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Nanostructured Liquid Crystalline Particles As an Alternative Delivery Vehicle for Plant Agrochemicals

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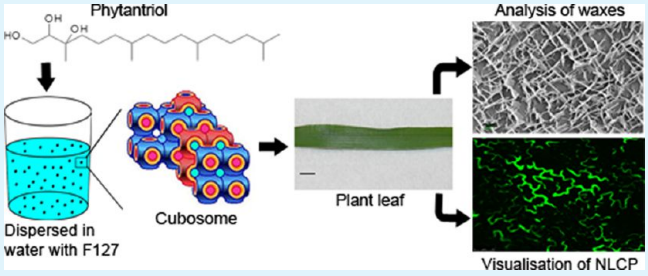
ABSTRACT: Agrochemical spray formulations applied to plants are often mixed with surfactants that facilitate delivery of the active ingredient. However, surfactants cause phytotoxicity and off-target effects in the environment. We propose the use of nanostructured liquid crystalline particles (NLCP) as an alternative to surfactant-based agrochemical delivery. For this, we have compared the application of commercial surfactants, di (2-ethylhexyl) sulfosuccinate and alkyl dimethyl betaine, with NLCP made from phytantriol, at concentrations of 0.1%, 1% and 5% on the adaxial surface of leaves of four plant species *Ttriticum aestivum* (wheat), *Zea mays* (maize), *Lupinus angustifolius* (lupin), and *Arabidopsis thaliana*. In comparison with the application of surfactants there was less phytotoxicity on leaves of each species following treatment with NLCP. Following treatment of leaves with NLCP analysis of cuticular wax micromorphology revealed less wax solubilization in the monocot species. The results clearly show that there are advantages in the use of NLCP rather than surfactants for agrochemical delivery.



KEYWORDS: *cubosomes, surfactants, image analysis, cuticular waxes*

INTRODUCTION

Surfactants are an integral part of agrochemical sprays that are applied to crops for their protection against disease and insect attack and for elimination of invasive weeds. Surfactants act by solubilization of leaf cuticular waxes, reducing the surface tension on droplets of water-based agrochemical formulations and by enhancing subsequent hydration of the cuticle.[1] A reduction in the contact angle of spray droplets on initially hydrophobic leaf surfaces[2] enables dispersion of the chemical solution across the leaf that leads to the enhanced uptake of the active ingredient by both stomatal penetration[3,4] and uptake through the cell walls of the epidermis.[5,6] Furthermore, surfactants increase the solubility of poorly water-soluble actives, effectively increasing the active ingredient concentration which reduces the volume of formulation required.[7] The mixing of agrochemicals with surfactants has been the most effective and widely used means by which these chemicals have been delivered into plants but this approach is not without serious disadvantages. On exposure to surfactants there are irreversible changes to the cuticle, especially to the epicuticular wax[8] of the fruits and leaves of many plant species.[9−11] Surfactants can also alter soil properties[12,13] and have been shown to be toxic to terrestrial invertebrates,[14,15] microbes,[14,15] and to aquatic organisms.[14−16]

The cuticle is a complex structure that consists of an insoluble polymeric cutin matrix and soluble intracuticular waxes that are generally referred to as cuticular waxes.[17,18] Cuticular waxes that come in direct contact with the atmosphere polymerize to form epicuticular waxes. Epicuticular and cuticular waxes form the cuticle proper and at their boundary are interspersed with the cutin matrix which lies below. It is generally accepted that the cuticle is composed of both cutin and the cuticle proper. The composition of cuticular waxes is dominated principally by fatty acids, primary and secondary alcohols, aldehydes, and esters and ketones,[17] and they may be mixed with secondary metabolites such as pentacyclic triterpenoids,[19,20] tocopherols,[21] and butanoids and propanoids.[22] The composition of cutin is very different from that of cuticular waxes by having both ω- hydroxy fatty acids and mid-chain hydroxy fatty acids[18,23] and glycerol along with unsubstituted epoxy and polyhydroxy fatty acids, α,ω- and polyhydroxy α,ω-dicarboxylic acids, fatty alcohols and phenolics.[18,24] Modification of the structural integrity of plant surface waxes, reduces the hydrophobicity of the cuticle, facilitating the adsorption of both microbial spores and anthropogenic substances leading to increased pathogenicity and phytotoxicity respectively.[11,25] Following the use of various commercial ionic and nonionic surfactants, phytotoxicity has

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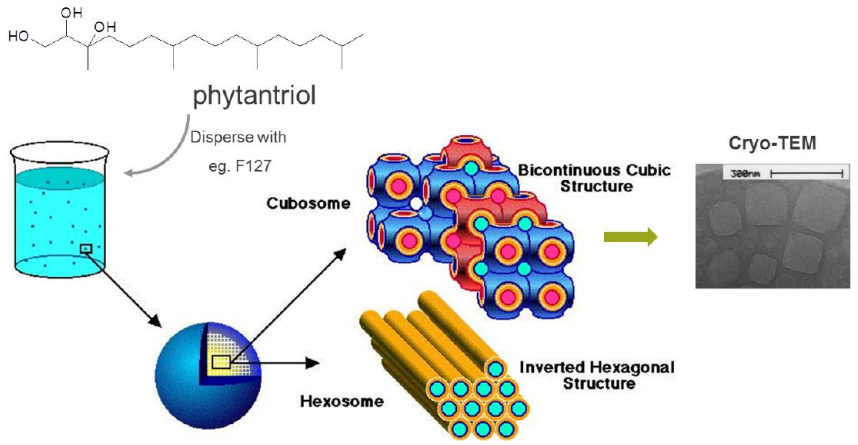

Figure 1. Overview of the synthesis of NLCP, their internal structure, and overall morphology. Briefly, synthesis involved dispersing phytantriol in water along with F127, followed by high energy shearing to obtain NLCP. NLCP can have either a bicontinuous cubic ("cubosome") or inverted hexagonal ("hexosome") nanostructure. Both cubosomes and hexosomes have nonintersecting hydrophilic and lipophilic regions. Cryo-TEM image on the far right of the figure shows the internal nanostructure of several cubosomes.

previously been reported on cucumber (*Cucumis sativus*),[26] barley (*Hordeum vulgare*),[27] soy bean (*Glycine max*), mung bean (*Phaseolus aureus*), sorghum (*Sorghum bicolour*), oat (*Avena sativa*), turnip (*Brassica rapa*), mustard (*Sinapis alba*), and sunflower (*Helianthus annuus*).[12] Cleary there is need for the development of alternative agrochemical delivery systems that can effectively deliver the active ingredient while avoiding the direct and indirect effects of surfactants on both crop plants and the environment. Recently, lipid-based nanocarriers have been shown to be adsorbed to several biorelevant surfaces[28] including those that are hydrophobic,[29] which opens up their potential as a novel way to apply agrochemicals that overcomes the disadvantages of the application of surfactant-based formulations.

Lipid-based nanoparticles have been of interest for delivery of pharmaceuticals for some time because they can be loaded with either hydrophilic or hydrophobic drugs and have the potential for sustained release.[30−32] The nanoparticles used are analogous to regular oily emulsion droplets but they possess an internal structure permeated with water channels of approximately 5 nm in diameter.[33−35] The lipids used to prepare the particles, self-assemble in water to form an ordered geometric structure, which in the case of the dispersed particles determines the type of internal structure that may be present. The lipid-based nanoparticles of most recent interest have an internal structure based on a bicontinuous cubic phase, and are known as "cubosomes", or an inverted hexagonal phase known as "hexosomes" (Figure 1).[34,36] To maintain colloidal stability so that phase separation does not occur, particles require dispersion in a polymeric stabilizer, most commonly the block copolymer Pluronic F127 (referred to hereafter as F127), which results in the formation of nanostructured particles typically 150−300 nm in diameter.[37] How these particles interact with plant surfaces and especially the hydrophobic plant cuticle is still not known and their suitability for delivery of agrochemicals is yet to be examined.

In the study reported here, we examined the effect on plant leaf surfaces of nanostructured liquid-crystalline particles (NLCP) derived from phytantriol (3,7,11,15-tetramethyl-1,2,3-hexadecanetriol) in comparison to that of the commercially available surfactants empimin and empigen. NLCP and surfactants were applied separately to the leaves of several crop and model species and the micromorphology of their surface waxes and cuticle layer as well as phytotoxicity was examined. Compared with the surfactant treatments, NLCP application led to less disruption of leaf surface wax morphology and lower levels of phytotoxicity demonstrating their suitability for agrochemical delivery.

MATERIALS AND METHODS

Plant Material and Growth Conditions. *Arabidopsis thaliana* ecotype Col-0 (*Arabidopsis*) seeds were obtained from Lehle Seeds (Round Rock, Texas, USA). Seeds were sterilized in 50% (v/v) ethanol and 1.5% (v/v) hydrogen peroxide for 5 min, rinsed three times in sterile dH_2O and transferred to 90 mm wide Petri plates containing MS basal medium (Sigma-Aldrich, Sydney, NSW, Australia) enriched with 3% (w/v) sucrose and 0.8% (w/v) bacteriological agar, at pH 5.7. The seeds were stratified in the dark for 48 h at 4 °C and were then transferred to a growth cabinet (Thermoline Scientific, Wetherill Park, NSW, Australia) maintained at 21 °C under cool white fluorescent light (100 μmol m^{-2} s^{-1}), with a 12/12 light/dark photoperiod.[38] After 14 days of growth the seedlings were transplanted to 100 mm wide plastic pots containing sterile potting mix (Potmate Premium Potting Mix, Debco, Tyabb, VIC, Australia), returned to the growth cabinet and watered regularly.

Lupinus angustifolius var. Wonga seeds (Naracoorte seeds, Naracoorte, SA, Australia) were surface sterilized by immersion in 80% (v/v) ethanol for 30 s, followed by washing in 2% (v/v) sodium hypochlorite for 2 min. The seeds were then rinsed with sterile dH_2O 5 times and transferred to a plastic tray containing a thin layer of absorbent cotton wool saturated with dH_2O. The tray was then incubated for two days in a growth cabinet (Thermoline Scientific) at 21 °C under high pressure sodium lights (300 μmol m^{-2} s^{-1}) with a 16/8 light/dark photoperiod. Germinated seedlings were then transplanted to 100 mm diameter plastic pots filled with sterile potting mix, placed back into the growth cabinet and watered regularly.

Zea mays var. 'Early Leaming' and *Triticum aestivum* cv. Wyalkatchem were obtained from Eden seeds (Lower Beechmont, QLD, Australia) and Nufarm Australia Limited, (Laverton North, VIC, Australia), respectively. The seeds were sterilized according to the procedures described above for *L. angustifolius* but were sown directly into 100 mm wide plastic pots filled with sterile potting mix. Seedlings were grown and maintained under the same conditions as described for *L. angustifolius*.

Preparation of Surfactants and Liquid Crystalline Nanoparticles. The surfactants, empimin, an anionic sodium di(2-ethylhexyl) sulfosuccinate, and empigen, a cationic and amphoteric betaine C_{12}−C_{14} alkyl dimethyl were provided by Nufarm Australia

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Limited. Surfactant solutions were prepared by dilution in dH_2O to concentrations of 0.1%, 1% and 5% (v/v) immediately prior to use.

NLCP were prepared as previously described.[37] Briefly, 900 mg of phytantriol (Roche Products Pty Limited, Dee Why, NSW Australia) was dispersed in 4.1 g of F127 (BASF Australia Ltd., Southbank, VIC Australia) solution (2.4% w/w) by ultrasonication (Misonix XL2000, Misonix Incorporated, Farmingdale, NY, USA) for 30 min in pulse mode (0.5 s pulses interrupted by 0.5 s breaks) at 40% of maximum power, resulting in a milky dispersion of NLCP. Particle size was characterized using a particle sizer (Malvern NanoS, Malvern Instruments, Malvern, UK) at 25 °C. The NLCP were stored at ambient temperature for a minimum of two days prior to use. For experimentation the concentrated NLCP (which contained 18% w/w phytantriol) were diluted to the equivalent phytantriol concentrations of 0.1, 1, and 5% v/v with dH_2O. NLCP concentration is expressed as the concentration of the NLCP forming lipid (phytantriol) in the dispersion (1% NLCP means 1% of phytantriol in solution). dH_2O was used as the control for all the surfactant and NLCP treatments. An F127 solution (2.4% w/w) diluted to 0.1% v/v was also used as a control for NLCP treatment. For preparation of fluorescently labeled cubosomes, 0.05 mg of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-carboxyfluorescein (Avanti Polar Lipids, Inc., Alabama, USA) was added per gram of phytantriol and used at a concentration of 0.1% v/v phytantriol .

Treatment of Leaves with Surfactants and NLCP and Macroscopic Analysis. Ten microliter droplets of surfactant or NLCP solution at each concentration were applied to the adaxial (upper) surface of leaves of 4 to 5 individual plants from each species using a micropipet. The plants were then carefully returned to the growth cabinet. After 48 h a minimum of eight leaves for each treatment were then excised and photographed using a digital camera. Images were then used to assess the level of phytotoxicity caused by each treatment on a scale of 0 to 5, where a score of 0 = no observed phytotoxicity (healthy, undamaged leaves), 1 = trace phytotoxicity (isolated speckling/necrosis on leaf), 2 = minor phytotoxicity (minor tissue damage but leaf generally healthy), 3 = moderate phytotoxicity (coalesced patches of necrosis), 4 = high level phytotoxicity (extensive or intense tissue damage) and 5 = severe phytotoxicity (complete necrosis). For scoring of phytotoxicity following treatment three independent assessments (including two blind) were made. Data obtained was subjected to statistical analysis using the Kruskal–Wallis test for significant differences between treatment groups (IBM SPSS Version 21, IBM Australia Ltd., St Leonards NSW, Australia).

Leaf Tissue Preparation and Examination by Light, Epifluorescence, and Confocal Microscopy. For examination of the cuticle of each species leaves were excised and then washed briefly in dH_2O followed by rinsing in embedding medium, (Tissue-tek OCT compound, ProSciTech Pty Ltd., Thuringowa, QLD, Australia). The leaves were then cut into smaller pieces to fit within a cryomold (15 mm ×15 mm ×5 mm, Tissue-tek Cryomold, ProSciTech Pty Ltd.). Leaf sections were placed into cryomolds containing embedding medium and immediately snap frozen in liquid nitrogen and then stored at −80 °C until further use. Transverse sections of 5–20 μm in thickness were then cut using a cryostat (Microm HM550 OMP, Thermo Scientific, Scoresby, VIC, Australia), and sections were placed on gelatin-coated slides[39] then rinsed in dH_2O. Sections were subsequently stained with 0.1% w/v Sudan IV or 0.1% Auramine O w/v (Sigma-Aldrich) to stain the cuticle as previously described.[40] Stained leaf sections were examined under bright light and epifluorescence microscopy (Axioskop 2 mot plus microscope, Zeiss, Göttingen, Germany) and images were captured with a digital camera attached to the microscope. Epifluorescence microscopy was conducted using ultraviolet (365 nm excitation, 420 nm emission) and blue light (450–490 nm excitation, 520 nm emission) filters. Confocal microscopy was conducted using a laser scanning confocal microscope (Leica TCS-SP5, Leica Microsystems Pty Ltd., North Ryde, NSW, Australia) and associated image capture software (LAS AF software version: 2.6.3.8173)

To examine the localization of fluorescently labeled NLCP leaves were treated as described above and were then excised 1 h after treatment and visualized using confocal microscopy.

Analysis of Leaf Surface Micromorphology Using Scanning Electron Microscopy. To examine the micromorphology of leaf surfaces, leaves of each species were excised at 48 h post treatment and were air-dried[41] at room temperature for 24–48 h in preparation for scanning electron microscopy (SEM). Four leaves from four plants (sixteen leaves in total) of each species for each treatment were selected. From each leaf a 9 mm^2 section of leaf was excised and mounted onto an aluminum stub. Each sample was coated with gold palladium for 120 s at 40 mA (BAL-TEC Sputter Coater SCD 050, Scotia, NY, USA) and imaged using a scanning electron microscope (Supra 55 VP, Carl Zeiss Pty Ltd., NSW, Australia) under an accelerating voltage of 5–10 kV. An average of 14 micrographs were obtained at random locations across the leaf sample surface for all treatment concentations and control groups. Image analysis was then performed on three randomly selected micrographs for each treatment group to assess the structural complexity of leaf epicuticular wax. Image analysis followed a two-step procedure. Step one involved the use of a software program[42] to determine the median number of white pixels for all images within each treatment group. In the second step, the number of dark objects which represent gaps between epicuticular wax structures were obtained using Pixcavator software. The number of dark objects was then scaled by the median number of white pixels for each treatment group to provide a measure of the structural complexity or relative density (RD) of wax structures. The data was then indexed against control groups. Data obtained was subjected to statistical analysis using analysis of variance (ANOVA) with Tukey's HSD posthoc test.

RESULTS

Cuticle Structure. Following staining of transverse leaf sections the cuticle was readily observed under light and fluorescence microscopy (Figure 2). The cuticle was present on both adaxial and abaxial surfaces of the leaves as a thin layer between 3 to 5 μm (except for *A. thaliana* which had a very thin cuticle, <1 μm, data not shown) directly adjacent to the external epidermal cell wall. Sudan IV staining showed the cuticle as a pink to red layer under the light microscope (Figure 2a, b), whereas auramine O staining allowed visualization of the cuticle under blue light excitation. A clear difference in the depth of cuticle was observed between periclinal and anticlinal cell walls, with regions between epidermal cells (and guard cells where present) displaying the thickest cuticle (Figure 2b–e). Different layers of the cuticle could be easily discerned including the epicuticular wax layer, an external cuticular layer and an internal cuticular layer (Figure 2f, g).

Effect of Surfactants and NLCP Treatment of Plants. Three concentrations (0.1, 1, and 5%) of each of the surfactants were tested separately on each plant species. Droplet behavior of applied surfactants and NLCP was noted to vary considerably between various treatments. When empigen was applied to the leaf surface of all four plant species, droplets were contained at the site of application. In contrast, empimin and NLCP droplets spread out across the leaf surface after application to cover a larger area. It also appeared that the effect of surfactant and NLCP treatments is concentration dependent and irreversible. The responses of leaves to surfactant or NLCP application are summarized in Table 1.

T. aestivum and Z. mays. With application of the surfactant empigen at a concentration of 0.1%, "ringlike" necrotic spots, limited to the site of application were observed on the monocot, *T. aestivum* (Figure 3a, c). Necrotic spots with uniform damage were further observed after increasing the concentration of the application to 1% and 5% (Figure 3e, g).

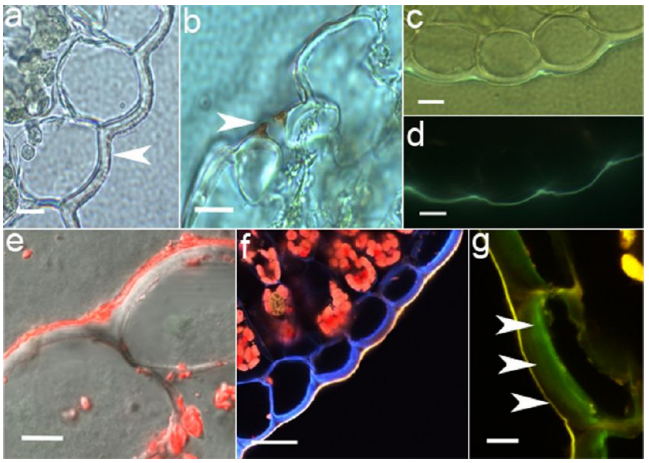

Figure 2. Structural features and components of leaf cuticles. Transverse sections of leaves were prepared and stained and then viewed with light, standard fluorescence and confocal microscopy. (a) Bright-field micrograph of *T. aestivum* stained with Sudan IV showing the cuticle stained a pink/red color (white arrow), (b) *L. angustifolius* section stained with Sudan IV. Note the cuticlar thickening on stomatal guard cells (white arrow), (c) *L. angustifolius* section stained with auramine O and visualized under a combination of epifluorescence and bright-field microscopy. The cuticle is shown as a thin, bright line on the external wall of epidermal cells and is a fluorescent green color, (d) The same image as c but under epifluorescence alone, (e) *L. angustifolius* section stained with auramine O and visualized with confocal microscopy. False color image of the cuticle on the external face of epidermal cells. Note chloroplasts in lower right of image, (f) *T. aestivum* section stained with auramine O and viewed using confocal microscopy. The cuticle can be seen as a prominent and continuous line along epidermal cell surfaces, (g) *T. aestivum* section stained with auramine O and viewed using confocal microscopy to show the internal layers of the cuticle. The top arrow indicates the internal cuticular layer, middle arrow shows the region of the external cuticular layer and bottom arrow the epicuticular wax layer. Scale bar on a = 32 μm, b = 40 μm, c = 36 μm, d = 36 μm, e = 8 μm, f = 23 μm, g = 4 μm.

Table 1. Phytotoxicity Scores after Surfactant or NLCP Treatment of Leaves of *T. aestivum*, *Z. mays*, *L. angustifolius*, and *A. thaliana*

treatment	plant species	0.10%	1%	5%
Empigen	*T. aestivum*	1.3 ± 0.2	4.2 ± 0.3	4.8 ± 0.1
	Z. mays	0.8 ± 0.1	2.3 ± 0.5	4.4 ± 0.3
	L. angustifolius	3.5 ± 0.8	4.9 ± 0.1	4.8 ± 0.1
	A. thaliana	3.3 ± 0.5	4.7 ± 0.2	5.0 ± 0.0
Empimin	*T. aestivum*	0.8 ± 0.1	3.6 ± 0.5	4.6 ± 0.2
	Z. mays	1.2 ± 0.5	3.0 ± 0.4	4.6 ± 0.3
	L. angustifolius	1.8 ± 0.3	4.3 ± 0.3	4.4 ± 0.3
	A. thaliana	3.1 ± 0.5	4.8 ± 0.3	4.8 ± 0.2
NLCP	*T. aestivum*	$0.3 \pm 0.2^{a,b}$	0.5 ± 0.3^{c}	1.6 ± 0.3^{c}
	Z. mays	0.0 ± 0.1^{c}	0.2 ± 0.2^{c}	0.2 ± 0.2^{c}
	L. angustifolius	0.6 ± 0.2^{c}	1.7 ± 0.5^{a}	$4.1 \pm 0.4^{a,b}$
	A. thaliana	0.5 ± 0.2^{c}	1.6 ± 0.8^{c}	3.1 ± 1.0^{c}

[a]indicates a significant difference ($P < 0.001$) between NLCP and empigen treatments of the same concentration. [b]indicates a significant difference ($P < 0.05$) between NLCP and empigen treatments of the same concentration. [c]indicates a significant difference ($P < 0.001$) betweenNLCP, empimin and empigen treatments of the same concentration.

In comparison to empigen, application of emipimin on the leaves of *T. aestivum* at similar concentrations of 0.1, 1, and 5% resulted in large areas of phytotoxicity. The results obtained for surfactant treatments on the leaves of *Z. mays* were equivalent to the observations made on *T. aestivum*. The necrotic spots formed, however, showed uniform damage after 0.1% empigen treatment. Under SEM, the adaxial surface of untreated control leaves of *T. aestivum* (Figure 3b) and *Z. mays* displayed vertically oriented, platelike epicuticular wax crystals. Treating leaves of *T. aestivum* (Figure 3d) and *Z. mays* with either of the surfactants, even at a low concentration of 0.1% resulted in marked solubilization of the fine structure of epicuticular waxes. As higher concentrations of surfactants (1 and 5%) were applied greater alteration of the wax microstructure was observed leading to almost complete wax solubilization on the leaves of both *T. aestivum* (Figure 3f, h) and *Z. mays* (data not shown).

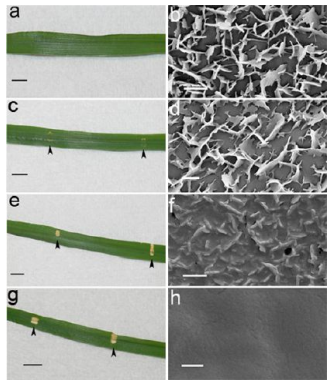

Figure 3. Leaves of *T. aestivum* following treatment with empigen and the corresponding epicuticular wax micromorphology. Whole leaves (left column) were treated with the following concentrations: (a) water-treated control, (c) 0.1% empigen, (e) 1% empigen, and (g) 5% empigen. Leaves showed increasing symptoms of phytotoxicity in a dose-dependent manner (sites of application are indicated with black arrows). Corresponding SEM images of the treated leaf surface (right column) show the progressive damage to epicuticular waxes with increased concentration of empigen. The structure of the platelike waxes on the adaxial surface of the leaves is evident in (b) the control. (d) Modification to the wax structure following treatment with 0.1% empigen. Pronounced solubilization of wax crystals can be observed in images after treatment with (f) 1% and (h) 5% empigen, respectively. Scale bar on a, c, e, g = 1 cm; b = 1000 nm, d = 740 nm, f = 1020 nm, h = 920 nm.

The NLCP displayed a similar spreading ability to application of the surfactant empimin, with spreading across the leaf surface. Interestingly, the application of NLCP on leaves of *T. aestivum* did not show any visual signs of necrosis when applied at a concentration of 0.1 or 1% (Figure 4a, c, e). Phytotoxicity symptoms, however, were observed on the *T. aestivum* leaves when the concentration of applied NLCP was increased to 5% but the intensity of necrosis was low when compared to that of surfactant treatments (Figure 4g). The phytotoxicity effects of NLCP on *Z. mays* are very similar to that of *T. aestivum*. There was little epicuticular wax solubilization following the NLCP treatments at 0.1 and 1% on the leaves of both *T. aestivum* (Figure 4b, d, f) and *Z. mays*. Solubilization of the wax micromorphology was, however, observed when the leaves of both *T. aestivum* and *Z. mays* were treated with NLCP at a concentration of 5% (Figure 4h). All of the observations made using SEM after NLCP treatments were consistent with the phytotoxicity symptoms observed earlier for both *T. aestivum* and *Z. mays*.

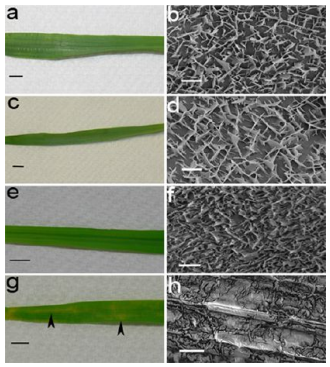

Figure 4. Leaves of *T. aestivum* following treatment with NLCP and the corresponding epicuticular wax micromorphology. Whole leaves (left column) were treated with the following concentrations: (a) water-treated control, (c) 0.1% NLCP, (e) 1% NLCP, and (g) 5% NLCP. Phytotoxicity symptoms on whole leaves could be observed only after 5% NLCP application (black arrows). Corresponding SEM images of the treated leaf surface (right column) show that the epicuticular waxes are intact in (b) the control samples and following treatment of leaves with NLCP at a concentration of (d) 0.1% and (f) 1% NLCP. (h) Leaves treated with 5% NLCP; some solubilization of epicuticular wax structure was observed. Scale bar on a, c, e, g = 1 cm; b = 595 nm, d = 732 nm, f = 520 nm, h = 1462 nm.

L. angustifolius. Phytotoxicity effects on the leaves of *L. angustifolius* after both the surfactants empimin and empigen were applied were similar to corresponding treatments on leaves of *T. aestivum* (Figure 5a, c, e, g). The wax micromorphology on the leaves of *L. angustifolius* was quite different from *T. aestivum* and *Z. mays* and was composed of fine threadlike structures along with underlying platelike wax crystals (Figure 5b). A similar trend of epicuticular wax solubilization on the leaves of *L. angustifolius* after various surfactant treatments (at 0.1, 1, and 5%) was found and followed that previously observed for *T. aestivum* and *Z. mays* (Figure 5 d, f, h) and are consistent with the phytotoxicity symptoms of these species. The phytotoxicity observed after NLCP treatment led to discoloration of leaves at 0.1%, which further intensified to sever phytotoxicity after 1 and 5% NLCP applications (Figure 6a, c, e, g). With wax solubilization, NLCP treatments on *L. angustifolius* leaves, at a concentration of 0.1% were sufficient to solubilize the fine structures of the wax layer leaving behind highly altered wax micromorphology (Figure 6b, d). The intensity of solubilization also increased with increasing concentration of NLCP (0.1−5%), leading to complete solubilization of epicuticular waxes at 5% (Figure 6f, g).

A. thaliana. After the application of surfactants (either empigen or empimin) on the leaves of *A. thaliana* there were no observable differences in the amount of phytotoxicity when compared to that of *T. aestivum*. Surprisingly, NLCP treatments were also similar to surfactant treatments for the tested concentrations (Figure 7 a, c, e, g). The epicuticular wax structure did not have any distinct wax crystals and appeared to be composed of an undifferentiated amorphous wax film (Figure 7b). Consistent with the visual symptoms, damage to the amorphous wax film caused by surfactant and NLCP treatments was concentration-dependent (Figure 7b, d, f, h).

Comparison of the Effect of NLCP and F127 on Phytotoxicty and Wax Structure. To determine the cause of phytotoxicity and wax solubilization on the leaves of various plants after NLCP treatments, we tested the effect of NLCP and the stabilizer, F127, alone. A concentration of 0.1% v/v F127 was tested as this is equivalent to NLCP solutions that contain 1% (w/w) phytantriol and 0.1% v/v F127. It was observed that under the tested conditions F127 alone elicited phytotoxicity symptoms only on the leaves of *Arabidopsis* (data not shown), whereas wax solubilization was observed following 0.1% F127 treatment on all the species tested (Figure 8c, f, i, l). The changes in epicuticular wax structure of *T. aestivum* and *Z. mays* following treatment with F127 alone were more pronounced than corresponding NLCP treatment that contained 1% phytantriol and 0.1% F127 (Figure 8a−f). These results indicate that F127 is responsible for solubilization of epicuticular waxes. For *L. angustifolius* and *A. thaliana* there were no observed differences in the intensity of wax solubilization following F127 and NLCP treatments (Figure 8g−l).

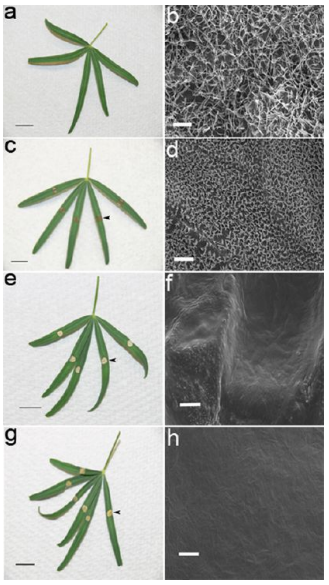

Figure 5. Leaves of *L. angustifolius* following treatment with empigen and the corresponding epicuticular wax micromorphology. Whole leaves (left column) were treated with the following concentrations: (a) water-treated control, (c) 0.1% empigen, (e) 1% empigen, and (g) 5% empigen. The intensity of phytotoxicity increased with increasing concentration of empigen (black arrows). Corresponding SEM images of the treated leaf surface (right column) show a dose-dependent increase in epicuticular wax solubilization. (b) Epicuticular waxes on untreated lupin leaves include fine threadlike structures that are closely packed throughout the adaxial surface. (d) Following treatment with 0.1% empigen, the fine structure of the waxes was greatly reduced and appear as small rodlike structures. Following treatment with (f) 1% and (h) 5% empigen, complete solubilization of waxes occurred. Scale bar on a, c, e, g = 1 cm; b = 16 μm, d = 8 μm, f = 7 μm, h = 74 μm.

Wax Solubilization Following Treatment of Leaves with Surfactants and NLCP. We have developed an arbitrary unit, the relative density (RD) of wax cover, to describe the structural complexity of epicuticular wax on the leaf surface. RD values equal to 1 correlate to intact and unaltered epicuticular wax cover and lower values of RD indicate a solubilization of waxes and a reduction in their structural complexity. The RD value found for surfactant treatments on the leaves of *T. aestivum* and *Z. mays* (Figure 9a and b) showed a dose-dependent solubilization of waxes that strongly correlate with our former observations of phytotoxicity and wax solubilization. Additionally, the RD values after 0.1% and 1% NLCP treatments on *T. aestivum* and *Z. mays*, were equivalent to that of controls indicating structural integrity of the wax

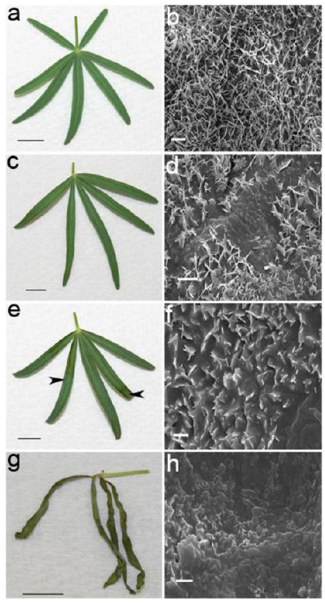

Figure 6. Leaves of *L. angustifolius* following treatment with NLCP and the corresponding epicuticular wax micromorphology. Whole leaves, composed of multiple leaflets (left column), were treated with the following concentrations: (a) water-treated control, (c) 0.1% NLCP, (e) 1% NLCP, and (g) 5% NLCP. Slight discolouration of leaflets was observed following treatment with 0.1% NLCP and became more intense following treatment with 1% NLCP (black arrows). (g) Complete wilting of leaflets was observed after treatment with 5% NLCP. Corresponding SEM images of the treated leaf surface (right column) show (b) the intact structure of epicuticular waxes and their disrupion after (d) 0.1% NLCP treatment. Complete solubilization of the epicutular waxes was observed after treatment with (f) 1% and (h) 5% NLCP. Scale bar on a, c, e, g = 1 cm; b = 1.6 μm, d = 14.6 μm, f = 0.19 μm, h = 1.9 μm.

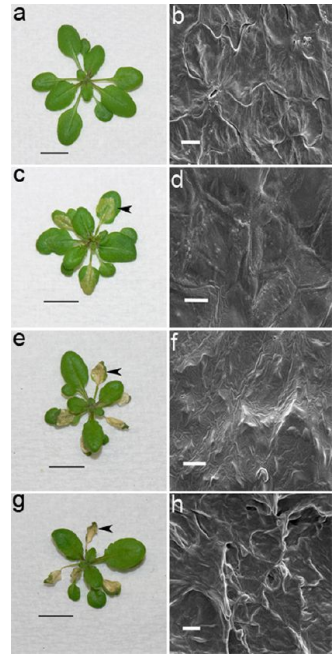

Figure 7. Leaves of *A. thaliana* following treatment with empimin and the corresponding epicuticular wax micromorphology. Whole leaves (left column) were treated with the following concentrations of empimin: (a) water-treated control, (c) 0.1% empimin, (e) 1% empimin, and (g) 5% empimin. Phytotoxicity observed on whole leaves of *A. thaliana* is indicated with black arrows. Corresponding SEM images of the treated leaf surface (right column) show the structure of the epicuticular wax "film" characteristic of *A. thaliana* leaves. Increased phytotoxicity and damage to the wax film after exposure to increased concentrations of empimin is evident. Scale bar on a, c, e, g = 1 cm; b = 19 μm, d = 24 μm, f = 23 μm, h = 18 μm.

micromorphology (Figure 9a, b). For *L. angustifolius* the RD values decreased with increased concentration of surfactants (Figure 9c) consistent with observations made for phytotoxicity and wax solubilization. For 0.1% NLCP treatment on *L. angustifolius* a significant reduction in RD value but to a lesser degree than that of surfactants was recorded. However, a concentration dependent decrease in RD was recorded for *L. angustifolius* when higher concentration of NLCP (1 and 5%) was applied. Consistent with observations made for phytotoxicity and epicuticular wax solubilization RD values for *A. thaliana* showed a concentration dependent decrease for both NLCP and surfactant treatments at all tested concentrations (Figure 9d). With 0.1% F127 treatments (Figure 9e) the RD values and wax solubilization correspond.

Interaction of Fluorescent NLCP with Leaf Surfaces. Fluorescently tagged NLCP were used to enable examination of the spatial distribution of the NLCP delivery to the leaf surface. Following the treatment, of fluorescently tagged NLCP at a concentration of 0.1 and 1%, the leaves of all species were observed to have localized fluorescence on the adaxial surface (Figure 10a, b). No such localization of fluorescence could be observed with the controls that were treated with unlabeled NLCP (data not shown). Leaves sampled at different time points of 5 min and 1 h showed a similar pattern of fluorescence indicating the rapidity with which NLCP is absorbed into the cuticle, with maximum intensity of fluorescence along anticlinal walls of the epidermal cells. 5% NLCP were not tested as this concentration caused phytotoxicity.

DISCUSSION

It has recently been shown that NLCP can efficiently adsorb to model hydrophobic surfaces made up of either tristearin or silicon.[28,29] As all aerial plant surfaces are generally hydrophobic, these previous studies opened up the possibility of NLCP being used as a vehicle for delivery of agrochemicals. In this study we have shown that application of NLCP to leaf surfaces caused significantly less disruption to epicuticular waxes and reduced phytotoxicity compared with two commonly used surfactants. We have also found, using image analysis, a direct and concentration-dependent correlation between solubilization of epicuticular waxes and level of phytotoxicity. Further, application of fluorescently labeled NLCP showed their adsorption to leaf surfaces that was most prominent at anticlinal cell walls. Together, our results show that these nanostructured lipid particles that possess unique properties may provide, in agricultural situations, an alternative to classical surfactant-based formulations. It must, however, be appreciated that the efficacy and efficiency of delivery of agrochemicals to plants using NLCP under controlled or field conditions has not yet been tested.

All species selected for this study showed dose-dependent phytotoxicity symptoms after surfactant (empigen and empimin) treatments, consistent with earlier reports.[11,43,44] There were clear differences between the two surfactant treatments; empigen caused distinct necrotic spotting of leaves

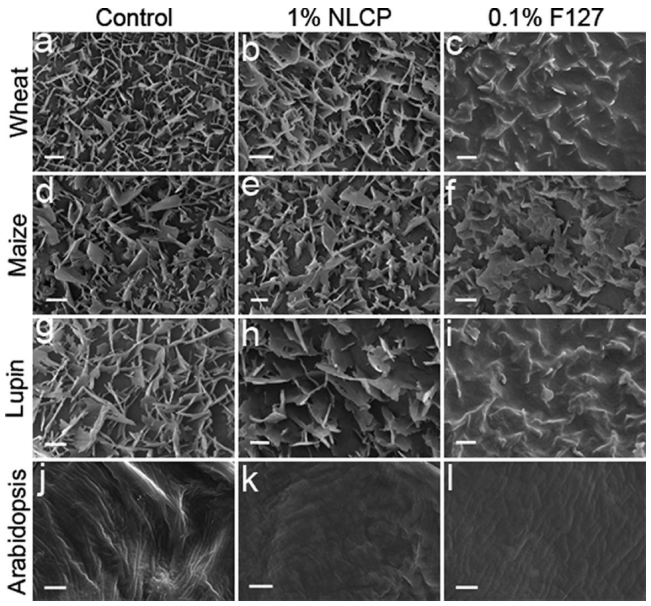

Figure 8. Micromorphology of the adaxial surface of leaves following treatment with 1% v/v NLCP (which contains 1.0% v/v phytantriol and 0.1% v/v F127) or 0.1% (v/v) F127 alone. (a–c) *T. aestivum* (wheat), (d–f) *Z. mays* (maize), (g–i) *L. angustfolius* (lupin), and (j–l) *A. thaliana* (*Arabidopsis*). Note the enhanced solubilization of surface waxes following treatment with 0.1% F127 compared with 1% NLCP for all species. Scale bar on a = 450 nm, b = 638 nm, c = 655 nm, d = 609 nm, e = 440 nm, f = 502 nm, g = 573 nm, h = 562 nm, i = 439 nm, j = 2.6 μm, k = 2.99 μm, l = 2.3 μm.

limited to the site of application, while empimin produced necrosis in a less defined manner. This difference in phytotoxicity between surfactants was consistent on all the species tested and can be explained as a consequence of the different physicochemical properties of the surfactants, empigen is a cationic, alkyl dimethyl betaine[45] and empimin is an anionic, sodium di(2-ethylhexyl) sulphosuccinate. It also appears that empimin is slightly more phytotoxic toward the species tested. Additionally, the observation of ringlike phytotoxicity, with a central undamaged area on the leaves of *T. aestivum* and *A. thaliana*, is a characteristic of 0.1% empigen treatment and may be a consequence of evaporation and surface tension of the solute (water in this case).[46] In contrast to surfactant treatments, NLCP treatments at 0.1 and 1% did not elicit any phytotoxic symptoms on the leaves of the monocots *T. aestivum* and *Z. mays*. These results demonstrate that phytotoxicity after NLCP treatment was greatly reduced in comparison to surfactant treatments. A further increase in concentration of NLCP to 5% caused phytotoxicity symptoms on *T. aestivum*; however, the leaves of *Z. mays* displayed no obvious effect. The absence of any phytotoxic symptoms in *Z. mays* may be due to the strong tensile strength of its leaves that enables them to resist greater amounts of NLCP.[47] The monocots *Z. mays* and *T. aestivum* are known for their anatomical toughness, while the dicots *L. angustifolius* and *A. thaliana* have a more fragile leaf structure.[48] This difference may also have contributed to the increased tolerance toward NLCP application shown by monocots. With *A. thaliana*, the phytotoxicity of NLCP treatments followed a similar trend to that which was observed with surfactant treatments, but was much reduced at every concentration tested. This clearly demonstrates that NLCP are a potentially much safer alternative to the conventional surfactants tested in this study, even on sensitive dicot plants such as *L. angustifolius* and *A. thaliana*.

The outermost layer of the cuticle consists of epicuticular waxes and differs greatly between species.[49,50] In the current study we observed the presence of platelike waxes on the adaxial surface of *T. aestivum* and *Z. mays* leaves. *L. angustifolius* displayed similar platelike waxes but also had fine "threadlike" structures on its surface while *A. thaliana* was observed to have an amorphous wax film on its leaf surface.[50] Epicuticular waxes are solubilized after the application of surfactants[8,9,51] and in order to further assess the intensity of wax alteration resulting from the various treatments used in this study, image analysis was conducted to quantify solubilization of epicuticular wax structures. Using this method we found that *T. aestivum*, *Z. mays*, and *L. angustifolius* showed significant differences in their response to treatment with NLCP in comparison with surfactants while for *A. thaliana* no such differences were observed.

The block copolymer F127 ensures the colloidal stability of the submicrometer-sized NLCP in water and as such, is an essential component in the NLCP dispersion. One percent phytantriol-based NLCP contains the same amount of F127 as a 0.1% F127 solution. Considering that it would not be possible to obtain NLCP without F127, phytantriol alone was not tested for analysis of phytotoxicity and wax solubilization. However, the presence of phytotoxicity symptoms (not wax solubilization) exclusively on *A. thaliana* after F127 treatment at 0.1% was surprising and may be a consequence of physiological sensitivity in comparison to other species tested.[48] For the monocots *Z. mays* and *T. aestivum*, the solubilization values for 1% NLCP indicated no wax solubilization, whereas for the equivalent F127 treatments, there was severe disruption. This comparison indicates that F127 alone is likely responsible for wax solubilization on monocots. On the dicots *L. angustifolius* and *A. thaliana*, both NLCP and F127 treatments elicited wax solubilization. This differential behavior of F127 to induce wax solubilization on monocots when applied as a pure solution may be due to the amount of F127 available for interaction with epicuticular waxes. In a 0.1% v/v phytantriol NLCP solution, F127 is bound to NLCP[28] on the surface, leaving behind no or very limited amount of free F127 to interact with plant surfaces, whereas in 0.1% w/v of F127 solution, all the F127 is freely available for any interaction, eventually leading to wax solubilization.

In this study, we have carried out detailed analyses on plants grown under defined conditions in plant growth chambers, however, the development of the cuticle on plant surfaces is more pronounced under natural conditions. We would therefore expect that NLCP will elicit even less impact on plant leaves when used in the natural environment. Further benefits of NLCP include their adhesive property which reduces chemical runoff,[28] the use of less water in agrochemical delivery and their potential for use with both lipid- and water-soluble actives. Furthermore, the constituents of the NLCP dispersion (phytantriol and F127) are commonly used in cosmetic and pharmaceutical formulations and, unlike many surfactants, are not toxic to the nontarget organisms of soil and water ecosystems.[16]

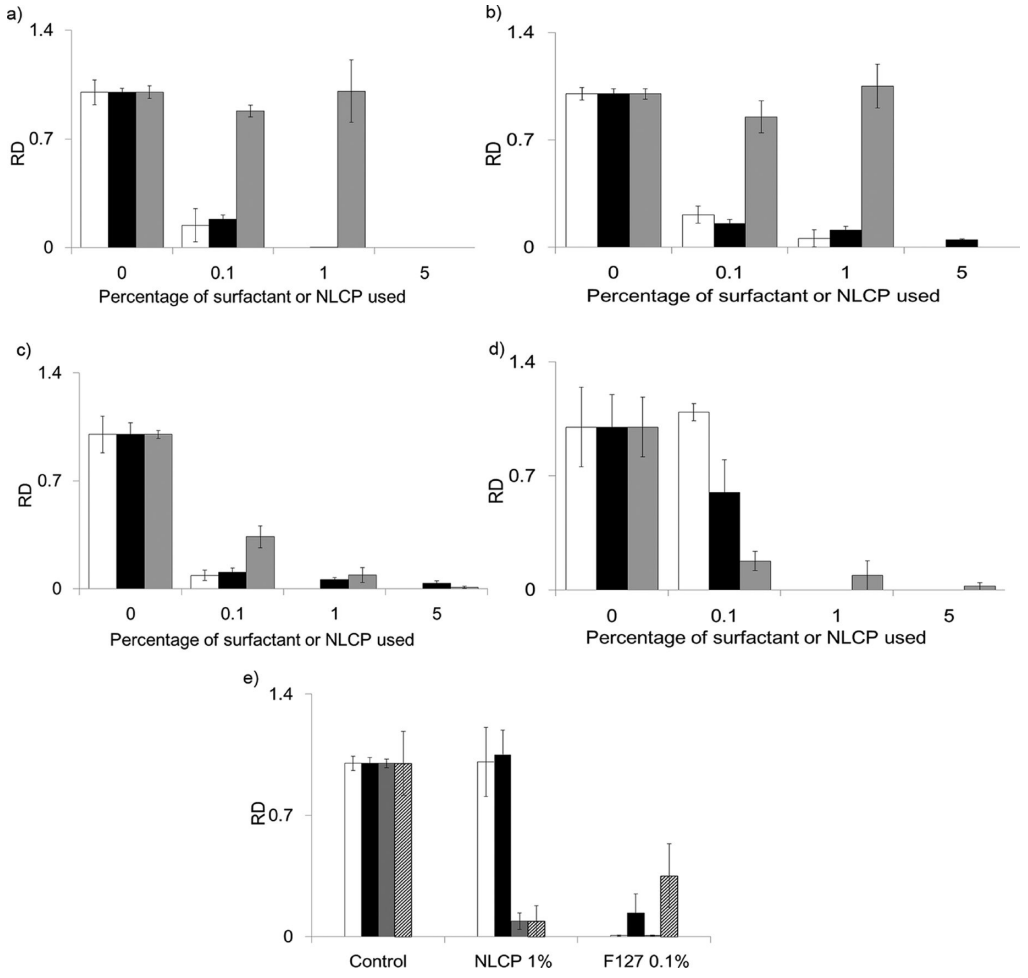

Figure 9. Relative density of wax cover (RD) after empigen (white bars), empimin (black bars), and NLCP (gray bars) treatment on leaves of (a) *T. aestivum*, (b) *Z. mays*, (c) *L. angustifolius*, and (d) *A. thaliana*. (e) Comparison of NLCP and F127 treatment of *Z. mays* (white bars), *T. aestivum* (black bars), *L. angustifolius* (gray bars), and *A. thaliana* (striped bars). Error bars represent the standard error of the mean. For (a and b) all treatments with NLCP were not significantly different ($p > 0.05$) from the controls, whereas all treatments with the two surfactants were significantly different ($p < 0.05$) from controls. For (c), all treatments were significantly different ($p < 0.05$) from controls. For (d), all treatments with the two surfactants and NLCP were significantly different ($p < 0.05$) from controls except for empigen and empimin at 0.1%. For (e), NLCP treatment on wheat and maize showed no significant difference ($p > 0.05$) from the control but all other treatments were significantly different ($p < 0.05$) from controls.

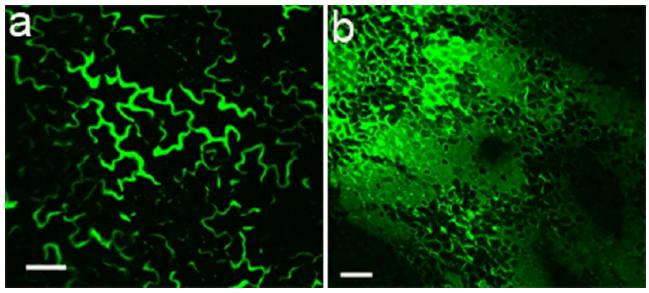

Figure 10. Distribution of fluorescence on leaves of *A. thaliana* and *L. angustifolius* following treatment with fluorescently tagged cubosomes. Confocal images of (a) fluorescently tagged NLCP adsorbed to the leaf surface of *A. thaliana* and (b) *L. angustifolius*. The fluorescence pattern in (a) shows uptake of the fluorescent lipid into the anticlinal cell walls of the epidermal cell layer on the adaxial epidermis. (b) Fluorescent NLCP adhered across the surface (to periclinal cell walls) as well as the anticlinal walls of cells in the epidermal layer of the leaf. Scale bar on a = 124 μm, b = 98 μm.

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Notes

The authors declare no competing financial interest.

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