Utilization of sophorolipids as biosurfactants for postemergence herbicides∗

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Sophorolipids are carbohydrate-based, amphiphilic biosurfactants produced by several species of the Starmerella yeast clade. Most sophorolipids are partially acetylated sophorose sugars O-β-glycosidically linked to 17-L-hydroxy-Δ9-octadecenoic acid, where typically the acyl carboxyl group forms a 400-lactone to the terminal glucosyl residue. Recently sophorolipids were discovered in which the sophorose is linked to the ω-carbon of the acyl group and occurs predominately in a non-lactone, anionic form. In this study we compared lactone sophorolipids produced by Starmerella (Candida) bombicola (Sb) and non-lactone sophorolipids produced by Candida kuii (Ck) against a synthetic polyethoxylated tallowamine surfactant (POEA) which is used in commercial postemergence herbicides. When mixed with the lipophilic contact herbicide lemongrass oil (LGO), stable emulsions with Ck lasted longer than with either POEA or Sb. Phytotoxicity (as measured by fresh and dry weights and visual damage three days after spraying) to sicklepod (Senna obtusifolia) by the Ck/LGO and Sb/LGO mixtures were similar to a POEA/LGO mixture, while visual damage to corn (Zea mays L.) was increased by the addition of all of the surfactants. When applied together with the water-soluble herbicide phosphinothricin, the Ck/LGO and Sb/LGO treatments caused decreases in sicklepod dry weights and herbicide damage ratings (HDR) compared to phosphinothricin applied without a surfactant ten days after treatment. With corn, POEA and Ck applied with PT had the greatest reductions in fresh and dry weights, and HDR values. These results indicate that sophorolipids have excellent promise as natural surfactants for postemergence herbicides.

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1. Introduction

Essentially all postemergence herbicide formulations contain adjuvants to allow greater adherence to the plant surface and for increased penetration of the plant cuticle (Monaco et al., 2002). Non-ionic surfactants, such as polyethoxylated tallowamines (POEAs), are the most widely used adjuvants (Foy and Pritchard, 1996), and are currently used in several postemergence herbicides including glyphosate (e.g. Monsanto's Roundup) and DL-phosphinothricin (e.g. Bayer CropScience's Liberty). Most surfactants including POEAs are petroleum-based (Hauthal, 2004; Van Bogaert et al., 2007), are among the most ubiquitous contaminants in aquatic systems, and have been implicated as contributors to the global decline of amphibian populations (Mann and Bidwell, 2000; Mann and Boddy, 2000; Mann and Bidwell, 2001). Several studies have shown POEAs as being toxic to a wide range of organisms (Folmar et al., 1979; Tsui and Chu, 2003; Howe et al., 2004; Relyea, 2005a, b; Brausch and Smith, 2007) as well as inducing necrosis and apoptosis in human cells (Benachour and Séralini, 2009).

Commercial interest in naturally-produced surfactants (also called biosurfactants) that are synthesized by living cells has increased in recent years due to their chemical diversity,
environmentally-friendly nature, feasibility of large-scale production, selectivity, performance under extreme conditions, and potential applications in environmental protection (Marchant and Banat, 2012). However, their use in agricultural applications is still rare (Sachdev and Cameotra, 2013). One particularly promising class of biosurfactants are sophorolipids, which consist of a hydrophobic fatty acid tail of 16 or 18 carbons and a carbohydrate head of the glucose disaccharide sophorose. Sophorolipids are synthesized by a phylogenetically diverse group within the Star-merella yeast clade (Van Bogaert et al., 2007; Develter and Laurysen, 2010; Kurtzman et al., 2010; Van Bogaert et al., 2011). Sophorolipids produced by the most commonly-used strain, Starmarella bombicola, are generally partially acetylated 2-O-β-D-glu-copyranosyl-D-glucopyranose units attached β-glycosidically to 17-L-hydroxyoctadecanoic or 17-L-hydroxy-∆9-octadecenoic acid and can be acetylated at the 6′- and/or 6″ positions (Fig. 1A; Tulloch et al., 1962, 1968). The fatty acid carboxyl group may be either free or internally esterified at the 4″-position, forming a lactone. Sophorolipids are highly biodegradable, and have acute and chronic toxicities more than ten-fold less than conventional surfactants (Van Bogaert et al., 2011). Additionally, sophorolipids can be produced in high yields in bioreactor culture (>400 g/L), and unlike rhamnolipid biosurfactants produced by Pseudomonas aeruginosa, sophorolipids are produced by nonpathogenic yeasts (Van Bogaert et al., 2007; Marchant and Banat, 2012).

Recently, a novel yeast strain, Candida kuii NRRL Y-27208, has been identified which synthesizes significant amounts of a novel sophorolipid containing an ω-hydroxy-linked acyl group and occurs predominantly in a non-lactone (i.e. acyclic open chain), anionic form (Fig. 1B; Kurtzman, 2012; Price et al., 2012). In this study we compare the non-lactone, anionic form from C. kuii, the lactone sophorolipid from S. bombicola and a commercial POEA surfactant for their efficacy as adjuvants in formulations with the postemergence herbicides phosphinothricin and lemongrass oil.

2. Materials and methods

2.1. Chemicals

DL-Phosphinothricin (PT; glufosinate-ammonium) was obtained from Sigma−Aldrich, St. Louis, MO, USA. PT is a water soluble compound whose herbicidal activity is due to it being a glutamine synthetase inhibitor, and the compound is translocated throughout the plant, giving it systemic activity (Lea et al., 1984). Lemongrass oil (LGO; steam distilled from Cymbopogon flexuosus and containing ~70% citral) was obtained from 100PureEssential Oils, Mechanicsburg, PA, USA. LGO is certified as an organic herbicide but unlike PT is not water soluble. Because LGO does not translocate, only the portions of the plant sprayed are affected, and its herbicidal activity is contact only and not systemic (Dayan et al., 2009). The commercial POEAs Surfonic T-5 (T-5; MW = 490 g/mol), T-10 (T-10; MW = 710 g/mol), and T-15 (T-15; MW = 908 g/mol) were obtained from Huntsman Corporation, Salt Lake City, UT.

2.2. Yeast cultivation and extraction of sophorolipids

C. kuii NRRL Y-27208 and S. bombicola NRRL Y-17069 were obtained from the ARS Culture Collection (NRRL), National Center for Agricultural Utilization Research, Peoria, IL, and maintained on YM medium (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 10 g/L glucose, 20 g/L agar). Inocula for sophorolipid production were prepared by growing each strain for 22 h in a 250-ml baffled flask containing 60 ml of sophorolipid medium (100 g/L glucose, 80 g/L oleic acid, 1.5 g/L yeast extract, 4 g/L NH₄Cl, 1 g/L KH₂PO₄, H₂O, 0.1 g/L NaCl, 0.5 g/L MgSO₄·7H₂O) using an Innova 44 rotary shaker (New Brunswick, Enfield, CT) set at 200 rpm and 30 °C. DASGIP 1500 ml bioreactor vessels (Jülich, Germany) containing 1000 ml of sophorolipid medium were then inoculated at 5% with the overnight cultures and grown with aerating at 0.9 vvm using an L-sparger, stirring at 700 rpm using two Rushton impellers offset by 4 cm, and pH control at 3.5 with 4 M NaOH. Samples were taken daily to analyze supernatant for glucose consumption using HPLC analysis with an HPX-87H column (Bio-Rad Laboratories, Hercules, CA) and a Waters 410 Differential Refractometer (Milford, MA). If residual glucose fell below 30 g/L, an additional 50 g of glucose was added to the bioreactor. If available oleic acid fell below 1–2% by visual assessment (i.e., oleic acid was a separate layer after centrifuging culture) or the presence of foaming, an additional 40 g of oleic acid was added to the bioreactor. Over the course of 6 days, cultures were supplemented with additional glucose on days 3 and 4; and additional oleic acid on days 4 and 5.

2.3. Chemical testing

The sophorolipid preparations were analyzed by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF/MS) and gas chromatography−mass spectroscopy (GC–MS) as described previously in detail (Price et al., 2012). Briefly, samples were mixed with 2.5-dihydroxybenzoic acid (2.5-DHB) matrix in acetonitrile and applied to the MS target. Spectra were acquired on a Bruker-Daltonics Microflex instrument in reflectron mode (Bruker-Daltonics, Billerica, MA, USA). Ion source 1 was set to 19.0 kV and source 2–15.9 kV (83.7% of IS 1), with lens and reflector voltages of 9.79 and 19.99 kV, respectively. C. kuii sophorolipids were characterized by m/z 729, 687 and 645 ions corresponding to open chain sophorolipids containing 2, 1 and 0 acetyl groups, respectively. S. bombicola sophorolipids were apparent as corresponding lactone-form ions at m/z 711, 669 and 623, 18 Da less than the open chain form due to the absence of a water molecule. Fatty acid compositions were determined by GC/
MS as the fatty acid methyl esters, and the sophorolipid preparations did not contain any residual oleic acid.

2.4. Recovery of sophorolipids from microbial cultures

Sophorolipids were recovered from the microbial cultures of *S. bombicola* and *C. kuoi*. The cultures were allowed to stand overnight at 4 °C. A dense molasses/oil separated at the bottom of the *S. bombicola* culture, and was recovered by decanting off the upper aqueous emulsion layer. Initially this layer was a viscous liquid, but it crystallized overnight in a refrigerator. The yield of this material was 44.7 g. It was shown to be sophorolipid in the lactone form by MALDI-TOF/MS and GC/MS. The remaining aqueous emulsion was extracted with ethyl acetate in a separatory funnel, and the layers were allowed to separate. The upper organic layer was drawn off and rotary evaporated at 60 °C. This material yielded 12.7 g of sophorolipids after standing overnight in a refrigerator.

The *C. kuoi* culture was a more stable emulsion, but separated off as an off-white solid on the surface after standing at 4 °C. This was recovered and resuspended in absolute ethanol (200 ml). After stirring with a glass rod to break up the solid, the eluent was filtered through a cotton pad and collected and evaporated to dryness with a yield of 31.4 g. MALDI-TOF/MS analysis indicated that it was the open-chain, non-lactone sophorolipid (termed Ck). Unlike the *S. bombicola* sophorolipid (termed Sb), it did not crystallize, but remained as a viscous liquid with the color and consistency of honey. Sophorolipids were stored at 4 °C and 10.0 g of each preparation were used for plant tests.

2.5. Emulsification of LGO with surfactants

The emulsion stability of 15% LGO in water mixtures were assessed visually at times 0 min, 15 min, 1 h, 3 h and 21 h and compared to those additionally containing 1% (v/v) POEAs (T-5, T-10 and T-15), Ck and Sb. The mixtures were vortexed in 7 ml glass stoppered tubes for 1 min and allowed to stand at a 45° angle for the specified times at room temperature (23 °C). The emulsion stabilities were assessed visually and photographed at the prescribed times.

2.6. Seeds, plant growth, herbicide treatments, and spraying apparatus

Non-transgenic corn (*Zea mays* L. ‘Silver Queen’) seeds were obtained from Johnny’s Selected Seeds, Winslow, ME, USA. Sicklepod (*Senna obtusifolia* (L.) H.S. Irwin & Barneby) seeds were collected from a wild population in Athens, GA, USA. These plants were chosen as bioassay species as they have very high germination rates and are normally harder to kill than many of the weedy species we have studied, making them better indicators of herbicidal activity.

Seeds of both species were planted in six-cell plastic landscape tray inserts (Sunshine Redi-Earth tray inserts (T.O. Plastics, Clearwater, MN) filled with a commercial potting substrate (Sunshine Redi-Earth® Professional Growing Mix, Sun Gro Horticulture, Seba Beach, AB, Canada) supplemented with Osmocote® 14-14-14 and MicroMax® chemical fertilizers (The Scotts Company LLC, Maryville, OH) at 23 and 3.5 g fertilizer/kg substrate, respectively. Each insert containing six plants constituted a replication. Plants were placed in a growth chamber set at a 16 h, 25 °C day/8 h, 20 °C night regime, and watered with distilled water as needed. Each treatment was replicated six times in a randomized complete block design, and the experiment was repeated using the same growth chamber. Postemergence herbicide applications were performed fourteen days after seedling emergence for both species using a Research Track Sprayer (DeVries Manufacturing, Hollandale, MN, USA) equipped with a model Teejet 808VS nozzle (Teejet technologies, Wheaton, IL, USA) with a conical pattern and 80° spray angle. The height of the nozzle to the insert soil level was 45 cm for all experiments. The spray head moved at a rate of 2.0 km/h at a pressure of 275 KPa and delivered a volume of 3.0 L/min.

2.7. Postemergence herbicidal activity of LGO and PT with or without surfactants

T-15 was chosen as the POEA for testing over T-5 and T-10 as it exhibited the best emulsifying abilities of the three surfactants. Treatments using LGO as the herbicide consisted of 1% (v/v) T-15, Ck and Sb alone and in combination with 4% (v/v) LGO. Treatments using PT as the herbicide consisted of 1% (v/v) T-15, Ck and Sb alone and in combination with 1.0 mg/ml PT. For both LGO and PT studies the control was distilled water applied without any surfactant. The treatments had pH values ranging from 6.5 to 7.0 for the two sophorolipid preparations and 7.5 to 8.0 for the T-15 preparations. For both LGO and PT, the herbicide concentrations were lower than the recommended rates in order to better evaluate the role of the surfactants in increasing herbicide efficacy. After spraying, plants were returned to the growth chamber, and after 3 days (for LGO treatments as phytoxic symptoms occur almost immediately after spraying and are maximal at this time period) or 10 days (for PT treatments as this herbicide is translocated and phytoxicity does not appear for several days). Herbicide damage ratings (HDR) using...
a 0–100 scale were determined based on visual phytotoxicity symptoms (Frans et al., 1986). Whole plants were harvested and fresh weights measured after rinsing off any substrate, and plants were then dried in an oven at 60 °C for 72 h to obtain dry weights.

2.8. Statistical design and analyses of postemergence herbicidal activity

All statistical analyses were performed using SAS Version 9.2 (SAS Institute, Inc., Cary, NC, USA). Plants were arranged in the growth chambers in a completely randomized design with five replicates per treatment and six plants per replicate, and each study was replicated over 2 experiments using the same growth chamber. A single-factor mixed model replicated experiment analysis of variance was used to analyze treatments separately for each herbicide and plant species. ANOVA treatment differences were considered to be significant at $p \leq 0.10$. Differences of least squares means was conducted at $p \leq 0.10$. Levene's homogeneity of variance test was applied to determine transformation necessity. All analyses were performed on transformed data where necessary, but raw data means are presented for ease of interpretation.

3. Results and discussion

3.1. Effect of surfactants on emulsification of LGO

The ability of T-15, Ck and Sb to emulsify LGO over an extended period of time is shown in Fig. 2. The emulsion stability of LGO-water mixtures was assessed visually and photographed over a time course of 0 min to 21 h. The emulsifiers POEA (T-5, T-10 and T-15), Ck and Sb were all compared to the surfactant-free control. The aqueous LGO mixtures were totally immiscible in the absence of an emulsifier (Fig. 2, column 1), and all five emulsifiers produced immediate emulsions. The three POEAs are high foam producers compared to the sophorolipids, resulting in stable head foams (Fig. 2, columns 2–4). The lactone form Sb was a noticeably less effective emulsifier, and by the 21 h time point the LGO-water layers had separated entirely (Fig. 2, column 6). By comparison, the non-lactone Ck performed as well as the commercial POEAs, with the additional advantage of creating reduced foaming (Fig. 2, column 5). It was also noted that Sb was significantly more difficult to solubilize than Ck, and it was also noted that Sb was prone to forming clumps that might result in blocking spray nozzles.

All emulsions consist of a continuous phase and a discontinuous phase, which in the present case water is the continuous phase while LGO is the discontinuous phase. Lipophilic herbicides such as LGO require an emulsifying agent to prevent the herbicide from separating into two distinct phases, which drastically affect the uniformity of application, causing problems with spraying equipment (Monaco et al., 2002).

Because of the amphiphilic nature of surfactants, they are typically classified by their hydrophilic–lipophilic balance (HLB), which is a measure of the water-lipid solubility, with a sliding scale range of 0–20, with values less than 10 being lipophilic and greater than 10 being hydrophilic. The ability of a surfactant to enhance herbicide penetration into the target plants is partially attributable to the HLB, with each herbicide having optimum HLB values. The HLB of sophorolipids is from 10 to 13, making them useful as emulsifiers for a wide range of herbicides (Van Bogaert et al., 2007).

Surface tension properties of the two sophorolipids had been previously determined using the pendant drop method, in which Ck had a critical micelle concentration (CMC) of 46.4 mg/L as compared to 5.6 or 6.9 mg/L for Sb (Price et al., 2012). Additionally, the absence of foaming by the sophorolipid preparations is highly desirable as antifoam agents are often added to herbicide formulations to suppress foaming and minimize air entrapment, which can cause pump and sprayer malfunctions.

3.2. Effect of surfactants on PT herbicidal activity

PT applied with the addition of T-15 and the two sophorolipids caused significant decreases in fresh weights, dry weights and HDR values of sicklepod as compared to the control (Table 2). Because the plants grew an additional 7 days, the absolute values are much higher than with the LGO treatments. Dry weights of plants treated with PT plus the surfactants were all less than PT without a surfactant, while HDR values for PT plus any of the surfactants was higher than PT applied alone.

In general, corn was much less affected by PT treatment than was sicklepod, with PT applied without any surfactant having similar herbicidal activity to the control and all three surfactants applied alone (Table 2). Only the PT/T-15 and PT/Ck treatments caused significant decreases in dry weights, although all treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sicklepod</th>
<th>Corn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh weight (g)$^a$</td>
<td>Dry weight (g)</td>
</tr>
<tr>
<td>Control</td>
<td>9.22 a</td>
<td>0.90 a</td>
</tr>
<tr>
<td>T-15</td>
<td>8.77 a</td>
<td>0.84 a</td>
</tr>
<tr>
<td>Ck</td>
<td>9.02 a</td>
<td>0.81 ab</td>
</tr>
<tr>
<td>Sb</td>
<td>8.24 a</td>
<td>0.73 abc</td>
</tr>
<tr>
<td>LGO</td>
<td>6.57 b</td>
<td>0.57 cde</td>
</tr>
<tr>
<td>LGO + T-15</td>
<td>5.16 b</td>
<td>0.46 de</td>
</tr>
<tr>
<td>LGO + Ck</td>
<td>4.52 b</td>
<td>0.44 e</td>
</tr>
<tr>
<td>LGO + Sb</td>
<td>4.68 b</td>
<td>0.51 cde</td>
</tr>
</tbody>
</table>

* Means within a column followed by the same letters are not significantly different based on differences of least-squares means at $p \leq 0.10$. 
produced synthetically (Van Bogaert et al., 2007). Surfactants and adjuvants used in agriculture are petroleum-based and prohibit the use of genetically modified organisms or compounds produced by these organisms, which would preclude the use of ‘new-to-nature’ sophorolipids (Van Bogaert et al., 2009; Saerens et al., 2011). As recently as 2009, sophorolipids had not yet found application in any commercial products (Hirata et al., 2009). However, in the past several years they have begun to be used in commercial products, particularly in housecleaning and cosmetic products where their anti-foaming and antimicrobial properties are attractive. Much of this progress in commercial utilization has been driven by the high yields of sophorolipids produced in culture, their ease in purification, and facile downstream processing which makes processing economically feasible (Marchand and Banat, 2012).

4. Conclusions

The Ck sophorolipids formed longer-lasting, stable emulsions with LGO than did either Sb or T-15, which is extremely important for practical use of these compounds with lipophilic herbicides. Phytotoxicity (fresh and dry weights) after three days to sicklepod by all three surfactant/LGO mixtures was similar to LGO applied alone, while the HDR value was greater with the two sophorolipid/LGO mixtures than with LGO applied alone or together with T-15. Injury to corn by LGO mixtures was generally much less, with only the LGO/Ck mixture significantly reducing dry weights. When applied with the water-soluble herbicide phosphinothricin, Ck and Sb caused decreases in sicklepod fresh and dry weights and HDR values compared to phosphinothricin applied without surfactants. For corn, T-15 and Ck applied with PT resulted in the greatest reductions in fresh and dry weights and HDR values. From these results it appears that both sophorolipids have excellent promise as natural surfactants/emulsifiers for postemergence herbicides.

Acknowledgments

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References


Table 2

Effect of surfactants, PT and PT + surfactants on fresh weights, dry weights, and herbicide damage ratings (HDR) of sicklepod and corn plants.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sicklepod</th>
<th></th>
<th>Corn</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh weight (g)</td>
<td>Dry weight (g)</td>
<td>HDR</td>
<td>Fresh weight (g)</td>
</tr>
<tr>
<td>Control</td>
<td>35.78 a</td>
<td>3.41 a</td>
<td>0 d</td>
<td>109.45 a</td>
</tr>
<tr>
<td>T-15</td>
<td>33.57 a</td>
<td>3.31 a</td>
<td>4 d</td>
<td>95.21 a</td>
</tr>
<tr>
<td>Ck</td>
<td>35.33 a</td>
<td>3.36 a</td>
<td>6 d</td>
<td>96.20 a</td>
</tr>
<tr>
<td>Sb</td>
<td>36.79 a</td>
<td>3.25 a</td>
<td>0 d</td>
<td>102.51 a</td>
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<tr>
<td>PT</td>
<td>27.26 ab</td>
<td>1.26 b</td>
<td>16 c</td>
<td>90.79 a</td>
</tr>
<tr>
<td>PT + T-15</td>
<td>14.94 bc</td>
<td>1.04 c</td>
<td>63 b</td>
<td>56.68 b</td>
</tr>
<tr>
<td>PT + Ck</td>
<td>8.79 c</td>
<td>0.77 c</td>
<td>93 a</td>
<td>53.00 b</td>
</tr>
<tr>
<td>PT + Sb</td>
<td>10.37 c</td>
<td>0.90 c</td>
<td>78 ab</td>
<td>87.43 a</td>
</tr>
</tbody>
</table>

* Means within a column followed by the same letters are not significantly different based on differences of least-squares means at p ≤ 0.10.


