>
<td>IMA 3.43 (2.75–4.30)</td>
</tr>
<tr>
<td>$C_{PW}$</td>
<td>FLU 0.67 (0.43–1.02)</td>
</tr>
<tr>
<td></td>
<td>IMA 3.83 (3.04–4.85)</td>
</tr>
<tr>
<td>$C_{CS}$</td>
<td>FLU 0.29 (0.15–0.58)</td>
</tr>
<tr>
<td></td>
<td>IMA 1.31 (0.99–1.65)</td>
</tr>
<tr>
<td>Combined toxicity</td>
<td></td>
</tr>
<tr>
<td>$C_{sa}$</td>
<td>MIX 2.70 (2.20–3.31)</td>
</tr>
<tr>
<td></td>
<td>1.17 (0.95–1.44) FLU</td>
</tr>
<tr>
<td>$C_{PW}$</td>
<td>MIX 3.50 (2.85–4.29)</td>
</tr>
<tr>
<td></td>
<td>1.53 (1.25–1.88) IMA</td>
</tr>
<tr>
<td>$C_{CS}$</td>
<td>MIX 2.20 (1.69–3.12)</td>
</tr>
<tr>
<td></td>
<td>0.27 (0.21–0.39) FLU</td>
</tr>
<tr>
<td></td>
<td>1.93 (1.48–2.73) IMA</td>
</tr>
</tbody>
</table>
1.93 μmol/L IMA on the basis of the series of concentrations used in the combined toxicity tests (Table 2). Consequently, the values of TU and TU₀ were 2.42 and 1.64, respectively, demonstrating the antagonistic action between FLU and IMA in their impairing effect on the growth of sorghum. The observed antagonism probably resulted from the competitive uptake of the herbicides by sorghum, and metabolism or detoxification inside the sorghum plant (Spurgeon et al. 2010), and the overlap of the toxic effect of IMA by that of FLU. This is in agreement with the result obtained for the mixture of the herbicides deduced from the herbicide concentration in soil pore water, rather than from the amended concentration.

3. Conclusions

The results obtained in the present study revealed that the concentration in soil pore water (C_PW) of FLU and IMA could represent their available fraction to sorghum. The size of C_PW was mainly governed by the affinity of the herbicides to soil. The co-existence of the herbicides in soil increased the values of C_PW of FLU and IMA when present individually, and thus enhanced their combined toxicity to sorghum. The interaction between FLU and IMA in terms of their toxic effect on sorghum growth observed in the test with the herbicides in solution, was similar to that obtained with C_PW of the chemicals in soil rather than that based on the amended concentration. This implies that the concentration of the herbicide in soil pore water is an effective tool for the assessment of the combined toxicity of herbicides in soil to non-target plants.

Acknowledgments

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