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ASRF Project - Seed Coat Permeability to Systemic Seed Treatment Uptake

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This ASRF project started on January 1, 2015 and with the approved co-cost extension to terminated on July 15, 2018.

The major accomplishment was that two papers were published in refereed scientific journals. Both papers cited the American Seed Research Foundation as a sponsor of the research.

I want to thank ASRF for funding this research or this research could have never been undertaken.

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RESEARCH

Systemic Seed Treatment Uptake during Imbibition by Corn and Soybean

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ABSTRACT

Systemic seed treatment uptake into soybean [*Glycine max* (L.) Merr.] and corn (*Zea mays* L.) seeds during imbibition has not been investigated over a broad range of application rates. The objectives of this study were to investigate the uptake capacity of seeds and assess the role of the seed coat on uptake. A fluorescent compound, coumarin 120 (7-amino-4-methylcoumarin), was applied as a model seed treatment in the range of 0.01 to 20.0 mg g^{-1} seed to study the dose effects on seed uptake of two corn lines and three soybean cultivars. In general, there was a large increase in seed uptake as dosage increased, followed by a saturated state at higher dosages. The uptake by two lines of corn seeds and three cultivars of soybean seeds showed a dose-dependent process that was described by an exponential model of $Y = y_0 - Ae^{-kx}$ ($r^2 \geq 0.93$), where y_0 is the uptake limit, A and k are constants, and x is the applied dose rate. The value of y_0 is an indicator of a seed's uptake capacity, and it differed between the two corn lines and the three soybean cultivars. The calculated dose rate at which the uptake achieved 95% of y_0 (x_{95}) also varied widely within the corn lines and soybean cultivars examined. A low value of x_{95} = 0.87 mg g^{-1} seed was measured for corn line B73. This result indicates that a seed treatment may reach its uptake limit at a low dosage. The seed-covering layers of corn lines and the black-seeded soybean cultivar attenuated the uptake of the seed treatment.

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Abbreviations: GCB, graphitized carbon black; HPLC, high-performance liquid chromatography; log K_{ow}, ratio of the concentration of a solute between water and octanol; MeCN, acetonitrile; PSA, primary secondary amine; PVA, polyvinyl alcohol; x_{95}, calculated dose rate at which the uptake achieved 95% of the uptake limit; y_0, uptake limit.

Seed treatments are widely used in agriculture to manage early-season insects and diseases, as well as seed-borne pathogens (Elbert et al., 2008; Sharma et al., 2015). For control of internal seed-borne pathogens and aboveground diseases and insect pests, the active ingredients must have systemic activity. For example, systemic neonicotinoid seed treatment insecticides are used worldwide to control foliar pests (Douglas and Tooker, 2015). Systemic triazole fungicide seed treatments are used to control *Tilletia* and rust diseases (Fletcher et al., 2010). There are few reports on the uptake of agrochemicals applied as seed treatments and generally under limited dosages that are used for pest management. For example, the uptake of selected herbicides by soybean [*Glycine max* (L.) Merr.] seeds was investigated over a 16-fold range (Rieder et al., 1970), and the fungicide triticonazole was tested over a 5.3-fold range (Quérou et al., 1998). The focus of the current study is on the uptake of a systemic compound into soybean and corn (*Zea mays* L.) seeds during imbibition over a much larger 2000-fold range, so our results have broad application for understanding commercial seed treatment uptake.

Plant roots take up systemic soil-applied compounds from the surrounding soil, and these chemicals are then transported

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upward into the aboveground tissues via the transpiration stream in the xylem. From the root to the xylem, these compounds must penetrate across several biological layers: the epidermis, cortex, endodermis, and pericycle (Collins et al., 2006). This process is driven by a water potential gradient, but those biological layers are selectively permeable (Santoni et al., 2000). This selective permeability is determined by the balance between the affinity and mobility of absorbed chemicals in plant tissues (Uchida, 1980). More hydrophilic chemicals are less able to permeate across the hydrophobic lipid membranes, whereas highly lipophilic chemicals are retained in lipid plant constituents. Therefore, for an organic compound to have systemic activity requires a balance between its hydrophilic and lipophilic properties. The degree of lipophilicity is determined by a compound's solubility in octanol and water, expressed on a log scale as the log K_{ow}, or partition coefficient (log P) (Satchivi, 2014; Trapp, 2004). Based on the log K_{ow}, the systemic ability of an organic compound's movement from root to shoot was mathematically described and termed the TSCF (transpiration stream concentration factory by Briggs et al., 1982). A normal or Gaussian distribution was revealed for the TSCF was TSCF = 0.784 exp−[(log K_{ow} − 1.78)^2/2.44], with the maximum systemic uptake of log $K_{ow} \approx 2.0$ using barley (*Hordeum vulgare* L.) as the model system. Later, additional Gaussian models were developed for the root-to-shoot uptake process in soybean and other species (Collins et al., 2006).

Alternatively, systemic active ingredients may be absorbed directly into crop seeds during imbibition from the treated seed surface to the embryo. For controlling internal seed-borne diseases and insect pests that attack the seed embryo, seed uptake of the active ingredients during imbibition is pivotal. Insufficient penetration of active ingredients into the seeds would fail to control these internal pathogens and insect pests. To better understand the potential for uptake of agrochemicals into seeds requires an understanding of the physical and chemical properties of the active ingredient. Previous studies from our laboratory revealed that seed coat permeability varied by crop seeds and were grouped into three categories: (i) permeable, (ii) selectively permeable, and (iii) nonpermeable (Taylor and Salanenka, 2012). These categories were developed based on uptake of selected fluorescent tracer compounds with known physical and chemical properties. For example, coumarin 1 or 151 is moderately lipophilic and nonionic in nature, whereas rhodamine B is moderately lipophilic and ionic in nature (Taylor and Salanenka, 2012). Seeds that allowed both tracers to diffuse through the seed coat to the embryo have the permeable characteristic, and soybean seeds were classified with permeable seed coats (Taylor and Salanenka, 2012). Seeds that allowed only coumarin 1 or 151 but restricted rhodamine B from diffusing through the seed coat and pericarp have selective permeability characteristic, and corn (*Zea mays* L.) seed is in this category (Dias et al., 2014). Therefore, in the case of seed treatment uptake, both the log K_{ow} and electrical charge of a molecule determine its ability to penetrate the seed coat. Finally, seeds that blocked both tracers from diffusing have the nonpermeable characteristic, and cucumber (*Cucumis sativus* L.) seed is an example (Salanenka and Taylor, 2011). Unfortunately, many questions remain on the uptake of systemic chemicals by seeds during imbibition. What is the uptake capacity of a seed to the seed treatment? Do the seed coat or seed-covering layers attenuate uptake? Finally, what is the maximum uptake efficiency of applied seed treatment that diffused into the soybean embryo or corn internal tissues (embryo + endosperm)? In this paper, a model fluorescent tracer, coumarin 120 (7-amino-4-methylcoumarin) with systemic properties was used to mimic an agrochemical seed treatment to investigate seed uptake over a wide range of application rates.

MATERIALS AND METHODS

Chemicals and Crop Seeds

Coumarin 120 is nonionic with a log K_{ow} of 1.1. According to another study from our laboratory, a nonionic compound with log K_{ow} 1.1 would diffuse through the seed-covering layers to the internal tissues of both soybean and corn (Yang et al., 2018). Therefore, this tracer is predicted to have systemic uptake (Briggs et al., 1982). Coumarin 120 (99%) was purchased from Sigma-Aldrich. Acetonitrile (MeCN, high-performance liquid chromatography [HPLC] grade) was purchased from Fisher Scientific. Primary secondary amine (PSA) was purchased from Agilent Technologies. Graphitized carbon black (GCB) was purchased from Agela Technologies. For seed coating, polyvinyl alcohol (PVA, Selvol 205) was purchased from Sekisui Specialty Chemical Company, and Triton X-100 was purchased from Electron Microscopy Sciences.

Preliminary uptake studies were conducted on nine lines of corn, and two were selected with low and high uptake (Avelar and Thomazella, unpublished data, 2016). Corn seeds of lines OH7B and B73 were provided by Dr. Dale Wilson (Valent BioSciences, Libertyville, IL). In addition, preliminary studies were conducted on 17 soybean cultivars, and three were selected with different uptake patterns (Avelar and Amirkhani, unpublished data, 2017). Two yellow-seeded soybean cultivars were examined: 'IAR 1902' from the Iowa State Research Foundation (Ames, IA), and 'TMG 132RR' from Tropical Melhoramento & Genética, Fundação MT (Unisoja, Brazil). Black-seeded soybean cultivar 'V12-1223' was provided by Dr. Bo Zhang, Virginia Polytechnic Institute and State University (Blacksburg, VA).

Seed Treatments with Coumarin 120

Suspension concentrates of coumarin 120 were prepared by dispersing coumarin 120 powder in an aqueous solution containing 4% PVA and 0.1% Triton X-100. The PVA was used as binder to adhere the coumarin 120 to the seed surface. Triton

X-100 was used as a surfactant to disperse the compound. Corn and soybean seeds were weighed individually, and the coumarin 120 suspension concentrate was loaded precisely onto the surface of each seed with a micropipette. The applied dosage of coumarin 120 for each treatment was calculated from the mean seed weight of each seed lot (Table 1). Each treated seed was dried in a gentle air stream immediately until a dry layer of coumarin 120 was observed on the surface of each seed. Four replicates (seeds) were examined for each treatment.

Decoated Coumarin 120 Seed Treatments

Corn seeds of both lots were soaked in water for 2 to 3 h, then the pericarp and testa were removed with forceps from each seed. The decoated seeds were dried in a forced-air oven at 30°C overnight. The intact seeds of both corn lots were soaked and dried in the same manner for direct experimental comparison. Seed coats of the three soybean cultivars were removed by hand without soaking. The decoated corn and soybean seeds were treated individually with the same method as described above. The applied dosage of coumarin 120 used in the decoated seed study was 0.5 and 10 mg g^{-1} seed.

Imbibition of Treated Seeds and Removal of Seed-Covering Layers

After treatment with different dosages of coumarin 120, seeds were equilibrated in a 70% relative humidity chamber to achieve a uniform water activity for all samples prior to sowing (Bay et al., 1995). Treated seeds were imbibed in silica sand with 20% moisture content at 20°C, and soybean and corn seeds were removed from the sand 12 and 20 h after planting, respectively. The imbibed seeds were thoroughly washed with water to remove any residual coumarin 120 on the surface of imbibed seeds. Then the soybean coat or corn pericarp and testa of intact seeds were removed with forceps. In the case of decoated seeds (embryos or embryos + endosperm) were thoroughly washed to remove residual tracer.

Sample Preparation for HPLC Analysis

Samples were extracted with the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method (Anastassiades et al., 2003). Briefly, the mortar and pestle were frozen with liquid N_2. For each replicate, one seed was placed into the frozen mortar, immersed in liquid N_2, and then were ground into a fine powder. The powder was transferred into a 50-mL centrifuge tube with a screw cap. Next, 10 mL of MeCN was added and the mixture was shaken for 2 min using a Vortex mixer at room temperature. After this step, a mixture of 3.5 g of $MgSO_4$ and 1.0 g of NaCl was added. The centrifuge tube was immediately shaken vigorously for 1 min to prevent the formation of $MgSO_4$ agglomerates and centrifuged at 3500 rpm for 5 min. A 4.5-mL sample of the supernatant was subjected to dispersive solid-phase extraction using a mixture of sorbents and $MgSO_4$ (corn: 30 mg GCB, 50 mg PSA, and 100 mg $MgSO_4$; soybean: 30 mg PSA and 100 mg $MgSO_4$). The tube was shaken vigorously for 1 min using a vortex mixer. Finally, the extract was filtered through a 0.22-µm syringe filter. The recovery was 86 ± 3.2% for the corn seed and 95 ± 3.8% for the soybean seed, respectively.

HPLC Analysis

Coumarin 120 was quantified using an Agilent 1100 HPLC equipped with a 1200 fluorescence detector using an ODS-3 column (GL Sciences, 5 µm, 4.6-mm × 75-mm column). The mobile phase (1 mL min^{-1}) used was 0 min 30% MeCN + 70% water, 6 min 40% MeCN + 60% water, 8 min 90% MeCN + 10% water, 10 min 30% MeCN + 70% water. A gradient system was used and the temperature of the column was 30°C. The injected volume was 20 µL. The wavelengths of the fluorescence detector were set at the maximum excitation and emission wavelengths of 342 (excitation) and 409 nm (emission). The retention time of coumarin 120 was 3.17 min.

Data Analysis

The application rate of coumarin 120 was expressed as milligrams per gram of seed, and using the mean seed weight for each seed lot, the application rate was converted to milligrams per seed (Table 1). Seed uptake was expressed as concentration of absorbed coumarin 120 as micrograms per gram of seed. The concentration of coumarin 120 in corn and soybean seed was calculated by regression analysis and was conducted using the built-in models in software Origin Pro 8.0 (OriginLab, 2008), using Eq. [1] below.

RESULTS

The concentration of absorbed coumarin 120 (tracer) into corn embryo + endosperm is shown for two corn lines treated with 0.01 to 20.0 mg g^{-1} seed (Fig. 1). The absorbed tracer concentration greatly increased from 0.01

Table 1. The applied dosage of coumarin 120 for each treatment of the corn and soybean seed.

	Applied dosage				
Applied dosage	B73	OH7B	IAR 1902	TMG 132RR	V12-1223
mg g^{-1} seed	mg $seed^{-1}$				
0.01	0.0023	0.0024	0.0015	0.0016	0.0019
0.05	0.011	0.012	0.008	0.008	0.009
0.1	0.023	0.024	0.015	0.016	0.019
0.5	0.113	0.024	0.076	0.079	0.094
1	0.23	0.24	0.15	0.16	0.19
5	1.13	1.18	0.76	0.79	0.94
10	2.25	2.35	1.53	1.58	1.88
20	4.50	4.70	3.05	3.15	3.75
Seed weight, g $seed^{-1}$	0.2250	0.2350	0.1525	0.1575	0.1875

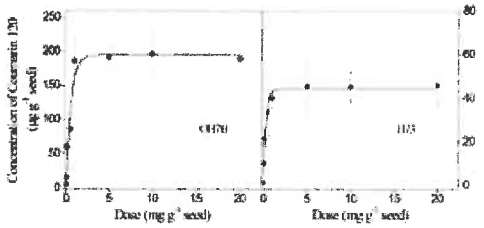

Fig. 1. The concentration of absorbed coumarin 120 in imbibed seeds of corn OH7B and B73 and their fitting curves with model $Y = y_0 - Ae^{-kx}$, where y_0 is the uptake limit, A and k are constants, and x is the applied dose rate. The bars represent ±SE from four replicates.

to 1.0 mg g^{-1} seed for both OH7B and B73, and both corn lines approached a saturated condition from 1.0 to 5.0 mg g^{-1} seed. Although the trend of uptake curves was similar for both lines, B73 reached tracer uptake saturation at a much lower concentration than OH7B. To further mathematically describe the uptake curves, dose-dependent relationships were analyzed using an empirical model:

$$Y = y_0 - Ae^{-kx} \quad [1]$$

where Y is the concentration of absorbed coumarin 120, y_0 is the calculated uptake limit, A and k are constants, x is the applied dose rate, and x_{95} is the calculated dosage in milligrams per gram of seed to achieve 95% of y_0. A is the background fluorescence in the sample, and a higher k value indicated that the uptake reached its saturated state at a lower applied dosage of tracer. The calculated constants and values from fitting Eq. [1] to the data are shown in Table 2. The uptake limit (y_0) of tracer for OH7B and B73 was 195 and 44.3 μg g^{-1} seed, respectively, whereas the x_{95} for OH7B and B73 was 1.74 and 0.87 mg g^{-1} seed, respectively. Therefore, the uptake limit (y_0) of OH7B was over fourfold higher than that of B73, and the x_{95} of OH7B was 50% higher than that of B73.

The role of the seed coat as a barrier for the uptake of coumarin 120 during imbibition was investigated using decoated seeds. For OH7B, the concentration of coumarin 120 increased from 82.7 to 148 μg g^{-1} seed at 0.5 mg g^{-1} seed dosage when the coat was removed, thus a 79% increase at this dose rate (Fig. 2). Similarly, the concentration of coumarin 120 increased from 166 to 514 μg g^{-1} seed at 10 mg g^{-1} seed dosage when the coat was removed, a 2.1-fold increase at this dose rate. For B73, the concentration of coumarin 120 increased by 28% at 0.5 mg g^{-1} seed dosage with a 2.6-fold increase from that of intact seed at 10 mg g^{-1} seed dosage (Fig. 2). Additionally, the concentration of coumarin 120 in the intact seeds after soaking and drying (Fig. 2) was compared with that of intact seeds without soaking and drying (Fig. 1) at 0.5 and 10 mg g^{-1} seed dosage, and no significant differences were measured for each dosage. Therefore, soaking and drying did not alter seed coat permeability.

The concentration of absorbed coumarin 120 (tracer) into soybean embryos is shown from seeds treated from 0.01 to 20.0 mg g^{-1} seed (Fig. 3). The concentration of tracer greatly increased at low applied dosages for TMG 132RR and IAR 1902 seeds, whereas the uptake curves of these two cultivars leveled off from 5.0 to 10.0 mg g^{-1} seed. In contrast, the tracer uptake rate of the black-seeded cultivar V12-1223 was much lower than the two yellow-seeded cultivars. The uptake curves of the three soybean cultivars also were described with the same Eq. [1] used for corn. The calculated x_{95} values from Eq. [1] using data of soybean seeds are shown in Table 2. The order of $x_{95\ \mathrm{TMG\ 132RR}} < x_{95\ \mathrm{IAR\ 1902}} << x_{95\ \mathrm{V12\text{-}1223}}$ revealed that TMG 132RR was approaching y_0 at a lower applied dosage than IAR 1902, whereas V12-1223 would approach y_0 at a dosage above the maximum dosage tested in this study when extrapolated from Eq. [1].

To understand the role of seed coat (testa) of soybean on tracer uptake, the testa was removed from the dry seed. The concentration of tracer in the embryo of V12-1223 increased from 14.0 (Fig. 3) to 114 μg g^{-1} seed at 0.5 mg g^{-1} seed dosage (Fig. 4), resulting in a sevenfold increase. At 10 mg g^{-1} seed dosage, the concentration of coumarin 120 increased from 97.7 (Fig. 3) to 237 μg g^{-1} seed dosage (Fig. 4) resulted in a 1.4-fold increase compared with the intact seed. However, the concentration did not differ between decoated and intact seeds for

Table 2. Constants from fitting Eq. [1] to the data of absorbed coumarin 120 from the seed treatment of corn and soybean and the calculated dose rate at 95% of the uptake limit (x_{95}).

Line or cultivar	Variable†				
	y_0	A	k	r^2	x_{95}
Corn					
OH7B	195	187	1.700	0.93	1.74
B73	44.3	40.3	3.340	0.95	0.87
Soybean					
TMG 132RR	238	221	0.458	0.96	6.39
IAR 1902	243	244	0.337	0.94	8.89
V12-1223	156	150	0.111	0.98	26.7‡

† y_0 is the uptake limit ,and A and k are constants.

‡ Extrapolated from Eq. [1].

The concentration curves of absorbed coumarin 120 for corn (Fig. 1) and soybean (Fig. 3) exhibited large increases as the applied dosage increased, followed by a saturated condition at higher dosages for two lines of corn and two yellow-seeded soybean seeds. The black-seeded soybean cultivar V12-1223 had a different dose response curve than the two yellow-seeded cultivars. The uptake by all five lots tested revealed a dose-dependent process that was described by the exponential Eq. [1]. The calculated values of y_0 varied widely within soybean and corn varieties examined (Table 2); however, the exponential model fit well for all five seed lots tested ($r^2 \geq 0.93$). In this study, we used a wide dose range (2000-fold) of coumarin 120 that for four of the five seed lots examined exceeded the maximum uptake limit that resulted in a saturated condition (Table 2). However, in previous reports on the uptake of agrochemicals by crop seeds, a linear model was developed to describe the uptake process as low dosages were applied over a relatively narrow range. For example, the uptake of herbicides (linuron, chlorpropham, atrazine, EPTC, and amiben) by soybean seeds was directly proportional to concentration of soaking solution in the range of 0.02 to 0.32 mM (Rieder et al., 1970). When the fungicide triticonazole was applied as a seed treatment to spring wheat (*Triticum aestivum* L.), the calculated amount absorbed by the caryopsis was linearly correlated to the applied rate in the range of 0.45 to 2.4 g kg^{-1} seed (Quérou et al., 1998). In our study, corn OH7B and three tested soybean cultivars also presented a linear relationship between seed uptake and applied dosage in the range of 0.01 to 0.5 mg g^{-1} seed with $r^2 > 0.93$. For corn B73, the linear dosage-dependent uptake was not measured even at low dosages. According to these two cited studies, linear models would not be suitable to predict a saturation condition on uptake of seeds.

As shown in Fig. 1 and 3, the saturation effect was observed over the uptake process of coumarin 120 by corn and soybean seeds after planting. This is the first report on the saturation effect of seed uptake. This observation would be useful to understand the uptake of seed treatment in crop protection. The value of y_0 in Eq. [1] is an indicator of the uptake capacity of a seed. A high value of y_0 indicates a seed has a high capacity of absorbing chemicals coated on the surface of a seed. This would benefit the pest management, especially the seed-borne pathogen management. On the other hand, if the applied seed treatment also presents the plant growth inhibitory effect (e.g., the triazole fungicides), the treated seeds would be at high risk of phytotoxicity. The value of x_{95} calculated by Eq. [1] is also an important parameter. A low value of x_{95} implies that uptake of a seed treatment would be saturated at a low dose rate. In our study, the value of x_{95} for corn B73 was 0.87 mg g^{-1} seed. This value is lower than the recommended dosage of many pesticides used as seed treatments (e.g., the recommended dosage of imidacloprid is 2~3 mg g^{-1} seed).

The uptake process of a tracer into a seed includes two stages: movement across the seed coat and further movement within the seed embryo (and endosperm). The decoating experiments were performed to separate the effect of the seed coat or seed-covering layers on uptake by the embryo or embryo + endosperm. The decoated seed had greater uptake of tracer than the intact seed of each corn line at each dosage (Fig. 2). Therefore, the pericarp–testa attenuated uptake and served as a barrier. In comparing decoated corn seeds of both lines, OH7B had greater uptake than B73 (Fig. 2). Therefore, as the seed-covering layers were removed, varietal differences in uptake were attributed to a greater absorption of the tracer by the embryo + endosperm of OH7B compared with B73. For soybean TMG 132RR and IAR 1902, the seed coat did not act as a barrier, as no differences in concentration were measured between intact (Fig. 3) and decoated seeds (Fig. 4). Therefore, the yellow-seeded cultivars were permeable. However, in the case of the black-seeded soybean, the seed coat both decreased the uptake limit (y_0) and required the highest dosage concentration to approach x_{95} (Table 2). The uptake of decoated V12-1223 showed no differences in comparison with the two permeable yellow-seeded cultivars. Therefore, uptake in the black-seeded cultivar was governed by seed coat permeability. Collectively, the maximum uptake of a cultivar or line was influenced by the seed coat that acted as a barrier, and/or internal tissues with their affinity for the systemic compound.

Soybean seed coat permeability was previously investigated due to its importance on seed quality and stand establishment under environmental stress. The permeable nature of many yellow-seeded soybean cultivars is attributed to the structural and compositional characteristic of the testa. Micropores were observed in permeable soybean seed coats (Chachalis and Smith, 2001) that would provide pathways for passive diffusion. A black-seeded soybean had slower imbibition rate, a thicker seed coat, and greater mechanical strength than a yellow-seeded cultivar (Tully et al., 1981). In addition, the composition of mature soybean seed coat varieties may differ and the cuticle of a nonpermeable variety contained a disproportionately higher amount of hydroxylated fatty acids in comparison with permeable varieties (Qutob et al., 2008).

In addition, to complete removal of the seed covering layers, mechanical injury that resulted in the loss of seed coat integrity also increased uptake in other studies. Mechanical damage to onion (*Allium cepa* L.) and leek (*Allium porrum* L.) seeds resulted in a perfusion of lanthanum applied during a seed soak (Beresniewicz et al., 1995). Lanthanum is a heavy metal that is electron dense and was imaged by electron microscopy. In the case of corn, mechanical damage to the pericarp–testa resulted in greater uptake of nonionic and ionic fluorescent tracers into seeds (Dias et al., 2014).

There are two kinds of diffusion processes for transportation across biological membranes for small-size molecules: passive diffusion and facilitated diffusion. Facilitated diffusion can be saturated (Cussler et al., 1989), whereas passive diffusion cannot. For example, glucose was shown to penetrate across cell membranes by both passive diffusion and facilitated diffusion (Renner et al., 1972; Carruthers, 1990). For the uptake of compounds by seeds, passive diffusion is generally the accepted mechanism, but given the results of this study, facilitated diffusion may take place simultaneously to the passive diffusion of coumarin 120. However, there is no evidence of a special transport protein carrier needed for facilitated diffusion, so this mechanism may not be responsible for the saturated condition. An alternate explanation is that the coumarin 120 would become saturated in the soil solution during imbibition as dosage concentration increased. The solubility of coumarin 120 is 6137 mg L^{-1}, so the soil concentration of coumarin would establish the gradient ultimately resulting in saturated condition in the seed.

The maximum uptake efficiency was at 0.1 mg g^{-1} seed for all corn and soybean seed lots examined (Fig. 5). Most likely the soil solution was not saturated at 0.1 mg g^{-1}, but this dosage yielded the highest percentage uptake of the total applied material. The seeds had a great affinity for coumarin 120 uptake when a small amount was available. However, as dosage increased, more coumarin 120 was available in the soil solution, but much of it remained in the soil solution and was not taken up. The result was that the maximum percentage uptake of total declined as dosage increased above 0.1 mg g^{-1}. A range of seed treatment uptake efficiencies were reported for imidacloprid and clothianidin, both used as neonicotinoid seed treatment insecticides. In a review on uptake of imidacloprid by seeds and plants, ~5% of the applied dose to seeds or soils was taken up, whereas 20% was reported in corn (Sur and Stork, 2003). A maximum of 1.34% of the initial clothianidin seed treatment was taken up by corn seedlings (Alford and Krupke, 2017). This wide range of percentage uptake may be related to application dosage and other seed and soil factors.

Insecticide seed treatments are commercially expressed as dosage per seed. Dosage rates were expressed throughout this paper as milligrams per gram of seed and were converted to milligrams per seed (Table 1). Therefore, commonly used seed treatment insecticide application rates are in the range of ~1 to 5 mg g^{-1} seed. In the case of commercially treated corn seed, this range would be in the saturated region (Fig. 1). The seed treatment application rate has implications in assessing seed lot quality and in agrochemical seed treatment efficacy. High application rates of selected seed treatments may induce phytotoxicity during germination (Taylor et al., 2001), and the initial increase phase is of significance in seed testing when evaluating the risk of phytotoxicity of agrochemicals applied as seed treatments. For example, the triazole fungicides are widely used as seed treatments, but they can inhibit germination due to their plant growth retarding effect (Buchenauer and Röhnet, 1981; Montfort et al., 1996). If the recommended dosage of a compound as a seed treatment was in the range of this initial uptake phase, overdosing or uneven application over the seed surface would increase the risk of phytotoxicity. There are many seed-borne pathogens that may reside within the seeds and could attack seeds during germination. Fungicides or bactericides must be able to permeate the seed coat and diffuse to the embryo to be efficacious to eradicate internal seed-borne pathogens. In our study, the maximum uptake concentration (y_0) ranged from 44.3 to 243 μg g^{-1} seed with the five lots studied (Table 2). Therefore, an internal seed-borne pathogen may not be eradicated if the concentration of an active ingredient is restricted to reach the embryo. Further, resistance may develop with suboptimal concentrations of active ingredient within the seed.

In summary, the maximum uptake limit (y_0) and the dosage concentration to achieve 95% of y_0, calculated as x_{95}, differed between the five seed lots investigated. In the case of corn, seed coat permeability reduced the uptake of both corn lines (Fig. 2). Further, the higher tracer uptake of OH7B was attributed to greater affinity of the embryo + endosperm to absorb or adsorb the tracer compared with B73. In the case of the two yellow-seeded soybean cultivars, both had permeable seed coats, and the seed coat was not a barrier to uptake. In contrast, the low seed coat permeability was the dominant contributor in the black-seeded soybean that limited uptake (y_0) and required the greatest dosage concentration to achieve x_{95}. However, embryo tissues of the three soybean cultivars had similar affinity for the tracer.

Conflict of Interest

The authors declare that there is no conflict of interest.

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Seed Science Research

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Relationships between compound lipophilicity on seed coat permeability and embryo uptake by soybean and corn

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Abstract

Systemic uptake of organic compounds from roots to leaves follows a Gaussian distribution in relation to the lipophilicity, as measured by the log *Kow*. Quantification of compound uptake with different lipophilicities, and applied as a seed treatment that diffuses through the seed coat into the embryo during imbibition, has not been reported. The aim of this investigation was to quantify the uptake of non-ionic compounds into seeds of soybean and corn. A series of fluorescent piperonyl amides were synthesized and a novel combinatorial pharmacodynamic technique was developed that provided a range of compounds from log *Kow* 0.02 to 5.7. Seeds were treated with a mixture of amides, imbibed and compounds chemically extracted and quantified by high-performance liquid chromatography using a fluorescence detector. The maximum uptake efficiency of the applied amide mixture from whole soybean and corn seeds was 67% at log *Kow* 2.9, and 43% at log *Kow* 3.4, respectively. The critical partition coefficient for uptake for both species was <4.2 log *Kow*. Seeds were dissected and separated as soybean embryo and testa, and corn internal tissues (embryo + endosperm) or seed covering layers (pericarp + testa), and >75% of the amides were found in the soybean embryo or corn internal tissues compared with the covering layers at log *Kow* <4.2. The distribution of amides showed that the corn seed covering layer had similar hydrophilic/lipophilic properties as internal tissues, while soybean tissues had different hydrophilic/lipophilic properties. Collectively, the Gaussian uptake pattern for systemic uptake into plants was not found for either seed species.

Introduction

Seed treatments are routinely applied worldwide to protect seeds and plants at the early stages of development from attack by insect pests and pathogens (Brandl, 2001). Active ingredients may have contact activity or be systemic in nature. More recently developed seed treatment agrochemicals, especially the neonicotinoid insecticides and the phenylpyrazole (fiprole) family of compounds, have systemic properties and are taken up by plants to manage aboveground insect pests (Maienfisch *et al.*, 2001; Aajoud *et al.*, 2006; Elbert *et al.*, 2008). Although systemic seed treatments are used commercially in agriculture, little is known about the uptake of active ingredients during imbibition from the applied seed treatment through the seed coat or seed covering layers such as the pericarp/testa in corn to the embryo.

The uptake of organic compounds by roots and the acropetal or apoplastic movement of systemic compounds in xylem have been described (Trapp, 2004; Satchivi, 2014). One of the physical/chemical properties that determines systemic movement in plants is the lipophilicity or hydrophilic/hydrophobic balance of an organic compound, measured as log *Kow*. Log *Kow* is the concentration ratio of a compound in octanol (o) and water (w) expressed on a log scale. The log *Kow* is also referred to in the literature as the log *P* or partition coefficient. There are two components in the pathway for uptake of a systemic compound from roots to leaves. First, the organic compound must have a lipophilic component to be taken up by roots and move to the xylem. Second, the compound must then move up within the transpiration stream to the shoot and leaves. The relationship of the log *Kow* and the transpiration stream concentration factor (TSCF) revealed a normal or Gaussian distribution with the maximum uptake at log *Kow* 1.8 for barley, *Hordeum vulgare* (Briggs *et al.*, 1982) and log *Kow* 3.1 for soybean (Hsu *et al.*, 1990) (Fig. 1). Thus, the movement of water-soluble, polar compounds with low log *Kow* are restricted through the lipid constituents, resulting in selective rejection of these compounds, whereas the passage of water is presumably not hindered (Briggs *et al.*, 1982). In contrast, highly lipophilic compounds have a great affinity for retention in plant roots, and cannot move further into other tissues (Collins *et al.*, 2006).

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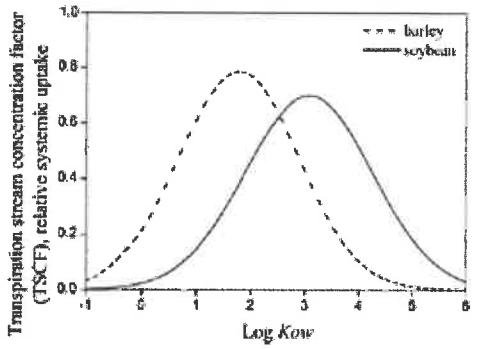

Figure 1. Effect of the log *Kow* of organic compounds on the uptake by roots and subsequent translocation to shoots (dashed line: TSCF, generated by $TSCF = 0.784 \exp -[(\log Kow - 1.78)^2/2.44]$) (equation from Briggs *et al.*, 1982), and soybean (continuous line: $TSCF = 0.7 \exp -[(\log Kow - 3.07)^2/2.78]$) (equation from Hsu *et al.*, 1990).

Our understanding of systemic seed treatment uptake is largely based on research on roots first reported (Briggs *et al.*, 1982)with the assumption that the relationship between the log *Kow* on systemic uptake of compounds is the same as diffusion of compounds through the seed coats to the embryo. However, research in our laboratory revealed that the general uptake pattern found in roots is more complicated in seeds and differed between seed species. Seed coat permeability was examined for eleven seed species representing seven plant families (Taylor and Salanenka, 2012 Dias *et al.*, 2014). Each seed species was grouped into three categories based on the compounds that could diffuse through the seed covering layers to the embryo: (1) permeable, e.g. soybean (Salanenka and Taylor, 2009), pea (*Pisum sativum*); (2) selectively permeable, e.g. corn (Dias *et al.*, 2014), onion (*Allium cepa*), tomato (*Solanum lycopersicum*), pepper (*Capsicum annuum*); and (3) non-permeable, e.g. cucumber (*Cucumis sativus*) and lettuce (*Lactuca sativa*) seeds. The distinction between permeable and selective permeable was that both ionic and non-ionic compounds diffused through the seed coat of permeable seed species, while only non-ionic compounds diffused through the seed coat of selective permeable species (Taylor and Salanenka, 2012). Furthermore, an aqueous pathway was proposed for movement of solutes across the pea (*P. sativum*) seed coat (Niemann *et al.*, 2013). However, it must be noted that pea seeds have a permeable seed coat that would allow either ionic or non-ionic compounds to move through the testa to the embryo (Salanenka and Taylor, 2009; Taylor and Salanenka, 2012; Dias *et al.*, 2014). Therefore uptake of compounds by pea seeds cannot be extrapolated to seed species with the selective or non-permeable seed coat characteristic.

The classification of seed coat permeability as permeable, selective permeable and non-permeable was experimentally determined by use of fluorescent tracer compounds that were applied as seed treatments. The fluorecent tracers eliminated the use of agrochemicals and labelled compounds, and served to mimic agrochemical seed treatment active ingredients. The fluorescent tracers represented a range of log *Kow* from −2.0 to 3.2, and were either non-ionic or ionic in nature (Salanenka and Taylor, 2011). The fluorescent tracer-treated seeds were sown in moistened sand and allowed to imbibe, but were removed from the sand prior to visible germination. The seed covering layers were removed and observed under UVA (365 nm) light. Permeablity was scored as postive, or negative, so all results were qualitative (Salanenka and Taylor, 2009). In addition, only one fluorescent tracer was applied and examined as a single seed treatment, so the log *Kow* and electical charge were fixed for each tracer. More recently, two fluorecent tracers (non-ionic and ionic) were co-applied to examine selective permeability in 27 lines of corn that represented dent, flint, sweet corn and popcorn endosperm types (Dias *et al.*, 2014). Uptake was observed under long UV light and the two tracers were differentiated by the fluorescence colour (blue for coumarin compound and red for rhodamine B).

The question remains if the Gaussian relationship found between the log *Kow* of compounds and systemic uptake in plants (Briggs *et al.*, 1982) is the same as diffusion of compounds through seed coats to the embryo. To examine the relationship between the log *Kow* and seed uptake, a method was used to provide quantitative data on uptake over a wide range of log *Kow* values, termed a combinatorial pharmacodynamic technique. Combinatorial optimization refers to finding an optimal object from a finite set of elements. In our case, determining the compound for optimal uptake from eleven test compounds was conducted. Phamacodynamics is defined as the study of uptake, movement, binding and interactions of pharmacological active molecules at their tissue site(s) of action (Rowland and Tozer, 2011). The compounds were non-ionic so both permeable (soybean) and selective permeable (corn) seeds can be studied. The combinatorial pharmacodynamic technique has been used on plant leaf uptake and transport, and insect topical and oral absorption studies (S. Donovan and B. Black, unpublished observations). This technique is in contrast to traditionally carrying out multiple experiments using heterogeneous compounds on multiple subjects, measuring their absorption, and trying to define the shape of the hydrophilic/lipophilic property space. The combinatorial pharmacodynamic technique gives a degree of precision not attainable by using a series of heterogeneous compounds in multiple experiments. As these amides are devoid of phytochemical toxicity and pesticidal toxicity, they do not perturb their absorption by an unwanted biochemical response. Moreover, ^{14}C-labelled compounds are not used and this therefore avoids waste disposal issues.

Materials and methods

Chemicals and crop seeds

A combinatorial pharmacodynamic technique was used which consisted of a series of 10 *n*-alkyl piperonyl amides that were custom synthesized to produce a series of *n*-alkyl piperonyl amides, *n*-alkyl = Cl, 2, 3, 4, 5, 6, 8, 10, 12 and 14 (Fig. 2). Brief experimental details for the synthesis of the piperonyl amides are as follows: to 3.0 g of piperonylic acid we added 5 ml of thionyl

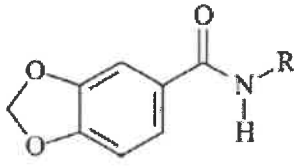

Figure 2. Chemical structure of piperonyl amides of fluorescent tracers ($R = n\text{-}C_nH_{2n+1}$, $n = 1$–14).

chloride and refluxed for 30 min. Then 25 ml toluene, 18.1 mM amines and 5 ml pyridine was added and refluxed for 1 h. The flask was cooled to room temperature, 50 ml ethyl acetate was added, washed with 5% HCl(aq), 5% NaOH(aq), saturated NaCl (aq), and dried over anhydrous sodium sulphate. The solution was filtered, and concentrated on a rotary evaporator. The recrystallization was carried out by adding 50 ml methylcyclohexane, refluxing, and cooling after complete solution was achieved. Some methylene chloride was added to C1, C2, C3 and C4 to achieve complete solution. The clear solution was allowed to slowly cool and then filtered by vacuum. All the reagents we used were Aldrich reagent grade.

There was 0.56 mM of each piperonyl amide in a solution of 70% acetone + 30% water. The HPLC log *Kow* and molecular weight of the *n*-alkyl piperonyl amides series is shown in Table 1. The method for determining the log of the octanol–water partition coefficient for each compound was by high-performance liquid chromatography (HPLC) that used a short octadecyl-poly(vinyl alcohol) column (Donovan and Pescatore, 2002).

Acetonitrile (MeCN) (HPLC grade) purchased from Fisher Scientific (Asheville, NC, USA) was used as the extraction solvent for the *n*-alkyl piperonyl amides. Primary secondary amine (PSA) was purchased from Agilent Technologies (Santa Clara, CA, USA), and graphitized carbon black (GCB) was purchased from Agela Technologies (Wilmington, DE, USA). Anhydrous $MgSO_4$ was from Avantor performance materials, Inc. (Center Valley, PA, USA). For seed coating, diatomaceous earth (DE) was from Celite Corporation (Lompoc, CA, USA), and polyvinyl alcohol (PVA, Selvol 205) was from Sekisui Specialty Chemical Company (Dallas, TX, USA).

Seeds of corn 'D-2901' were provided by the New York Seed Certification program, Cornell University (Ithaca, NY, USA). The seeds of soybean IAR 1902 SCN were obtained from the Iowa State University Research Foundation (Ames, IO, USA). All seeds were not previously treated with agrochemicals, and were stored at 5°C and 40% relative humidity until used for uptake studies.

Coating corn and soybean with piperonyl amide mixtures

A thin adsorbent seed coating was first applied to single seeds to facilitate the high loading rates of the fluorescent tracer series in a single seed treatment. Twenty grams of DE was dispersed in 80 g of 4% PVA aqueous solution to prepare a 20% DE suspension concentrate. Soybean or corn seeds were dipped into 20% DE suspension concentrate one by one to form a continuous coating surrounding each seed with a thickness of <0.5 mm. The wet DE layer on the surface of seeds was dried immediately with a gentle air stream to dry the suspension. The piperonyl amide mixture was then applied with a micropipette to single DE coated seeds of soybean (20 µl) and corn (40 µl), resulting in 11.2 and 22.4 nmol per soybean and corn seed, respectively. The treated seeds were blown with a gentle air stream immediately until acetone and water totally evaporated (<1 min).

Imbibition and separation of seed tissues

Coated seeds were sown in sand moistened to 20% and maintained at 20°C. The soybean and corn were removed from the moistened sand prior to visible germination, 12 and 20 h after sowing, respectively. Each imbibed seed was then washed with water to remove the coating that contained any residual piperonyl amides. The testa of 10 soybean seeds were separated from the embryos, and were pooled together as one sample (rep). The pericarp + testa of 10 corn were separated from endosperm + embryo, and were pooled together as one sample (rep). The pericarp + testa were termed the covering layers, while the endosperm + embryo were termed internal tissues. There were four replicates for each treatment.

Preparing soybean embryos and corn internal tissues samples for HPLC analysis

The soybean embryo and corn internal tissues samples were extracted with the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method with modifications for soybean and corn seeds (Anastassiades *et al.*, 2003). Briefly, ten soybean embryos or ten corn embryo + endosperm were placed into a frozen mortar and frozen with liquid nitrogen, and ground into a fine powder. The powder was transferred into a 50 ml centrifuge tube with a screw cap, and 8 ml of MeCN was added and the mixture was shaken for 2 min using a Vortex mixer at room temperature. Following this, a mixture of 2.5 g of $MgSO_4$ and 1.0 g of NaCl was added. The tube was immediately shaken vigorously for 1 min to prevent the formation of $MgSO_4$ agglomerates, and centrifuged at 3500 r.p.m. for 5 min. Then 3.0 ml of the supernatant was subjected to dispersive SPE (solid phase extraction) using a mixture of 8 mg GCB, 50 mg PSA and 100 mg $MgSO_4$. The mixture was shaken vigorously for 1 min using a Vortex mixer. Finally, the extract was filtered through a 0.22 µm syringe filter. In developing the HPLC method, the per cent recoveries were determined for the eleven amides from soybean embryo + testa, and corn endosperm + embryo and pericarp + testa. The recovery at ≤3.82 log *Kow* for both seed tissues was >82% for soybean and >85% for corn.

Preparing soybean testa and corn seed covering layers for HPLC analysis

The covering layers samples were pretreated with a modified QuEChERS method (Anastassiades *et al.*, 2003). Briefly, the testa of ten soybean or covering layers of corn were immersed in 1.5 ml of acetonitrile overnight. The extract was transferred into a tube containing 20 mg PSA, 5 mg GCB and 50 mg $MgSO_4$. The mixture was shaken for 1 min. Finally, the extract was filtered through a 0.22 µm syringe filter. Preliminary studies revealed that recovery at ≤3.82 log *Kow* was >84% for soybean and >92% for corn.

HPLC analysis of amides content

The amides content was determined using an Agilent 1100 HPLC equipped with a 1200 fluorescence detector (FLD) using an ODS 3 column (GL Sciences Inc., 5 µm, 4.6 mm×75 mm column). The mobile phase used was 0 min 30% MeCN + 70% water, 22 min 40% MeCN + 60% water, 25 min 80% MeCN + 20% water, 40 min 90% MeCN + 10% water. The temperature of the column was 30°C. The injection volume was 20 µl. The wavelengths of FLD were set at 292 nm (excitation) and 340 nm (emission). The retention time in minutes for each amide is shown in Table 1.

Table 1. The HPLC log *Kow*, molecular weight and retention time on the HPLC column of eleven n-alkyl piperonyl amides

n-alkyl	C1	C2	C3	C4	C5	C6	C7	C8	C10	C12	C14
Log *Kow*	0.02	0.66	1.43	2.23	2.88	3.39	3.82	4.18	4.78	5.26	5.66
Molecular weight	179.2	193.2	207.2	221.3	235.3	249.3	263.3	277.4	305.4	333.5	361.5
Retention time (min)	3.63	5.94	10.38	15.43	18.59	21.04	23.15	24.96	27.70	31.08	35.58

Data calculation

Per cent uptake in relation to the maximal log *Kow* (relative amount) =

$$\frac{\text{Concentration of each amide in tissue}}{\text{Concentration of the amide at the maximal log } Kow \text{ in the same tissue}} \times 100\%.$$

Per cent uptake based on amount applied (uptake efficiency) =

$$\frac{\text{Amount of each amide absorbed by a seed}}{\text{Applied amount of each amide}} \times 100\%.$$

Per cent distribution within seed =

$$\frac{\text{Amount of each amide in the covering or internal tissues}}{\text{Sum amount of each amide in the covering + internal tissues}} \times 100\%.$$

Results

Effects of log Kow on maximal uptake of piperonyl amides by soybean and corn seed tissues

The relative amount of piperonyl amides in soybean seed tissues is shown in relation to the maximal (100%) log *Kow* value (Fig. 3). The soybean embryo had a different uptake pattern compared with the testa (Fig. 3). The maximum embryo content of amides was at log *Kow* 2.88, while the testa had 39 ± 3.3% at the same log *Kow*. Meanwhile, the maximum amount in the testa was at log *Kow* 4.18, while only 47 ± 1.0% was in the embryo. The soybean testa revealed a decreased permeability to hydrophilic amides from 0.02 to 2.88, then increased permeability to moderately lipophilic amides from 2.88 to 4.18 log *Kow*, then a sharp decrease in permeability >4.18 log *Kow*.

The relative amount of piperonyl amides in corn seed internal (endosperm + embryo) tissues and covering (pericarp + testa) layers is shown in relation the maximal log *Kow* value (Fig. 4). The curve of internal tissues had a similar trend to seed covering layers, and both tissues had the same maximum at log *Kow* 3.39. However, the curves of internal tissues and covering layers were asymmetric. At log *Kow* 0.02, the relative amount of piperonyl amide was 56 ± 1.4% in the covering layers, and 36 ± 1.9% in the internal tissues. At >3.82 log *Kow*, the permeability was greater in the covering layers than the internal tissues, and at log *Kow* 4.78 the relative amount was 36 ± 1.4% in the covering layers compared with only 4 ± 0.2% in the internal tissues.

Per cent uptake efficiency of piperonyl amides compared with amount applied in soybean and corn seeds

A known dosage was applied to single seeds so the uptake efficiency or per cent recovery was calculated for each piperonyl amide for both soybean and corn. The uptake efficiency of piperonyl amides by whole soybean seeds was 50 ± 3.1% at log *Kow* 0.02 (Fig. 5). The maximum uptake was 67 ± 4.6% at log *Kow* 2.88 and then decreased to 35 ± 2.8% at log *Kow* 4.18. The uptake efficiency of piperonyl amides is shown for corn seeds (Fig. 5). The uptake efficiency increased from 17 ± 0.9 to 43.0 ± 0.8% as

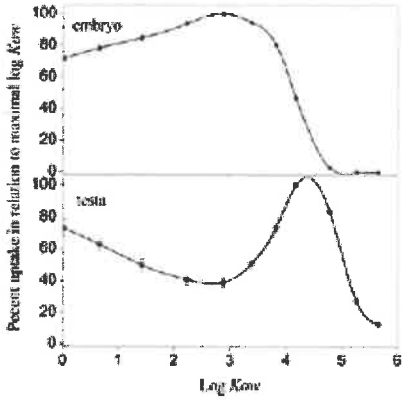

Figure 3. The uptake of piperonyl amides into soybean seeds in relation to maximal log *Kow*. The bars represent ±SE from four replicates.

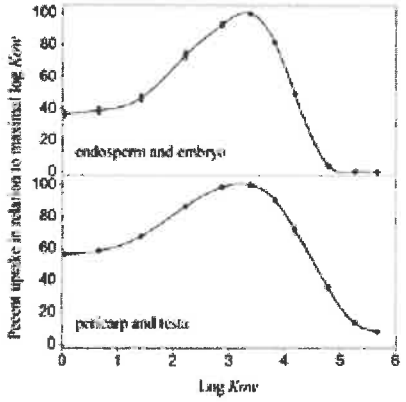

Figure 4. The uptake of piperonyl amides into corn seeds in relation to maximal log *Kow*. The bars represent ±SE from four replicates.

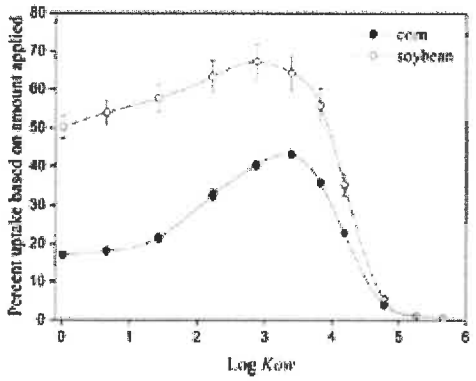

Figure 5. Per cent uptake of piperonyl amides compared with amount applied in soybean and corn seeds. The bars represent ± SE from four replicates.

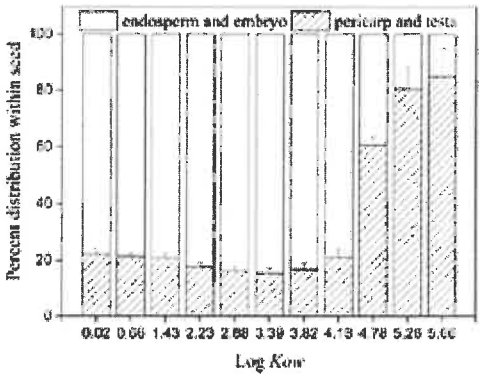

Figure 7. Distribution of absorbed piperonyl amides in seed tissues of corn seeds. The bars represent ± SE from four replicates.

log *Kow* increased from 0.02 to 3.39, respectively. Uptake efficiency decreased from 36 ± 0.6% at log *Kow* 3.82 to 0.6 ± 0.2% at log *Kow* 5.66. Overall, soybean had greater per cent uptake efficiency than corn.

Distribution of piperonyl amides between seed tissues of soybean and corn seeds

The distribution of absorbed piperonyl amides was determined between the embryo and testa of soybean (Fig. 6). The highest percentage of amides (>90%) was measured in the embryo from log *Kow* 0.02 to 3.82 compared with the testa. Then a shift in the distribution occurred at log *Kow* >4.18, and <35% of the amides were detected in the embryo at log *Kow* 4.78 to 5.66.

A similar distribution trend was measured for corn seeds (Fig. 7) as found in soybeans. The highest percentage of amides (>78%) was found in the internal tissues from log *Kow* 0.02 to 4.18 compared with the covering layers. Then the distribution changed with <39% in the internal tissues at log *Kow* 4.78 and higher, compared with the covering layers.

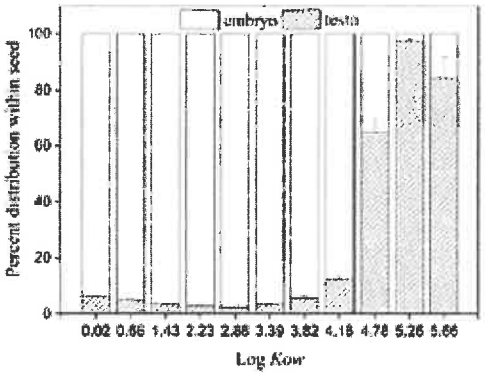

Figure 6. Distribution of absorbed piperonyl amides in seed tissues of soybean seeds. The bars represent ± SE from four replicates.

Discussion

This investigation focused on the lipophilicity of organic compounds in relation to uptake into the embryo and testa of soybean, and internal tissues and covering layers of corn seeds. Lipophilicity, measured by the log *Kow* or log *P* is the affinity of compounds for the lipid phase of plant tissues (plasma membrane, waxes, cutin, suberin, etc.) (Braumann, 1986). To examine a range of log *Kow* compounds in a single treatment, a homologous series of piperonyl amides were synthesized that increased the hydrophobicity of the molecule from log *Kow* 0.02 (C = 1) to 5.66 (C = 14) (Table 1). The log *Kow* values were measured by a HPLC technique, which is a more precise measure of hydrophobicity since it is from a chromatographic index, as opposed to a measure of lipophilicity when measured from a biphasic index (Donovan and Pescatore, 2002). There are a number of reports from investigators who support that HPLC-based log *Kow* derived values can and should correlate better to biological QSAR (quantitative structure–activity relationship) than the shake flask log *Kow* derived values (Braumann, 1986; Hsieh and Dorsey, 1995).

The mechanistic description of the uptake of an organic compound was first described for oral pharmaceuticals. Lipinski's rule of five or simply the rule of five (RO5) is a rule of thumb to evaluate drug-likeness or determine if a chemical compound with a certain pharmacological or biological activity has properties that would make it a likely orally active drug in humans (Lipinski *et al.*, 2001):

- An octanol-water partition coefficient log *Kow* not greater than 5;
- A molecular mass less than 500 daltons;
- No more than five hydrogen bond donors (the total number of nitrogen–hydrogen and oxygen–hydrogen bonds);
- No more than 10 hydrogen bond acceptors (all nitrogen or oxygen atoms);

The RO5 was coined as all numbers are multiples of five, although there are only four rules. In the field of agrochemical discovery, Lipinski's RO5 approach was quickly adopted to profile agrochemicals (Tice, 2001; Hao *et al.*, 2011). Tice (2001) studied herbicides, and soil applied herbicides share the same common

environment of treated seeds sown in the field. The number of rotatable bonds (<12) was added as a rule resulting in the 'Agricultural Rule of Five' (Avram *et al.*, 2014). Rotatable bond is defined as any single non-ring bond, bound to a non-terminal heavy (i.e. non-hydrogen) atom (Veber *et al.*, 2002). All piperonyl amides with log *Kow* <5 are in agreement with all RO5, and have less than 12 rotatable bonds, thus are in agreement with this additional rule or criterion. Therefore, the RO5 pertains to physical-chemical properties of molecules that are pharmaceuticals, agrochemicals or other organic compounds. The range of log *Kow* 0.02 to 5.66 was chosen for this investigation as it encompasses the log *Kow* range suggested by the RO5. Piperonyl amides are not agrochemicals, but are representative molecules with a common flurochrome for all compounds that permit detection with a HPLC with a fluorescent detector. Collectively, this combinatorial pharmacodynamic technique provided a valuable tool to conduct research in seed biology.

Uptake in relation to the maximal log *Kow* compound (designated as 100%) differed between the embryo and testa of soybean (Fig. 3), and there were contrasting trends between seed tissues over the range of log *Kow* from 0.02 to 4.18. Uptake increased in the embryo and decreased in the testa from log *Kow* 0.02 to 2.88, while the trend reversed between tissues from log *Kow* 2.88 to 4.18. At low *Kow* >4.18, there was decreased uptake by both tissues. This indicated that the testa and embryo of soybean seeds had different hydrophilic/lipophilic properties. Collectively, the embryos of soybean had a high permeability to hydrophilic amides (log *Kow* 0.02~3.39), but limited permeability to lipophilic amides (log *Kow* >3.82). In contrast, the soybean testa allowed unrestricted transport of polar compounds to the embryo, but acted as a barrier and/or had greater retention of lipophilic compounds in the range of log *Kow* 3.82 to 4.78.

There may be two modes of transport in the soybean testa. The first decrease from log *Kow* 0.02 to 2.88 may be attributed the amide water solubility (Fig. 3). As log *Kow* decreased, the aqueous solubility increased, so hydrophilic amides would move more readily with water uptake during imbibition. In support, an aqueous pathway was found in pea seeds (Niemann *et al.*, 2013), and pea, like soybean, has the permeable seed coat characteristic (Taylor and Salanenka, 2012). The optimum uptake between log *Kow* 4.18 and 4.78 may be due to the hydrophobic characteristic of the testa favouring uptake in this region. The overall net effect would be the sum of the two modes of uptake.

Corn showed similar trends between the seed covering and internal tissues and the maximal uptake for both seed parts was at log *Kow* 3.39 (Fig. 4). This result indicated that the covering layers of corn seeds were similar in permeability to internal tissues, but curves of both tissues were not symmetrical. Covering layers had greater permeability to highly lipophilic amides (>4.78 log *Kow*) compared with internal tissues. Tomato seeds, like corn, have selective seed coat permeability (Taylor and Salanenka, 2012) and were examined in a similar experiment using the combinatorial pharmacodynamic technique (Yang *et al.*, 2017). The uptake pattern was more normally distributed in tomato than corn, and even varietal differences were measured for tomato. Differences in uptake patterns are attributed to the seed coat composition. The inner layer of the tomato seed coat was composed of suberin (Beresniewicz *et al.*, 1995), and resembled the lipophilic nature of roots, thus reducing uptake of organic compounds with log *Kow* <2.

The overall uptake trend was similar for soybean and corn on the whole seed basis and uptake gradually increased from log *Kow* 0.02 to about 3.0, followed by a steep decline at >3.82 log *Kow* (Fig. 5). Therefore, a normal distribution was not measured for seed uptake of both crops in relation to log *Kow*. Moreover, soybean seed had greater permeability to the piperonyl amides than corn seed over the range of log *Kow* 0.02 to 4.18. Whole plant uptake revealed a Gaussian distribution for uptake in both barley (Briggs *et al.*, 1982) and soybean (Hsu *et al.*, 1990). In subsequent studies, other Gaussian equations were established to describe the root-to-shoot translocation of plants (Collins *et al.*, 2006). In contrast, the log *Kow* dependent uptake curves of soybean and corn seeds were asymmetrical so do not satisfy a Gaussian model. Furthermore, the maximal TSCF was at 1.8 log *Kow*, while the maximal per cent uptake of soybean and corn seeds was log *Kow* 2.88 and 3.39, respectively. Collectively, the uptake characteristics of seeds were not related systemic uptake patterns in whole plants. This was attributed to chemical composition of seed covering layers and internal tissues that affected permeability and affinity of the piperonyl amide compounds.

The distribution of piperonyl amides between testa or seed covering layers and embryo or internal tissues revealed similar patterns (Figs 6 and 7). In soybean, <15% of the piperonyl amides was measured in the testa from log *Kow* 0.02 to 4.18 (Fig. 6), compared with <25% in covering layers of corn (Fig. 7). A sharp shift in distribution occurred at >4.78 log *Kow* with >60% in the seed covering layer(s). Therefore, hydrophobic amides were retained in the seed coat covering layer(s) or due to their limited water solubility did not diffuse through the seed coat. In support of distribution of tracers in different seed parts, the affinity and mobility of fungicides were related to the partition or binding in the tissues of plants (Uchida, 1980), and the lipid content is one of the controlling factors (Schwab *et al.*, 1998).

The greater distribution of the piperonyl amides with log *Kow* <4.18 in soybean embryo compared with the testa (Fig. 6), and corn internal tissues compared with the covering layers (Fig. 7) was partially attributed to <10% of the total seed weight being composed of seed covering layers. Due to the small contribution of the soybean testa to the total seed weight (Figs 3 and 6), there was little effect on the whole seed trend (Fig. 5). In corn, the average concentration of each amide was 37 nmol g^{-1} in the seed covering layers and 13 nmol g^{-1} in the internal tissues. Therefore, there was a concentration gradient from the seed covering layers to the internal tissues. Conversely, in soybean, the average concentration of amides <4.18 log *Kow* was 8.6 nmol g^{-1} in the testa and 23 nmol g^{-1} in the embryo. So the soybean seed coat is permeable and does not act as a barrier to the movement of the amides with lower log *Kow* values. Water uptake is not uniform over the entire seed surface, and water was found to be taken up rapidly through the raphe region to the hilum of soybean seed (Koizumi *et al.*, 2008; Koizumi and Kano, 2014). In corn seeds, rapid water uptake was measured through the black layer (McDonald *et al.*, 1994). The uptake of organic compounds may follow specific pathways through the seed coat. Diffusion of a non-ionic fluorescent tracer from the environment to the cucumber embryo was through the micropylar canal (Salanenka *et al.*, 2009). Aqueous pathways dominated the permeation of solutes across pea seed coats by diffusion and bulk flow of water (Niemann *et al.*, 2013). Assuming that soybean seed coat permeability was similar to pea seeds, then hydrophilic compounds could readily pass from the environment though the water-filled narrow aqueous pathways to the embryo.

Our data provide a new understanding of seed coat permeability and uptake over a range of log *Kow* values. This investigation was

conducted with one low dosage, so cannot be extrapolated to the wide range of dosages used in commercial agrochemical seed treatments. Therefore, more work is needed to understand the effect of dosage rate on the uptake of a single non-ionic tracer in seeds. Collectively, this paper provides insight on the expected uptake of agrochemicals with known log *Kow* applied as seed treatments.

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