NANOSTRUCTURED LIQUID CRYSTALLINE PARTICLES AS NEXT
GENERATION AGROCHEMICAL DELIVERY VEHICLES

by

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ABSTRACT

Modern day agricultural practices are heavily dependent on the use of surfactant-based agrochemical formulations to eliminate invasive weeds, pests or infective pathogens. Surfactants are often used to modify the physicochemical properties of the active ingredient, enhance the spray dispersion of the formulation and act on the plant surfaces. The surface hydrophobicity configured by the cuticle, which is a major protective barrier on the plant surface is irreversibly altered by the surfactant based agrochemical spray formulations. Surfactants are often used to plasticise the cuticle for an efficient and systemic delivery of the agrochemical active. The use of surfactants in an agrochemical formulation can, however, be lead to various associated disadvantages. These include crop phytotoxicity, off-target effects like disruption of soil properties, water contamination, toxicity towards terrestrial microbes and invertebrates and aquatic organisms, all of which are a major concern. Off-target effects coupled with crop phytotoxicity were shown to effect plant development, which further lead to yield losses and socioeconomic disparities. To overcome these problems associated with the use of conventional surfactantsnanostructured liquid crystalline particles (NLCP) are proposed as an alternative to the classic surfactant based agrochemical delivery.

The cuticle and the phylloplane of the economically important crop plants Triticum aestivum (wheat), Zea mays (maize), Lupinus angustifolius (lupin) and model plant Arabidopsis thaliana were micrographed to understand the structural organisation of the consecutive layers of the cuticle. Epicuticular waxes that form the outmost layer of the cuticle were examined under high resolution scanning electron microscopy (SEM) and novel image analysis procedures were developed to quantify the epicuticular wax micromorphology. The NLCP were then applied at varying concentrations of 0.1%, 1.0% and 5% (v/v) on the plant surfaces in comparison to the commercial agricultural surfactants empigen and empimin. It was found that unlike the
surfactants empigen and empimin, 0.1% and 1.0% NLCP applications did not have any effect on the wax micromorphology of *T. aestivum* and *Zea mays*. Though the applied 0.1% NLCP to *L. angustifolius* did not solubilise the epicuticular waxes, a higher concentration of 1.0% NLCP and all the surfactant applications severely altered the epicuticular waxes. *A. thaliana* however, did not have any epicuticular waxes and was sensitive to all the NLCP and surfactant treatments. The stress against NLCP and surfactants spray applications on *Z. mays*, *L. angustifolius* and *A. thaliana* was further investigated by quantifying the stress markers, callose a β-1,3 glucan and H$_2$O$_2$ a reactive oxygen molecule, production in the presence of UV-C radiation. It was found that, in the presence of UV radiation, *L. angustifolius* callose response was significantly less for NLCP spray treatments, in comparison to surfactant applications at similar concentrations. *Z. mays* was resistant to both NLCP and surfactant applications followed by UV treatments, while *A. thaliana* was sensitive to all the applied formulations and UV. Additionally, the infection of downy mildew *Hyaloperonospora arabidopsis* on *A. thaliana* after NLCP or surfactant treatments was observed to remain similar to control plants.

To test the efficacy of NLCP to control *A. thaliana*, an auxin herbicide 2,4–dichlorophenoxyacetic acid (2,4–D) was loaded onto NLCP. At 0.1% 2,4-D, NLCP herbicidal delivery was found similar to that of surfactant based 2,4-D delivery. Field trials were conducted using 2,4-D NLCP to curb the weed *Raphanus raphanistrum* in the crop fields of *T. aestivum* and *Hordeum vulgare*. When tested against commercial auxin herbicide estercide 800, 0.03% 2,4-D NLCP elicited better control of the weed and a 15% yield increase for *T. aestivum* was registered. Together, it was shown for NLCP to interact with the plant surfaces without damaging the surface architecture of the leaves. As NLCP are made from ingredients that are already used in the manufacture of cosmeceuticals it is more likely for them to be non-toxic to the flora and fauna. NLCP can undoubtedly be an alternative to classical surfactant
based delivery of agrochemicals however, further, research is much needed for better understanding the NLCP plant interactions.
Chapter 1: LITERATURE REVIEW

1.1 General introduction

To maximise the yield, agricultural practices across the globe utilise various agrochemicals at different stages of crop development. Pesticides, herbicides and insecticides are examples of traditional agrochemicals that are used routinely in agriculture. The chemicals that are applied to plants are usually a complex amalgam of different components: active ingredients, surfactants, stabilisers, adjuvants and emulsifiers. Of these, the active ingredients and surfactants together form the functional part of commercial agrochemical spray formulations. The active ingredient elicits the corresponding herbicidal, pesticidal or microcidal effects while the surfactant assists to enhance the functionality of the active ingredient. Stabilisers, adjuvants and emulsifiers contribute to enhance the efficacy of the formulation by maintaining viscosity and surface tension of droplets and they also prevent frothing. Though surfactants augment the action of the active ingredient their use is not without disadvantages. Surfactants pose threats to the crop plants they are applied and to human health, along with the potential to effect terrestrial and aquatic, flora and fauna. The plant cuticle is a thin hydrophobic layer that covers all the aerially exposed surfaces of the plant and thus it is the first point of contact for agrochemical sprays. The cuticle is a complex layer made up of fatty acids, primary and secondary alcohols, aldehydes, ketones, esters, alkanes and secondary metabolites and it acts as a primary physical barrier that protects the plant from various environmental stresses, pathogen attacks and water loss. Further, the cuticle comes in direct contact with all the anthropogenic substances that are applied or sprayed to plants surfaces. One of the most commonly applied substances on the plant surfaces in addition to irrigational water are agrochemicals. Surfactants that are mixed with agrochemicals are used to solubilise the
complex cuticular layer to enable systemic and efficient delivery of the active ingredient. Cuticular solubilisation is not only irreversible but also stresses crop plants making them more susceptible to pathogen attack and environmental factors such as drought that ultimately lead to yield loss. Additionally, due to their chemical nature there are environmental concerns with the use of surfactant based agrochemicals. Alternatives to traditional surfactants are eagerly sought by both the aspiring industries and the farmers that use them. In this thesis, nanostructured liquid crystalline particles (NLCPs) are proposed as an alternative to surfactant-based agrochemicals and that have the potential to deliver an active ingredient without solubilising the cuticle. Further, NLCPs will be less toxic to the environment and its associated biota, will be safe for humans and will not contaminate ecosystems.

The NLCPs are obtained by dispersing self-assembling lipids in water along with a stabilising agent and are similar to oil in water emulsions. The self-assembly of phytantriol followed by a high energy dispersion determines the three dimensional structure and results in formation of either cubosomal or hexosamal shaped nanoparticles. Based on this structure, the lipid-based nanoparticles may be called as cubosomes or hexosomes. The cubosomes have an internal bi-continuous cubic structure with non-intersecting water and lipid channels. Hexosomes on the other hand, have an inverted hexagonal structure along with the non-intersecting water and lipid channels. Lipid and water channels within the cubosomes or hexosomes can either be loaded with hydrophilic or hydrophobic molecules which can then be released in a controlled manner. NLCP have found considerable use in the pharmaceutical industry where their unique properties that enable delivery of drugs has been well established. Despite their wide use with pharmaceuticals they have not been used for delivery of molecules to plants.

Chapter 1 Literature review
This chapter therefore, provides a review on the use and effect of agrochemical surfactants on the cuticle and their phytotoxicity. The use of nanoparticles as an alternative to agrochemical surfactant are described and the micromorphology of the cuticle and its role in interacting with various environmental will be discussed.

1.2 Surfactants

1.2.1 Role of surfactants on plant surfaces

In the context of agrochemicals, often there is ambiguity associated with use of terms such as surfactants and adjuvants. Hazen (2000), defined surfactants as “amphipathic molecules having the ability to orient at interfaces to modify surface properties, such as interfacial tension”, and an adjuvant as, “a material added to a tank mix to aid or modify the action of an agrochemical, or the physical properties of the mixture”. Broadly, surfactants are a type of adjuvants but the reverse is not true (Hazen, 2000; Castro et al., 2014). Some of the commonly used surfactants are Silwet L-77, an organo silicone, Teric GN8 an alkylphenol ethoxylate and Isorchem 113/S-Na a linear alkylbenzene sulfonate. Furthermore, they are classified into non-ionic or ionic surfactants (Hazen, 2000; Castro et al., 2014). Adjuvants can be various types of oils, humectants, solvents, polymers and salts (Hazen, 2000).

Surfactants containing both hydrophiles and lipophiles are added to most agricultural formulations. Hydrophiles and lipophiles facilitate the entry of the active ingredient through the cuticle, via many well-known mechanisms such as hydration of the cuticle, modification of the physicochemical properties of both the agrochemical and plant surfaces and solubilisation of the cuticle (Rutter et al., 1990; Stevens et al., 1991; Stock and Holloway, 1993; Castro et al., 2013; Chitband et al., 2013). For example, rapid penetration of radiolabelled deoxyglucose through stomata in bean leaves was demonstrated through the use of organosilicone surfactants.
Similarly, use of various surfactants allowed infiltration of *Pyrus communis* (pear) leaf stomata by aqueous solutions due to decreased surface tension (Greene and Bukovac, 1974). Through hydration of the cuticle surfactants also assisted the penetration of radiolabelled deoxyglucose into the leaves of *Avena sativa* (oat), *Triticum aestivum* (wheat), *Citrus aurantium* (bitter orange) and *P. communis* (Riederer and Schönherr, 1990). The role of surfactants in reducing the contact angle of applied droplets and thereby improving the activity of herbicides was investigated in weeds (Sanyal et al., 2008). The use of surfactants was also reported to reduce both the cost and application volumes of agrochemicals in *Solanum tuberosum* (potato) farming. However, the effectiveness of a surfactant is dependent on many factors (Castro et al., 2013) such as micro-roughness of the plant surfaces (Zhang et al., 2006; Castro et al., 2013), hydrophilic-lipophilic balance (Hess and Foy, 2000) and physicochemical properties of the surfactant (Kirkwood, 1993; Zhang et al., 2006; Idziak et al., 2013; Basi et al., 2014).

### 1.2.2 Potential impacts of the use of surfactants in agriculture

#### 1.2.2.1 Phytotoxicity

Surfactant-induced phytotoxicity of crop plants and agroecosystems is well established along with concern of their use in various agricultural practices (Temple and Hilton, 1963; Parr and Norman, 1965; Krogh et al., 2003; Ying, 2006; Castro et al., 2014). A wide range of horticultural and broad acre crop plants have shown surfactant phytotoxicity and associated yield losses. Severe damage to the root system was reported for *Hordeum vulgare* after the use of alcohol ethoxylate surfactants (Krogh et al., 2003). The addition of Linear alkylbenzene sulphonates to potting soil used for growing *Sorghum bicolor* (sorghum), *Helianthus annuus* (sunflower) and mung bean (*Phaseolus aureus*), at a respective concentration of 167, 289, and
316 mg/kg was shown to be toxic (Ying, 2006). A reduction in the shoot fresh weight of *Avena sativa* (oat), turnip (*Brassica rapa*) and mustard (*Sinapis alba*) after application of Linear alkylbenzene sulphonates at various concentrations was also reported (Ying, 2006). Similarly, use of sodium alkyl ether a surfactant commonly used in agriculture negatively affected the root growth and leaf development of *T. aestivum* (Yilmaz and Dane, 2013). Wide range of ionic and non-ionic surfactants were shown to be toxic to *Cucumis sativus* (cucumber) when tested at a concentration ranging 0.1% to 1.0% (Temple and Hilton, 1963). Non-target phytotoxicity effects for five different herbicidal active ingredients when sprayed along with non-ionic surfactant Agral 90® was investigate for *Lactuca sativa* (lettuce), *Raphanus sativus* (radish), *Fragaria ananassa* (strawberry), *Zea mays* (corn), *Solanum lycopersicon* (tomato) (White and Boutin, 2007). Similarly, label recommended doses of herbicides applied in combination with (non-ionic) surfactant Agral 60 were toxic when applied to *Brassica napus* (canola), *Pisum sativum* (field pea), *Lupinus angustifolius* (lupin) and *Vicia faba* (faba bean), (Lemerle and Hinkley, 1991). Surfactant-induced phytotoxicity was also extended to the aquatic species of green algae, diatoms, cyanobacteria and floating vascular plants (Freemark and Boutin, 1994; Peterson et al., 1994; Clark et al., 2004; White and Boutin, 2007). It has also been reported that surfactant-induced phytotoxicity was not limited to yield loss of crop plants, but included ecological and economic disparities (Lemerle and Hinkley, 1991; White and Boutin, 2007).

1.2.2.2 Disruption of the leaf cuticle

Disruption and damage to leaf epicuticular wax micromorphology following treatment with surfactants has been reported for many plant species. For example for *Nerium oleander* (oleander) and *Pinus pinea* (stone pine) the solubilisation of waxes was reported after the use of the anionic surfactants alkylbenzene sulphonate and sodium dioctyl sulfo succinate

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respectively (Bussotti et al., 1997; Gonthier et al., 2010). On Brassica spp (broccoli) leaves, the non-ionic surfactant, Triton X-100 at a concentration of 0.1%, had a solubilisation effect resulting in deformation of epicuticular wax structures (Tamura et al., 2001). In contrast, the application of Triton X-100 on the leaves of Ipomoea purpurea (morning glory), Portulaca oleracea (common purslane) and Spirodela polyrhiza (giant duckweed) had no effect on the micromorphology of the amorphous waxes (Falk et al., 1994). The authors claim of an absence of epicuticular wax solubilisation was not substantiated by the scanning electron microscopy (SEM) images presented in their report, that clearly showed amorphous wax solubilisation on the surfactant treated phylloplane (Falk et al., 1994). Unfortunately, data and SEM images collected after the application of seven other surfactants on each of the three species was not presented (Falk et al., 1994). In another study with Tween-20 differential phytotoxicity on Brassica spp, Hordeum vulgare (barley), T. aestivum and V. faba was determined to be associated with the microroughness, anatomical and physiological characteristics of the plants and the chemical and physical properties of the surfactant tested (Knoche et al., 1992). After the application of the surfactant monosodium methanearsonate, changes in the epicuticular wax composition was reported for Xanthium strumarium (cocklebur) and Gossypium hirsutum (cotton) (Keese and Camper, 2006). The damage to epicuticular waxes of P. pinea was also reported to occur following the accumulation of natural sea sprays contaminated with surfactants (Nicolotti et al., 2005). Accumulation of various contaminants and decrease in the hydrophobicity of plant surfaces has also been described in the literature (Parr and Norman, 1965; Neinhuis et al., 1992). Neinhuis et al., (1992) also reported the loss of hydrophobicity and self-cleaning properties and accumulation of fungal spores on the plant cuticles after the application of Triton X-100 on the leaves of Brassica oleracea var. gongylodes (kohlrabi). In
summary, the use of surfactants for agrochemical delivery is strongly associated with phytotoxicity, loss of cuticle integrity and susceptibility to pathogens.

1.2.2.3 Impact on the environment

It is generally true for many chemicals including surfactants that their use may cause irreversible damage to the environment due to their slow or low degradability and their overuse (Castro et al., 2014). For example, the reduction in numbers of Pinus spp in the coastal forests of Italy has been shown to be closely associated with the presence of surfactants in sea sprays (Nicolotti et al., 2005). Linear alkylbenzene sulphonates, an anionic surfactant, was reported to be toxic to terrestrial plants such as grasses, vegetables and oil seeds (Ying, 2006). Excessive usage of surfactants is also reported to induce herbicide resistance in the weed Eleusine indica (Chuah et al., 2013). Similarly, the toxicity of 23 different pesticides to aquatic plants and planktons was reported in Canada (Peterson et al., 1994). Surfactants such as nonylphenol and octylphenol are reported to act on the endocrine systems of different fishes (Ying, 2006) and chronic and sublethal toxicity for a range of anionic, cationic and non-ionic surfactants has been investigated in invertebrates and fishes (Lewis, 1991). A detailed study on toxicity associated with the non-ionic surfactants alcohol ethoxylates and alkylamine ethoxylates was presented by Krogh et al (2003). Use of surfactants can also alter natural soil properties that alter the water holding capacity and result in lower soil water content (Nagy and Deák, 2013). Human health toxicity after the use of glyphosate (Castro et al., 2014), adverse endocrine disruption and sterility in humans, faminisation of young male alligators (Alligator mississippiensis), carcinogenic effects in mice, reduction in number of brown pelicans (Pelecanus occidentalis) have all been reported with the use of various agrochemicals (which are known to contain surfactants) in Latin American countries (Henriques et al., 1997).
addition, aquatic bodies are always contaminated with more than one surfactant, agrochemicals or anthropogenic substances, forming binary and ternary amalgams (Mustaffa et al., 2014). The co-occurrence of anthropogenic substances is known as synergism. The effects of synergism was reported for algae and marine bacteria due to the presence of both surfactants and chlorinated compounds (Rosal et al., 2010).

Clearly, there are number of problems for the wide scale use of surfactants in agriculture. Alternatives to traditional surfactant-based agrochemicals that can effectively deliver the active ingredient systemically to the plant and yet that are safe to the environment have been actively investigated. Recent developments in the field of nanotechnology has opened new vistas in using nanoparticles for sustained release of biomolecules and agrochemical actives systemically to plants (Hussain et al., 2013).

1.3 Nanoparticles

1.3.1 Introduction

A widely accepted definition for nanoparticles is, “an anthropogenic particle that is engineered to have unique properties that were earlier not found in its bulk and has a size ranging between 1-100 nm” (Auffan et al., 2009; Petros and DeSimone, 2010; Taylor et al., 2013; Kobayashi et al., 2014). Some, on the other hand, argue that nanoparticles can even be up to a size of 500 nm (Bawa, 2007; Buse and El-Anreed, 2010) others support a maximum size limit of 30 nm (Auffan et al., 2009) ultimately leading to a reported size range for their great diversity of 1 – 500 nm.

The shapes of nanoparticles are diverse and often their names are derived from them such as nanowires, nanorods, hollow spheres (Mondal and Srivastava, 2010), nanotubes, (Journet et
al., 1997) cubosomes or hexosomes (Boyd et al., 2007) or even as solid spheres (Panácek et al., 2009). Similar to their diversity in size and shape, a wide variety of materials have been used in their fabrication. Graphite was used for producing single-walled carbon nanotubes (Journet et al., 1997), phytantriol (a commercial lipid) and glycerol-based lipids like monoolein and mono-linolein (a monoglyceride) in preparing hexosomes (Dong et al., 2006; Boyd et al., 2007), monoolein, for cubosomes (Spicer et al., 2001), zinc sulphate (ZnSO₄) for making ZnO nanoparticles (Zhu and Zhou, 2008), biological polysaccharide materials like chitosan have been used (de Salamanca et al., 2006) and bovine serum albumin in combination with various other chemicals have been used for making cationic bovine serum albumin nanoparticles (Lu et al., 2005).

The application of nanoparticles has extended to a diverse array of biological and engineering disciplines. For example, silicon nanowires are used in the manufacture of photovoltaic devices for the conversion of solar energy into electricity (Peng and Lee, 2011), tin dioxide nanowires for gas sensing (Strelcov and et al., 2008), the growth inhibitory properties of silver nanoparticles have been used against fungi such as *Candida albicans* (Panácek et al., 2009). Nanoparticles have found use for gene delivery (Yuan et al., 2010; Prabha et al., 2014) such as mesoporous silica nanoparticles for delivery of chemicals and DNA into plants (Torney et al., 2007), nanocapsules synthesized using oligomeric ethylene glycol have been used for drug delivery (Shen et al., 2010) while a wide range of surface-modified nanoparticles have been utilised for medical and diagnostic applications (Kobayashi et al., 2014; Prabha et al., 2014).

1.3.2 Nanotechnology in plant-biotechnology

The application of nanotechnology to plants has gained increasing attention but their application has been slow to be realised. Insect pest control using diatomaceous earth microparticles of sizes ranging between 5 - 30 μm was reported on stored products such as
plywood (Mewis and Ulrichs, 2001) and though the use of diatomaceous earth is really microtechnology it can be considered as an early use of micron-sized particles that led the way towards nanotechnology. Similarly, in earlier work, an approach for implementing nanotechnology was made with Kaoline, popularly known as china clay. Kaoline was used in combination with various plastics to prepare a nano-subnanocomposite material that could enhance controlled release of fertilizers. Though the pores in the nanocomposite material ranged between 10-20 nm, it cannot be truly considered as a nanotechnology leap because the report described the use of nanoporous sheets rather than nanoparticles (Liu et al., 2006). The size of the nanoporous sheets was however not clear. The use of mesoporous silica nanoparticles both for delivery of genes and chemicals into protoplasts of tobacco as demonstrated by Torney et al (2007), was one of the very early in-vitro proof of the concept of nanoparticle applications in the plant sciences. However, there are few other reports of the application of nanoparticles to plants. The shielding property of nanoparticles against UV light was explored by using porous hollow silica nanoparticles (PHSN). These PHSN of diameter 60-100 nm prevented photodegradation of avermectin, a pesticide used in crop protection (Li et al., 2007). The uptake of much smaller mesoporous silica nanoparticles (MSNs) of size 2-20 nm without causing any phytotoxicity was reported for Arabidopsis thaliana (Hussain et al., 2013). Symplastic and apoplastic accumulation of MSNs was also reported for leaves and roots of T. aestivum, Z. mays, L. angustifolius and Arabidopsis thaliana (Sun et al., 2014). Gene silencing using biolistic delivery of Cre protein loaded MSNs in immature corn embryos was also recently reported (Martin-Ortigosa et al., 2014). Leaves infiltrated with single-walled carbon nanotubes (SWNTs) were used to demonstrate enhanced photosynthetic activity (Giraldo et al., 2014). The same group also reported higher rates of electron transport when chloroplasts were assembled on to SWNTs, creating a new vista of nanobionics. Engineered
Potato virus X (PVX) nanoparticles used to image Nicotiana benthamiana (Shukla et al., 2014) further demonstrated the applicability of nanoparticles in the plant sciences.

1.3.3 Toxicity of nanoparticles to plants

With increasing application of nanotechnology, studies on the toxicology of nanoparticles to plants has also developed. For example, it was reported that zinc nanoparticles made of either direct metal zinc or zinc oxide, inhibited both the seed germination and root elongation of six different plant species namely, Brassica napus (rapeseed), Raphanus sativus (radish), Lolium sp (ryegrass), L. sativa (lettuce), Z. mays and C. sativus (cucumber), when incubated in distilled water containing the nanoparticles (Lin and Xing, 2007). Similarly, a significant reduction in the biomass of Cucurbita pepo (zucchini) was reported with the presence of multiwalled carbon nanotubes (MWCNTs), copper nanoparticles and silver nanoparticles, when used in hydroponic solution (Stampoulis et al., 2009). An increase in oxidative stress was also reported after treating V. faba seedlings with MWCNTs (Wang et al., 2014). The reduction of surface waxes on leaves, and alteration of various physiological processes in various plants, along with various toxic effects on fungi and algae has been the subject of a review (Navarro et al., 2008).

Aerially applied CeO2 nanoparticles on the leaves of C. sativus was demonstrated to systemically accumulate throughout the plant and had negative impacts on plant health (Hong et al., 2014). Toxicity towards aquatic organisms was also reported for titanium oxide, zinc oxide, silver oxide, fullerene and carbon nanotubes (Gottschalk et al., 2009). However, nano-toxicology points to the need for the development of safer technology, which can enhance agricultural productivity. Husen and Siddiqui, (2014) have explained that, functionalization of nanomaterials can reduce the phytotoxicity. Use of fullerol (carbon-based nanoparticles) led to an increase in water retaining capacity, fruit yield, biomass and secondary metabolites like terpenes, carotenoids and saponins in Momordica charantia (bitter melon) (Kole et al., 2013).
Extensive investigation of nanoparticles and nanomaterials is therefore fundamental to the development of safe and novel uses of nanotechnology.

1.3.4 Lipid nanoparticles

A lipid is defined as, “a small water-insoluble molecule generally containing fatty acids, sterols, or isoprenoid compounds”, (Nelson and Cox, 2000) and lipid nanoparticles are formed via the use of such lipids. Self-assembly, large surface areas, non-toxicity, biocompatibility, biodegradability (Sawant and Dodiya, 2008), low commercial cost, ease of availability, and their unique structural properties, make them promising candidates for nanotechnology applications and especially in the fields of nanomedicine, cosmetics (Chen et al., 2014; Lohani et al., 2014) and the agrochemical industry. Lipid nanoparticles are broadly grouped into solid lipid nanoparticles (SLN) and nanosturctured lipid carriers or liquid crystalline nanostructures. SLN initially appeared to be promising for drug delivery, but critical evaluation led to identification of some key problems, in particular, low pay-load, undesired drug expulsion and high water content (Müller et al., 2002), that made their practical use limited. Novel cosmetic formulations that can enrich skin hydration (Wissing and Müller, 2003), ibuprofen lipid nanoparticles for oral delivery in treating inflammations (Potta et al., 2011), capotothecin loaded lipid nanoparticles for melanoma (Huang et al., 2008), are some of the few examples that demonstrate the great prospect of lipids as drug carries. However, nanostructured liquid crystalline particles (NLCP) also hold great potential and are described in the following sections.

1.3.5 Liquid crystalline nanostructures

The majority of the lipids are insoluble in water, amphiphilic in nature (and so generally referred to as amphiphiles), with a polar, hydrophilic head group and a non-polar, hydrophobic
tail group and this amphiphilic nature renders them to a unique property of forming liquid crystalline structures. Amphiphiles, when exposed to a polar environment tend to reduce free energy by positioning and self-assembling themselves into the so called mesophases or liquid crystalline structures and this phenomenon is referred to as the hydrophobic effect (Rizwan et al., 2010). These phases are analogous to oil in water. Further, the hydrophobic effect itself is dependent on many factors including, concentration, shape and ratio of the hydrophilic and hydrophobic groups of the amphiphiles (Kaasgaard and Drummond, 2006; Chen et al., 2014). Therefore, the relation between the hydrophilic and hydrophobic groups with respect to reducing the free energy of the system, is defined by the equation; \( P = \frac{v}{a l} \), where \( P \) is the critical packing parameter, \( v \) is the volume of the hydrophobic tail, \( a \) is the surface area of the hydrophilic head group and \( l \) is the length of the hydrophobic tail (Israelachvili et al., 1976; Kaasgaard and Drummond, 2006). The packing parameter, \( P \), is used to predict the type of liquid crystal phases (or mesophases) being obtained with the use of specific amphiphile (Fig 1.1). This thesis focuses on the application of \( H_{II} \) and \( Q_{II} \) shown in Fig 1.1, which are called cubosomes and hexosomes respectively. Further, the liquid crystals can be divided into thermotropic liquid crystals and lyotropic liquid crystals (Lancelot et al., 2014). For thermotropic liquid crystals, as the name suggests, temperature plays a major role in their structural and physical properties. On the other hand, many factors such as molecular concentrations of amphiphiles and their shapes, along with pressure, pH and temperature control the structural and physical properties of lyotropic liquid crystals (Rizwan et al., 2010; Lancelot et al., 2014).
1.3.5.1 Cubosomes and hexosomes

Though the mesophases or liquid crystals range in size from 150 – 300 nm they can be referred to as liquid crystalline phases or nanostructured liquid crystalline particles (NLCP) as they have repeating crystalline units of size 4 nm – 13 nm. Cubosomes and hexosomes are obtained with $P$ values ranging approximately between 1 and 2 (Fig 1.1). $Q_I$ and $H_I$ denote normal phases of cubosomes and hexosomes, while $Q_{II}$ and $H_{II}$ are used to denote inverse phases of the same. Further, inverted and non-inverted phases represent the orientation of the hydrophiles and lipophiles in the phase structure. In a normal phase the hydrophobic chains radiate inward with hydrophilic heads forming the circumference of the circle and ultimately forming a cart-wheel like structure. In an inverted phase the hydrophobic chains radiate outward, leaving the hydrophilic heads in the centre resulting in a spiked-wheel like structure.

Both the cubosomes and hexosomes have hydrophilic and lipophilic channels, but their orientations differ to give them specific phase structures. In cubosomes ($Q_I$ and $Q_{II}$ in Fig 1.1), there are three continuous and non-intersecting channels, extended three dimensionally in a matrix. Two of the three channels found in cubosomes are hydrophilic (or lipophobic) and the other is a hydrophobic (or lipophilic) channel. As there are two different types of channels, cubosomes are also referred to as bicontinuous cubic phases (Rizwan et al., 2007). In contrast, the hexosomes are not well described in the literature. They are however cylindrical micelles, parallel to each other, packed together in a hexagonal three dimensional array (see $H_I$ and $H_{II}$ in Fig 1.1) (Cullis et al., 1986; Gustafsson et al., 1997).

1.3.5.2 Applications of cubosomes and hexosomes

The application of nanostructured liquid crystalline particles (NLCP) has been extensively studied in the past and reviewing the applications of mesophases is out of the scope of this
Figure 1.1 Liquid crystals (or mesophases) formed with various values of $P$ (critical packing parameter). Not all the crystal phases are observed when an amphiphile is exposed to a polar environment, but the progression of mesophases from $L_2$ to $L_1$ via $L_{a}$ is retained. $L_1$, $I_1$, $H_1$ and $Q_1$ represent the normal (or non-inverted) phase and $L_2$, $I_2$, $H_{II}$ and $Q_{II}$ represent inverted phase. Figure adapted with permission from Dong (2009).
literature review. However, Lancelot et al., (2014); Chen et al., (2014); Mulet et al., (2013); Fong et al., (2012); Guo et al., (2010) and Rizwan et al., (2010) have all recently reviewed the application of liquid crystalline particles for drug delivery. Lohani et al., (2014) listed the patents including popular cosmeceutical companies L’Oreal and Nivea that were investigating the use of cubosomes in various personal care formulations. Together, cubosomes and hexosomes were proven as delivery vehicles for the treatment of cancer, genetic diseases, vaccination, autoimmune disorders, anti-inflammation and cataract, that can be administered through oral, nasal, ophthalmic, intravenous and topical routes. Though the use of NLCP was described exhaustively for the delivery of health care and personal care formulations, agrochemical delivery using cubosomes and hexosomes, to my knowledge, has not been reported in the literature. It would therefore seem to be reasonable to examine NLCP interactions with plants and their potential to deliver molecules of interest. But the different chemicals such as phytantriol, propylene glycol, pluronic F127, pluronic F68, glycerol monooleate, vitamin E acetate and phosphatidylcholine (Guo et al., 2010; Lancelot et al., 2014) that could be used in the preparation of NLCP are yet to be tested.

1.4 The plant cuticle

1.4.1 The role of the plant cuticle

Cuticula is a Latin word which means ‘thin skin’, from which the French botanist Brongniart, derived the term ‘cuticle’ in 1934 (Jeffree, 2007). The cuticle, as the name suggests, forms a thin layer on all aerially exposed plant surfaces, has a thickness that ranges from 0 nm to 11500 nm and is absent on roots (Jeffree, 2007). The cuticle helps to protect the plant from microbes (Jenks et al., 1994; Metraux et al., 2014), water loss (Riederer and Schreiber, 2001) and other environmental stresses. The cuticle may also be responsible for resistance of plants against
pests, for example, cabbage and wheat against *Plutella xylostella* (Diamondback moth) and *Mayetiola destructor* (Hessian fly) respectively (Eigenbrode et al., 1991; Kosma et al., 2010). It was also reported that the cuticle is responsible for the suppression of floral organ fusion and thereby mediate development of epidermal cell architecture (Chen et al., 2003; Luo et al., 2007).

### 1.4.2 Structural components of the cuticle

The cuticle is divided into three different layers: cuticle proper (CP), external cuticular layer (ECL) and internal cuticular layer (ICL) (Fig 1.2). CP (or epicuticular wax film, EWF) harbours the epicuticular waxes and is the outermost surface of the cuticle that directly comes in contact with the atmosphere. The CP is rich in the soluble cuticular components that are generally referred to as cuticular waxes. The composition of cuticular waxes was dominated by fatty acids, primary and secondary alcohols, aldehydes, ketones and esters and is detailed in section 1.4.3. The cuticular layer below the CP consists of an external cuticular layer (ECL) and an internal cuticular layer (ICL) (Fig 1.2). The ECL is sandwiched between CP and ICL, while ICL is external to the cell wall. Along with cuticular waxes, insoluble polymeric cutin, and polysaccharides, phenolics are generally found in the ECL and ICL. Therefore, the cuticle consists of an insoluble polymeric cutin matrix and soluble cuticular waxes (Domínguez et al., 2008; Pollard et al., 2008b; Samuels et al., 2008) (Fig 1.2).

### 1.4.3 Composition of the cuticle

The chemical composition of cuticular waxes is complex though it is dominated principally by fatty acids, primary and secondary alcohols, aldehydes, ketones, esters and ketones (Kunst and Samuels, 2003) (Table 1). Various pentacyclic triterpenoids such as amyrins and others were reported separately by Jenks et al., (1995) and Buschhaus et al., (2007) in the leaf cuticle of
Arabidopsis and Rosa canina (rose) respectively. Further, there are reports of the presence of tocopherols along with triterpenoids in Rubus idaeus (Red raspberry) and Crataegus monogyna (Hawthorn) flowers (Griffiths et al., 2000). Butanoids and propanoids were found in Taxus baccata (English yew) leaves (Wen et al., 2006). The presence of polysaccharides in the cuticle was summarised for Quercus ilex (oak), Eucalyptus globulus (Tasmanian blue gum), Populus spp (poplar) and Pyrus communis (pear) (Fernández et al., 2014; Guzmán et al., 2014). Structural differences between cuticles of different plants have been widely described in the literature and the chemical composition of the cuticle often varies with the different layers of the cuticle (Guzmén et al., 2014). Cutin that forms the polymeric matrix is chemically very different from cuticular waxes by having both ω- hydroxy fatty acids and mid chain hydroxy fatty acids (Kolattukudy, 1981; Samuels et al., 2008). The presence of glycerol along with unsubstituted, epoxy and polyhydroxy fatty acids, α,ω- and polyhydroxy α,ω-dicarboxylic acids, fatty alcohols and phenolics was also reported (Pollard et al., 2008a; Samuels et al.,
Figure 1.2 Diagram of the cuticle that has been modified and adapted with permission from Dominguez et al., (2011), depicts the structure of the cuticle. The epicuticular waxes are lodged on amorphous epicuticular wax film (EWF). Internal to EWF, external cuticular layer (ECL) is rich in phenolics. Internal cuticular layer (ICL) is in-turn sandwiched between ECL and the cell wall. Often cell wall polysaccharides are interspersed into the ICL. The orange colour represents the soluble cuticular waxes.
2008). The complexity of the cuticle is further increased by the presence of unmistakable
differences in compositions of the leaf versus stem cuticle as displayed by *Arabidopsis thaliana*
(Jenks et al., 1995). Similarly, differences between the adaxial and abaxial surfaces of leaves
was reported for *Taxus baccata*, where adaxial surface was dominated by alkanediols and
abaxial surface was rich in nonacosan-10-ol (Wen et al., 2006; Fernández et al., 2014).
Developmental changes in the composition of the cuticle on the leaves of *Prunus laurocerasus*
(English laurel) was also investigated to show that young leaves are rich in alcohols while the
chemical composition of older leaves is dominated by alkanes (Jetter and Schaffer, 2001).

1.4.4 Biosynthesis of the cuticle

Developmental and environmental signals tightly regulate cuticle biosynthesis which is also
under stringent transcriptional control (Samuels et al., 2008; Go et al., 2014). Cuticle
biosynthesis starts with the anabolism of long chain fatty acids that occurs in the plastids and
is common to plants. Fatty acid synthase sequentially repeats the condensation, reduction,
dehydration and reduction reactions that generate long chain fatty acyl groups (C\text{\text{16-C18}})
utilising malonyl – CoA as a 2 carbon donor (Stryer, 1995). The fatty acids formed are then
transferred to the endoplasmic reticulum by long-chain acyl-CoA synthetase (LACS) (Kachroo
and Kachroo, 2009). These long chain acyl-CoA synthetases then become the substrate for the
fatty acid elongase (FAE) multienzyme complex (comprised of four enzymes), for generating
very long chain fatty acids (VLCFAs) in a sequential elongation process. This elongation
includes several repeats of condensation, reduction, dehydration and reduction reactions (Kunst
and Samuels, 2009; Borisjuk et al., 2014) (Fig 1.3). Chains of length C\text{34} are obtained by
different FAE’s that have unique substrate chain length specificities. Fatty acyl-CoA reductase
and wax synthase, then utilise VLCFAs to generate primary alcohols and wax esters
respectively in an acyl reduction pathway. Alternatively, in a decarbonylation pathway VLCFAs become substrate to some so far unknown enzymes to produce alkanes, which are subsequently acted upon by midchain alkane hydrozylases to yield secondary alcohols and ketones (Kunst and Samuels, 2009). The transport of the wax precursors to the plasma membrane and then to the cell exterior is still enigmatic, however, an ABC (ATP binding cassette) transporter located on the plasma membrane is shown to be important for wax export (Bird et al., 2007; Yeats and Rose, 2013) along with a glycosylphosphatidylinositol-anchored lipid transfer protein (LTPG) (DeBono et al., 2009) (Fig 1.3).

1.4.5 Photoprotective properties of plant cuticles

All plant cells except those of roots need protection from harmful radiation for which the contribution of the cuticle is significant (Kokilavani and Rajendiran, 2014). However, it is important to note that, plant waxes or cutin monomers or even the cell walls do not have the ability to absorb UV radiation (Day et al., 1992; Pfündel et al., 2007). This raises the question of how plants are actually able to screen harmful radiation. Compounds like phenolics, betalains and carotenoids present either as covalently bound to epicuticular waxes and the cuticle or distributed among various epidermal cell organelles help to overcome harmful radiation (Pfündel et al., 2007). These compounds consist of conjugated double bond systems that can effectively absorb short-wavelength and high energy radiation such as ultraviolet (UV) radiation (Table 1.2).

Phenolic compounds can be divided into hydroxycinnamic acids, flavonoids, flavanols and anthocyanins. These phenolics are known to be separately present in cellular organelles like the vacuoles of epidermal cells, in fruit cuticles along with being present as covalently linked
Table 1.1 Major chemical classes found in epicuticular waxes among several plant species and their predominant chain lengths (*-class reported but carbon number was not reported; - class not reported)

<table>
<thead>
<tr>
<th>Plant species, organ and reference</th>
<th>Major chemical class and their respective chain lengths</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Fatty acids</td>
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<tr>
<td>Arabidopsis (Stem) (Lai et al., 2007)</td>
<td>-</td>
</tr>
<tr>
<td>Pea (Leaves) (Gniwotta et al., 2005)</td>
<td>26-28</td>
</tr>
<tr>
<td>Rose (Leaves) (Buschhaus et al., 2007)</td>
<td>-</td>
</tr>
<tr>
<td>Arabidopsis (Leaf) (Chen et al., 2003)</td>
<td>16-30</td>
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<tr>
<td>Arabidopsis (Leaf) (Jenks et al., 1995)</td>
<td>16-30</td>
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<tr>
<td>Arabidopsis (Stem) (Rashotte et al., 1997)</td>
<td>16-30</td>
</tr>
<tr>
<td>Arabidopsis (Stem) (Li et al., 2008)</td>
<td>24-30</td>
</tr>
<tr>
<td>Arabidopsis (Stem) (Millar et al., 1999)</td>
<td>16-30</td>
</tr>
</tbody>
</table>
Figure 1.3 Process of fatty acid elongation. Adapted with permission from Kunst and Samuels (2009). The synthesis of cuticle components begins in plastids with the synthesis of fatty acids. Fatty acids were then transferred to FAS in endoplasmic reticulum, where VLCFAs were synthesised. The cuticle precursors were synthesized from VLCFAs and transferred from ER to cell exterior via an unknown pathway. The FAS: Fatty acid synthase, FAE: Fatty acid elongase, VLCFA: Very long chain fatty acids, ER: Endoplasmic reticulum, PM: Plasma membrane, LTPG: Glycosylphosphatidylinositol-anchored Lipid Transfer Protein.
compounds (in different combinations) that help the plant to protect itself from various radiations (Pfündel et al., 2007; Kaling et al., 2014). Hydroxycinnamic acid is reported to be found covalently linked to epicuticular waxes (Liakopoulos et al., 2001). In contrast, flavonoids were not only reported to be present in the cuticle and other cell organelles but also covalently linked to hydroxycinnamic acid (Schnitzler et al., 1996). Flavanols, were also shown to be present in various fruit skins (Pfündel et al., 2007). Similar to flavonoids, anthocyanins were also found to be present in various cells, covalently linked to hydroxycinnamic acid. Together, all the phenolics help the plant to absorb high energy radiations such as UV-A and UV-B along with high intensity visible light (Karabourniotis et al.; Kaling et al., 2014).

Betalains are known to be very similar in function to that of anthocyanins and anthocyanins are involved in many plant properties and processes such as pigmentation of fruits and flowers, osmoregulation and protecting plants from harmful radiation (Stintzing and Carle, 2004). Betalains are also described as protecting plants from both UV and visible radiation and, for example, was shown for *Mesembryanthemum crystallinum* where incident radiation stimulated the accumulation of betalains and flavonoids (Vogt et al., 1999). However, there are few reports of the presence of betalains in plant cuticles and cell walls.

Carotenoids are produced in plastids and in turn plastids are known to be absent in epidermal cell layers except in guard cells (Pfündel et al., 2007). Thus, it is unlikely that carotenoids would be found associated with the cuticle. The reflective properties of the cuticle are well documented and plants protect themselves from radiation by reflecting it through epicuticular waxes which was termed as leaf specular reflectance (Barker et al., 1997).
1.4.6 The cuticle as a barrier against environmental stress

A plant's reaction to both its biotic and abiotic environment involves a highly intricate process in which the cuticle plays an important role as the primary physical and chemical barrier. The role of the cuticle in controlling water loss, for example, was demonstrated by comparing low cuticular water permeable leaves of epiphytes from tropical climates, to that of plants from Mediterranean climates. It was shown that the water loss for epiphytes was reduced in comparison to plants from tropical climates (Riederer and Schreiber, 2001). Water loss was also reduced in transgenic *Medicago sativa* (alfalfa) that overexpressed the transcription factor WRKY that was associated with the formation of a thick cuticle (Tang et al., 2014). The cuticle may also resist thermal stress by having a high specific heat that helps the cuticle to remain at a relative constant temperature avoiding fluctuations and therefore thermal stress (Casado and Heredia, 2001; Antonio, 2003). Plants respond to harsh environmental conditions by altering the chemical constituents of the cuticle, for example, the cutin content in the cuticle of *Oryza sativa* (rice) was reported to be increased by almost 48.6% and 65.9% in the leaves and panicles respectively after a drought stress (Zhou et al., 2014). Similar increases in alkane content after drought stress was reported for *Gossypium hirsutum* (cotton) (Bondada et al., 1996). It remains relatively unclear however, how a change in cuticular chemical composition may enable a plant to avoid drought stress.

1.4.7 Role of the cuticle in protection against pathogens

Hydrophobic plant cuticles help to prevent the adhesion of fungal spores and act as the first physical barrier to prevent pathogen attack (Carver and Gurr, 2007). Hydrophobicity is predominantly contributed by epicuticular wax crystals and their arrangement on the leaf surface. Leaf surface hydrophobicity together with phyllotaxy act also as a topographical
barrier to spore germination (Carver and Gurr, 2007). The plant cuticle also helps to trigger a systemic response to pathogen attack. The role of cutin monomers in defence responses was shown in suspension culture of potato cells. Addition of cutin monomers to the potato cells in culture triggered alkalinization of the medium, production of ethylene and associated defence genes (Chassort and Métraux, 2005).

The involvement of cutin monomers has recently been shown to play a vital role in defence against *Xanthomonas campestris* and *Botrytis cinerea* in *Solanum lycopersicum* (Buxdorf et al., 2014). The chemical constituents of waxes on the fruit surface of *Pyrus bretschneideri* (pear) were shown to be essential for the resistance of *Alternaria alternate* infection (Li et al., 2014). Further, cuticle breakdown products were shown to trigger systemic plant responses against pathogen attack (Metraux et al., 2014). There is little information on how cuticle damage may affect subsequent attack by pathogens and how modification of the leaf surface by chemicals and surfactants may influence this interaction.
Table 1.2 Compounds that protect plants against visible and UV radiation.

<table>
<thead>
<tr>
<th>Type of compound</th>
<th>Radiation shielded</th>
<th>Level of protection</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>i. Phenolics</td>
<td>UV-A, UV-B, Visible</td>
<td>High</td>
<td>(Schnitzler et al., 1996; Liakopoulos et al., 2001; Pfundel et al., 2007)</td>
</tr>
<tr>
<td>- Hydroxycinnamic acids</td>
<td>UV-A, UV-B, Visible</td>
<td>High</td>
<td>(Schnitzler et al., 1996; Liakopoulos et al., 2001; Pfundel et al., 2007)</td>
</tr>
<tr>
<td>- Flavonoids</td>
<td>Visible</td>
<td>High</td>
<td>(Pfundel et al., 2007; Kaling et al., 2014)</td>
</tr>
<tr>
<td>- Flavonols</td>
<td>Visible</td>
<td>High</td>
<td>(Pfundel et al., 2007)</td>
</tr>
<tr>
<td>- Anthocyanins</td>
<td>Visible</td>
<td>Low</td>
<td>(Pfundel et al., 2007; Kaling et al., 2014)</td>
</tr>
<tr>
<td>ii. Betalains</td>
<td>Visible</td>
<td>Low</td>
<td>(Vogt et al., 1999; Stintzing and Carle, 2004; Pfundel et al., 2007)</td>
</tr>
<tr>
<td>iii. Carotenoids</td>
<td>Visible</td>
<td>Low</td>
<td>(Pfundel et al., 2007; Singh and Singh, 2014)</td>
</tr>
</tbody>
</table>
1.4.8 Barrier properties of the cuticle against herbivorous insects

Plants have adopted physical and systemic strategies to reduce insect attack. At the physical level, the micromorphology of the cuticle plays a critical role in deterring insect adhesion and protecting plants against herbivorous insect pests (Müller, 2007; Fürstenberg-Hägg et al., 2013; Al Bitar et al., 2014). In addition the bioactive secondary and primary metabolites associated with the cuticle, epicuticular wax components target the metabolic systems of the insect pest (Fürstenberg-Hägg et al., 2013). *Myzus persicae* (Green peach aphid) and *Rhopalosiphum padi* (Oat aphid) settling was reported to be reduced due to the presence of various fatty acids on the leaf surface of *Clytostoma callistegioides* (Bignonia) (Castillo et al., 2010). Epicuticular wax components on *Brassica oleracea* (Cabbage) leaves were also shown to increase resistance towards *Plutella xylostella* (Diamondback moth) (Eigenbrode and Pillai, 1998). At a systemic level, the adhesion of *Pieris brassicae* (Cabbage Butterfly) eggs on the surface of *Arabidopsis thaliana* triggered chemical changes in the cuticle composition of the leaf (Blenn et al., 2012). Another report found that systemic accumulation of cutin monomer content along with its associated gene products was critical for resistance against *Mayetiola destructor* (Hessian fly) larva infestation on the leaves of *T. aestivum*. (Kosma et al., 2010). As with pathogens the interactions of the aerial parts of plants with insects are likely to be influenced by the integrity of the cuticle.

1.4.9 The cuticle of *Arabidopsis thaliana*

*Arabidopsis thaliana* has widely contributed to plant research and not surprisingly it has contributed greatly to our understanding of plant cuticles. Scanning electron microscopy studies on the cuticular waxes of *Arabidopsis*, revealed an absence of wax crystals on the leaf surfaces while being present on the surface of stems and siliques (Jenks et al., 2008). Often
wax crystal morphology on stems is associated with the presence of rods, tubes, vertical plates, dendritic and umbrella-like structures and is dominated by the presence of columnar crystals (Jenks et al., 2008). The epicuticular waxes of floral parts and seeds of Arabidopsis has not been described to date. However, this could be achieved by comparing the eceriferum cuticle mutants available for Arabidopsis. Eceriferum in Latin means “without wax” (a term derived from stone art). The eceriferum cuticular mutants for Arabidopsis commonly referred to as cer, were first described by Dellaert et al., (1979) at both phenotypic and genotypic levels. The contribution of cer mutants in cuticular studies was proved many times in the literature (Kunst and Samuels, 2003; Pollard et al., 2008b; Borisjuk et al., 2014; Go et al., 2014). The cuticular mutants of Arabidopsis along with cer mutants were described in detail by Tanaka and Machida, (2007). The growth and development of the cuticle was shown to, not only involves stringent gene regulation but was also influenced by many biotic and abiotic factors.

1.4.10 Cuticle studies on other plants

The cuticular mutants of Zea mays (Maize) and Hordeum vulgare (Barley) are described as glossy mutants and cer mutants respectively. The glossy mutants have contributed significantly to cuticular research and were described by Post-Beittenmiller (1996). Cuticle studies in other plants are limited to the characterization of the chemical composition of different wax layers (Jetter et al., 2000; Buschhaus et al., 2007; Fernández et al., 2014), hydration and thermal stress (Stark et al., 2008; Tang et al., 2014), cuticle-insect interactions (Griffiths et al., 2000; Gniwotta et al., 2005; Castillo et al., 2010; Kosma et al., 2010; Al Bitar et al., 2014) and cuticle-microbe interactions (Marcell and Beattie, 2002; Buxdorf et al., 2014).
1.5 Techniques for evaluation of the plant cuticle

It is important to understand the structure, composition and the properties of the cuticle in the context of the research presented in this thesis as the cuticle is the first plant surface to come into contact with either nanoparticles or surfactants when sprayed on the plant surface. There are many available techniques that can be used to study and understand the cuticle, for example, scanning electron microscopy, transmission electron microscopy fluorescence microscopy and gas chromatography (GC). Microscopy provides critical insight into the structural organisation while gas chromatography gives analytical precision to the component analysis of the cuticle.

1.5.1 Microscopy of plant cuticles

1.5.1.1 Scanning electron microscopy and transmission electron microscopy

SEM has been extensively used in determining the ultra-structure of the cuticle (Elliott-Kingston et al., 2014). Based on SEM analysis almost 13000 plant species were examined to classify wax crystals into 23 types (Barthlott et al., 1998). Similarly, there are other reports that determined the structure of wax crystals on Arabidopsis along with the eceriferum mutants that had altered or reduced wax micromorphology (Koornneef et al., 1989; Jenks et al., 1996; Rashotte et al., 2004; Go et al., 2014). SEM studies on other plants include characterisation of tubular wax crystals on needles of Taxus baccata (English yew) (Wen et al., 2006), examining the diversity of cuticular waxes on Salix and Populus species (Cameron et al., 2002) and observing the developmental changes in Prunus laurocerasus (laurel) leaf surface waxes (Jetter and Schaffer, 2001). Further, contrasting differences between adaxial and abaxial leaf surfaces was found for P. sativum (Gniwotta et al., 2005). In Z. mays surface wax distribution was studied in detail to show that the surface hydrophobicity was dependent on the cuticular wax (Beattie and Marcell, 2002). These reports together demonstrate the applicability of SEM to
cuticular studies, however, there is a principle difficulty which needs to be surmounted in respect to NLCP-cuticle interactions. NLCP lose their morphological properties during dry mounting, a commonly used technique for SEM sample preparation. One possible solution is the use of cryo-field emission scanning electron microscopy (cryo-FESEM), which has been successfully employed to characterise the NLCP (Rizwan et al., 2007). This technique has also been used for analysis of root hair developmental patterns in *Echium plantagineum* (Paterson's curse) and extracellular matrix assembly in diatoms (Tsai et al., 2003). The importance of sample preparation that emphasises elimination of artifacts and technical advances in sample preparation for SEM was reviewed by Pathan et al., (2008) and Ensikat et al., (2010).

TEM on the other hand has been extensively employed by many researchers to elucidate the ultra-structure of the cuticle. For example, the difference in the thickness of the cuticle on leaves of *O. sativa* cuticle mutants was demonstrated by TEM (Zhou et al., 2014). TEM has also been instrumental in describing the cuticle of the fossil plant *Ruflorinia orlandoi* (Carrizo et al., 2014). Similarly the differences in the leaf and stems of both wild type and cuticular mutants of *Arabidopsis thaliana* was investigated by number of research groups (Bu et al., 2014; Go et al., 2014). Similar to SEM, investigation of the cuticle using TEM requires stringent sample preparation protocols. Guzman et al., (2014) recently showed the effects of sample preparation on the thickness of the epicuticular wax layer found in TEM imaging of the leaves of *E. globulus*, *P. communis* (pear) and *Populus sp* (poplar).

1.5.1.2 Fluorescence microscopy

Though SEM provides major details on the surface architecture of cuticles it gives no information about sub-wax-crystal layers or their constituents. Due to the very small width of the cuticle high powered light, fluorescence and confocal laser scanning microscopy (CLSM)
must be employed for this purpose (Fernández et al., 1999). The greatest advantage of CLSM over other fluorescence microscopy techniques is that it can generate three-dimensional imaging which facilitates understanding of, and quantification of, cuticle architecture in detail (Buda et al., 2009; Wuyts et al., 2010). However, it has to be noted that dye saturation and photo-bleaching effects on samples with low fluorescence intensities are two disadvantages of CLSM over wide-field fluorescence microscopy.

Aforementioned examples for SEM, TEM and epi-fluorescence imaging techniques clearly provide valuable information about the surface architecture of the cuticle. Therefore, they can be very useful for investigating the interactions of the nanostructured liquid crystalline particles (NLCP) with plant surfaces.

1.5.2 Analysis of plant cuticles using gas chromatography

Gas Chromatography (GC) has been instrumental in cuticular research. Some examples include, determining changes in epicuticular wax composition of leaves, upon the invasion of fungus *Curvularia eragrostidis* (Wang et al., 2008), identifying developmental changes of epicuticular waxes of *Prunus laurocerasus* (English laurel) (Jetter and Schaffer, 2001), analysis of the chemical composition of wax crystals of *Taxus baccata* (English yew) needles (Wen et al., 2006), comparing the compositional changes of both, intracuticular and epicuticular waxes of leaves of *Rosa canina* (rose) (Buschhaus et al., 2007) and *Pisum sativum* (pea) (Gniwotta et al., 2005), and comparing cuticular wax composition of *Rubus idaeus* (raspberry) and *Crataegus monogyna* (hawthorn) (Griffiths et al., 2000). GC can also be employed for chemo taxonomy, where plants species are taxonomically identified and classified based on their epicuticular wax composition (Ray et al., 2014). For *Arabidopsis thaliana*, almost 40 different ecotypes were screened and an average stem wax profile for stems was proposed, along with a
detailed epicuticular wax composition for each of the *eceriferum* mutants studied (Jenks et al., 1996; Rashotte et al., 2004). The efficiency of GC is further increased with the choice of detectors namely Mass spectrometry (MS) and Flame Ionisation Detectors (FID). Thus, GC is an invaluable tool in assessing the chemical changes associated with plant surfaces in respect to this study.

### 1.6 Conclusion

This literature review clearly identifies that surfactant-based agrochemicals are heavily used in agriculture at various stages of the crop development for controlling weeds, pathogens or pests and thereby help enhance crop yield. Surfactants, however, act by changing the physiochemical properties of both the active ingredient and the plant surface. These changes on the plant surfaces are irreversible, makes the plants increasingly susceptible to biotic and abiotic stress and may lead to yield losses. Further, surfactants pose deleterious off-target effects and pollute the environment. On the other hand, nanotechnology is being successfully employed in a number of areas in the sciences. Specifically, nanostructured liquid crystalline particles (NLCP) made up of lipids, have been tested widely for the delivering of the oral drugs and cosmetics. Also the literature presented here identifies the ability to load both hydrophilic and hydrophobic drugs onto the NLCP. Therefore, NLCP have been proposed as an alternative delivery vehicles to the classical surfactant based agrochemical delivery and is the focus of the research presented in this thesis.

### 1.7 Objectives of this thesis

The research presented in this thesis investigates the use of nanostructured liquid crystalline particles (NLCP) as an alternative delivery vehicle for agrochemicals. For this the surface interactions of NLCP were compared with that of traditional agrochemical surfactants on four
different plant species *Triticum aestivum* (wheat), *Zea mays* (maize), *Lupinus angustifolius* (lupin) and *Arabidopsis thaliana*. The response of the plants to NLCP and surfactants were also evaluated in the presence of biotic and abiotic stresses. Finally, the efficiency of NLCP to deliver herbicides was tested and compared to that of surfactants.

This research was therefore structured to focus on four key areas as follows:

Chapter 2. **Investigation of the micromorphology of plant cuticle.** This research was aimed at understanding the intricate micromorphology of the cuticle and arrangement of the epicuticular waxes on the leaf surface of the four plant species *T. aestivum*, *Z. mays*, *L. angustifolius* and *A. thaliana*. Methods to evaluate the structural integrity of epicuticular waxes were also investigated.

Chapter 3. **The potential of NLCP as next generation agrochemical delivery vehicles.** For this study the NLCP were compared at different concentrations against traditionally used agrochemical surfactants. Phytotoxicity and the integrity of epicuticular wax micromorphology was simultaneously evaluated, to understand the impact of the surfactants and NLCP formulations on plant surface roughness.

Chapter 4. **Biotic and abiotic stress response on plants treated with NLCP and surfactant spray applications.** In this chapter two key stress response components were quantified to evaluate the physiological performance of the plants following treatment with surfactants and NLCPs. Novel image analysis techniques were employed to quantify data obtained.

Chapter 5. **Screening the efficiency of NLCP to deliver an auxin herbicide.** For this chapter NLCP delivery of an auxin herbicide was compared to that of traditional surfactant-based
herbicide delivery at different concentrations. Field trials were also conducted to evaluate the performance of NLCP to kill invasive weeds.
Chapter 2: ELUCIDATION OF THE MICROMORPHOLOGY OF THE CUTICLE AND EPICUTICULAR WAXES ON THE PHYLLOPLANE

2.1 Introduction

All the plant surfaces except those of the roots are covered with a continuous hydrophobic layer of cuticle that protects the plant from external stress such as water loss, pathogen attacks and UV light (Jenks et al., 1994; Pfundel et al., 2007). There is comprehensive literature available on the biochemical and biophysical properties of the cuticle which aim to understand the structural and biological properties of plant cuticles (Jetter et al., 2007; Pfundel et al., 2007; Riederer and Friedmann, 2007; Schreiber, 2007). Broadly, the cuticle is divided into three consecutive layers namely the cuticular proper, the external cuticular layer and the internal cuticular layer. Cuticular proper forms the outermost layer of the cuticle and it harbours epicuticular wax crystals that come in direct contact with the atmosphere (Jeffree, 2007). Cuticular proper is continuous with external cuticular layer which in turn is stacked on internal cuticular layer external to cell wall.

The plant cuticle is made up of a complex mixture of chemicals including fatty acids, aldehydes, primary and secondary alcohols, alkyl esters and ketones (Jenks et al., 1995; Kunst and Samuels, 2003). The composition and number of chemical classes that can be observed in a plant cuticle is highly variable. Cutin that forms the backbone of the cuticle is made up of mid-chain hydrox and epoxy C_{16} and C_{18} fatty acids and the cuticular waxes that are interspersed in cutin matrix are predominantly made up of C_{20} to C_{40} aliphatic compounds and secondary metabolites like terpenoids, flavonoids and phenylpropanoids (Samuels et al., 2008). On the leaf surface of *Taxus baccata* (English yew) the composition of the epicuticular wax
crystals on the adaxial leaf surface is different to that of abaxial surface by having 33% more
alkanediols. Similarly the abaxial surface had 8% greater nonacosan-10-ol (Wen et al., 2006).
Similar differences for the cuticular composition of stem and leaf surfaces of Arabidopsis were
also reported (Jenks et al., 1995). The term, “epicuticular wax crystals”, could sometimes be
misleading because it does not describe the crystalline or amorphous nature of the wax crystals.
Nevertheless, the amount of crystallinity in the epicuticular waxes was reported to be higher
when compared to other layers of cuticle and thus they may be called as epicuticular wax
crystals (Stark and Tian, 2007). Further, a mosaic arrangement of amorphous and crystalline
regions in different layers of cuticle was reported (Jetter et al., 2007; Stark and Tian, 2007).
The formation of amorphous zones in the cuticle was explained as a consequence of hydrogen
bonding between different chemical components of cuticle. For example, alcohols and esters
(Casado and Heredia, 2001). The presence of such regions is proved to be important for
physical strength and selective permeability of the cuticle (Casado and Heredia, 2001; Jetter et
al., 2007).

Modern agricultural practices are heavily dependent on the usage of agrochemical actives for
the control or removal of insect pests, weeds or pathogens. Agrochemical actives due to their
physicochemical nature may have minimal capacity to be absorbed through the cuticle.
Surfactants are therefore added as a vital component to agrochemical spray formulation to
enhance the ability of active ingredient. These surfactants facilitate maximum absorption of
active ingredient into the cuticle by hydration or solubilisation or by changing physicochemical
properties of cuticle (Stevens et al., 1991; Stock and Holloway, 1993). But often surfactants
permanently damage the cuticle and epicuticular waxes. Severe to moderate alteration of the
epicuticular wax micromorphology after agrochemical surfactant sprays has been reported for

Chapter 2 Elucidating the micromorphology of the cuticle and epicuticular waxes
a number of weeds (Falk et al., 1994) and crop plants (Tamura et al., 2001; Gonthier, 2010). Though these reports show a direct evidence for epicuticular wax crystal damage, the amount of damage to the wax micromorphology was not quantified. The prime reason for this might be unavailability of methods to quantify the damage to wax micromorphology. Hence, it is important not only to develop a method for the precise quantification of damage to epicuticular waxes but also to compare and understand the nature and intensity of the micromorphological change on the phylloplane.

While significance progress has been made into understanding the biochemical and biophysical properties of cuticle, the micromorphology of the cuticle on the plant surface remains relatively unexplored. Bright filed and fluorescence microscopy techniques have been employed to image plant cuticles in combination with stains like Sudan IV, Nile blue and Auramine O. Though Sudan IV staining was extensively reported for staining pericarp and endosperm of cereal seeds (Krishnan and Dayanandan, 2003; Czerednik et al., 2012) it is rarely used to the stain cuticle on the plant surface. Sudan IV staining for the visualisation of the cuticle is limited to leaf and fruit surfaces of *Fragaria* sp (Lisek et al., 2002) and *Solanum lycopersicum* respectively (Domínguez et al., 2008; Buda et al., 2009). *T. aestivum, Z. mays, L. angustifolius* and *A. thaliana* were never described for cuticle staining using Sudan IV. Similarly, Auramine O was also reported many times in the literature for the study of pollen grains (Cook and Oparka, 1983; Chaudhury et al., 1994; Peirson et al., 1996). The use of Auramine O for the visualisation of the cuticle was not extended to plants beyond *Z. mays* and *Solanum lycopersicum* (Lequeu et al., 2003; Buda et al., 2009). Clearly, the potential of Sudan IV and Auramine O to selectively stain the cuticle has not been fully explored. The current chapter therefore aims to study the cuticular structure and arrangement on leaves of *T. aestivum, Z. mays, L. angustifolius* and *A.
thaliana using a variety of microscopy approaches. For this cuticular staining was performed using Sudan IV, Auramine O and Calcofluor. Bright-field and epifluorescence microscopy techniques were then employed to compare the structural arrangement of the cuticles. Epicuticular waxes were also visualised under high power scanning electron microscopy (SEM) and novel image analysis techniques were developed to quantify the data obtained with SEM. This study clearly demonstrates that the micromorphology of the plant cuticle could be studied in great detail by applying simple staining techniques in combination with basic microscopy and image analysis tools.

2.2 Materials and Methods

2.2.1 Plant growth and maintenance

Of the four plant species selected for the study T. aestivum and Z. mays are monocot crop plants with high economic importance. The dicots selected for this research are L. angustifolius (Lupin) and A. thaliana, the former is an emerging crop in Australia and the latter is a model used extensively in plant research.

A. thaliana ecotype Col-O seeds obtained from Lehle seeds (Round Rock, Texas, USA) were carefully transferred to 1.5 ml Eppendorf tubes for surface sterilisation. Seeds were sterilised in 50% (v/v) ethanol and 1.5% (w/v) hydrogen peroxide (Sigma-Aldrich, Sydney, NSW, Australia) for about 5 minutes while inverting the tubes regularly. After centrifugation for 20 seconds the supernatant was discarded and the seeds were rinsed in sterile distilled water for at least 3 times to remove any traces of hydrogen peroxide. 0.15% bacteriological agar was added to suspend the seeds before transferring them to MS basal media (Sigma-Aldrich, Sydney, NSW, Australia) in 90mm petri plates enriched with 30% sucrose along with 0.8% bacteriological agar at pH 5.7. Seeds were transferred to growth cabinets after a stratification
for 48 hours at 4°C. The seeds were maintained in growth cabinets for 14 days at 21°C, under cool fluorescent light (approximately 100 μmol/m²/s) with a photoperiod of 16/8 light/dark period (Thermoline Scientific, Wetherill Park, NSW, Australia). Healthy seedlings were carefully transferred to sterile potting mix (Potmate Premium Potting Mix, Debco, Tyabb, VIC, Australia) in 4 cm wide × 8 cm tall pots. 3 plants/pot were planted and number of plants per pot varied according to experiment.

*L. angustifolius* variety ‘Wonga’ seeds (Naracoorte seeds, Naracoorte, SA, Australia) were sterilised by continuously string seeds in 80% ethanol for 30 sec. The seeds were directly transferred to 2% v/v sodium hypochlorite solution in which they were stirred for 2 min. At the end of 2 min seeds were immediately washed in distilled water at least for 5 times to clear any traces of sodium hypochlorite. They were then transferred to growth cabinet for incubation, in plastic trays containing a thin layer of cotton wool saturated with water. The incubation continued for two days at a temperature of 21°C under high pressure sodium lights (approximately 300 μmol/m²/s) with 16/8 light/dark photoperiod in growth cabinets (Thermoline Scientific). For further development healthy seedlings were transferred to sterile potting mix (Potmate Premium Potting Mix) in 10cm wide × 10 cm tall pots and maintained under same conditions described earlier for *L. angustifolius* seeds.

For *Z. mays* variety Early Leaming (Eden seeds, Lower Beechmont, QLD, Australia) and *T. aestivum* cultivar Wyalkatchem (gift from Nufarm Australia Limited, Laverton North, VIC Australia) seeds were sterilised following the procedures earlier described for *L. angustifolius* but were directly transferred to sterile soil in 10cm wide × 10 cm tall plastic pots. Pots were regularly watered and maintained in conditions earlier described for *L. angustifolius*. 

*Chapter 2 Elucidating the micromorphology of the cuticle and epicuticular waxes*
2.2.2 Sample fixation and cryosectioning

Four plants per pot were potted for each of *A. thaliana*, *T. aestivum*, *Z. mays* and *L. angustifolious* and were maintained as described earlier. After 21 days of potting (seven days for *A. thaliana*) healthy leaves from plants were excised and washed in distilled water to remove any particulate matter. The leaves were fixed following the method described by Buda et al., (2009) with several modifications. Briefly, leaf tissue was floated on FAA fixative containing 37% formaldehyde (v/v), 5% glacial acetic acid (v/v), 45% ethanol (v/v), 45% distilled water (v/v) and vacuum-infiltrated for 15 min. The leaf tissue was transferred to 4 °C and left overnight on fresh FAA fixative. For cryoprotection the tissue was vacuum-infiltrated for 15 min in 10% sucrose (w/v) in 100Mm PBS containing 11.5 g/L Na$_2$HPO$_4$, 2 g/L NaH$_2$PO$_4$, 90 g/L NaCl, at pH 7.2, followed by a 15 min vacuum infiltration in 20% sucrose(w/v) in 100Mm PBS. Subsequently, the tissue was rinsed in embedding medium (Tissue-tek OCT compound, ProSciTech Pty Ltd., Thuringowa, QLD, Australia) and placed in cryomolds containing fresh embedding medium (15 mm × 15 mm × 5 mm, ProSciTech). The cryomold were then very slowly immersed in liquid nitrogen and the frozen cryomolds were stored at -80 °C until further processing. Sometimes, tissue was directly cryoprotected without fixing it in FAA. A Cryostat (Microm HM550 OMP, Thermo Scientific, Scoresby, VIC, Australia) was employed to cut transverse sections of 5 μm – 30 μm thickness. The sections were carefully transferred to gelatine coated slides and stored at 4° C until further use.

2.2.3 Staining of tissue sections

Three stains namely Sudan IV, Auramine O and Calcofluor white M2R (Sigma-Aldrich) were used to facilitate visualisation of the cuticle in bright filed and fluorescence microscopy. Sudan IV stain was prepared by dissolving 0.1% Sudan IV (w/v) in isopropyl alcohol. This solution

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Chapter 2 Elucidating the micromorphology of the cuticle and epicuticular waxes
was diluted with water in a ratio of 3:2 before filtering it through a 0.4 μm syringe filter (GE Healthcare Pty. Ltd. Australia, Rydalmere, NSW, Australia). Cryosections were then stained for 10 min before gently washing away unbound stain with 50% isopropyl alcohol. After very gently washing with distilled water a cover slip was mounted and edges were sealed with transparent nail polish (Buda et al., 2009).

Auramine O stain was prepared by dissolving 0.1% Auramine O (w/v) in 0.05 M Tris/HCl at pH 7.2. The sections were stained in this freshly prepared stain for 15 min. After washing very gently with distilled water slides were mounted in DABCO (Sigma-Aldrich) or 50% glycerol and sealed with transparent nail polish. Utmost care was taken while handling Auramine O as it is highly carcinogenic (Buda et al., 2009).

Calcofluor white M2R stain was prepared by dissolving 0.1% (w/v) in distilled water. After staining with Calcofluor for 2 min and gently rinsing with water the cryosections mounted on slides were then successively stained with Auramine O. Mounting was performed as elaborated above for Auramine O (Buda et al., 2009).

2.2.4 Bright field and fluorescence microscopy

Bright field microscopy and epifluorescence microscopy were performed using an Axioskop 2 mot plus microscope (Zeiss, Göttingen, Germany). A digital camera attached to the microscope was employed to capture images. For collecting images ultraviolet epifluorescence filter with 365 nm excitation, 420 nm emission bands was used and for blue light a filter with 450-490 nm excitation, 520 nm emission bands was used. For confocal microscopy a Leica TCS-SP5 microscope (Leica Microsystems Pty Ltd., North Ryde, NSW, Australia) running with image capture software (LAS AF software version: 2.6.3.8137) was used. For Auramine O an excitation from 458 nm argon laser was used and emission was collected between 491 nm and

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563 nm. Calcoflour was excited using laser from 405 nm near-UV diode and emission was collected between 415 nm and 448 nm (Buda et al., 2009). Before proceeding to z-stacking at a magnification of 1000×, 2D imaging wax performed by adjusting the zoom. Auto-programming based on individual sample was used to collect the z-stacks with a step size of 0.5 μm. 3D movies were generated using LAS AF software version: 2.6.3.8137. To improve, the resolution brightness, contrast and midtones on all the microscopy images were adjusted using Microsoft ® office picture manager 2010 (Microsoft Pty. Limited, North Ryde, NSW, Australia). Adobe ® Phtoshop ® CS6 version 13.0 ×64 was used to present the images for this research (Adobe Systems Pty. Ltd., Sydney, NSW, Australia).

2.2.6 Sample preparation and gold coating for scanning electron microscopy (SEM)

Fresh leaves were collected from plants that were grown as described in sections 2.2.1. A healthy and mature leaf was randomly sampled from each plant giving a total of four leaves per species. The excised leaves were maintained at room temperature for 24 to 48 hours to slowly reduce the amount of water (Pathan et al., 2008). A small area of 3 × 3 mm² area was randomly cut using a clean scalpel blade from each leaf. The tissue was mounted on to a clean aluminium stub using an adhesive conducting carbon tape and stored in vacuum chamber until further use. The samples were coated gold palladium for 120 seconds at 40 mA (BAL-TEC Sputter Coater SCD 050, Scotia, NY, USA) before performing scanning electron microscopy (Supra 55 VP, Carl Zeiss Pty Ltd., NSW, Australia). A voltage between 5-10 kV was maintained while imaging the samples. For each plant a total average of 35 images was obtained.

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2.2.7 Image analysis

ImageJ version 1.46r (Schneider et al., 2012) was used to calculate the area of wax crystals and the perimeter of wax crystals on SEM images. An additional software programme, “Pixcavator” (www.inperc.com) was used in combination to ImageJ for calculating the relative density (RD) of wax crystals. Data obtained was statistically analysed using IBM SPSS Statistics 21 (IBM Australia Ltd, St Leonards NSW, Australia).

2.2.8 Gas chromatography

To extract the cuticular waxes on the surface of leaves 200 mg of fresh leaf tissue was dipped in 2 ml chloroform containing 100μg of triacontane (Sigma-Aldrich) for 15 sec, 30 sec, 45 sec and 60 sec at room temperature separately (Broun et al., 2004). After removing the plant tissue, chloroform was removed under continuous stream of nitrogen gas. Prior to derivatization, the extract was dissolved in 100 μl of N,O-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (Sigma-Aldrich) and 100 μl pyridine (Sigma-Aldrich). Derivatization was carried at 100 °C for 1 hour. To analyse the samples, HP5MS (30 m, 0.25 mm i.d., 0.25 μm film thickness; Supelco) column fitted to Agilent 6890 running on a flame ionisation detector was used. The initial temperature of oven was set at 150°C followed by ramping at 4°C/minute to 320°C (10 minutes hold). At an injection temperature of 280°C a sample volume of 1 μl was injected and flow rate of helium gas was adjusted at 1.5 l/minute (Hannoufa et al., 1993; Millar et al., 1999). Peaks were identified by comparing the retention times with known commercial standards (Sigma-Aldrich). Even numbered long chain saturated fatty acids between C16 to C30, even numbered long chain primary alcohols between C24 to C30 and long chain alkanes between C20 to C40 were used to identify respective peaks (Hannoufa et al., 1993; Millar et al., 1999).
2.3 Results

2.3.1 The structure of the cuticle on the leaf surfaces

Three different stains namely Sudan IV, Auramine O and Calcofluor, were employed to study the structure of the cuticle on the leaf cross sections of *Triticum aestivum*, *Zea mays* and *Lupinus angustifolius*. Both Sudan IV and Auramine O are lipophilic stains, while Auramine O additionally has strong affinity to stain unsaturated fats and cutin precursors, and it is also a fluorescent stain. Sudan IV stains the cuticle bright red to pink layer that can be readily observed using bright field microscopy. Auramine O stains the cuticle yellow to green colour and has differential affinity towards different layers of cuticle facilitating high resolution imaging. Calcofluor is another fluorescent stain with strong affinity towards cellulosic material in cell walls. It stains cell walls in deep blue colour. Dichromatic staining to differentiate cuticle region to cellulosic regions was achieved by using Auramine O in combination with Calcofluor. Both epifluorescence microscope and confocal microscope were used to collect images from the dichromatically stained samples.

Following Sudan IV staining the, cuticle was observed as a continuous bright red to pink layer on both adaxial and abaxial surfaces of the leaves of *T. aestivum* (Fig 2.1). It was observed that anticlinal cell wall regions had a thickest cuticle cover, which tapered towards periclinal cell walls and the cuticle on epidermal cells of leaf edges seemed to be thicker than that of epidermal cells on the phylloplane (Fig 2.1 a, b and c). The cuticle on the stomatal guard cells was found to be projecting outward (Fig 2.1 d). Fluorescence microscopy on Auramine O stained leaf cross sections of *T. aestivum* helped to resolve the cuticle into different layers namely cuticle proper (CP), external cuticular layer (ECL) and internal cuticular layer (ICL) (Fig 2.2). All the layers of cuticle CP, ECL and ICL together followed the contour of the...
epidermal cell walls forming the silhouette of the epidermal cell layer. The CP was seen as a conspicuous bright layer over a dull fluorescent ECL (Fig 2.2 a and b) and the boundaries of CP were observed to be distinct to that of ECL and ICL (Fig 2.2 c and d). ECL was sandwiched between two bright layers CP and ICL, and ICL was observed to be a continuous layer with the underlying cell wall of epidermis (Fig 2.2 b, d and f). Consistent with Sudan IV staining, the thickness of the cuticle was observed to be higher in anticlinal cell junctions in comparison to the cuticle on periclinal cell regions of the epidermis (Fig 2.2 d and f). Cuticular overhangs seen earlier with Sudan IV staining were also confirmed with Auramine O staining (Fig 2.2 e). After dichromatically staining cryosections with Auramine O and Calcofluor a bright yellow to red fluorescence could be visualised (Fig 2.3). The yellow to red colour from the periclinal cell wall of the epidermis adjacent to the cuticle was extended to the anticlinal cell walls of the epidermis (Fig 2.3 a). It was interesting to observe a sudden shift in colour from yellow red to deep blue colour, as we move from the anticlinal pegs to lower periclinal cell wall of epidermis adjacent to parenchyma (Fig 2.3 a). A yellow to green colour was also observed in the leaf edges and stomatal guard cells instead of the yellow to red colour as observed on epidermal cells of phylloplane (Fig 2.3 c and e). The confocal images of the cuticle however showed a distinct demarcation between cuticular and cell wall layers (Fig 2.3 b, d and f). The cuticle could clearly be resolved into distinct layers namely CP, ECL and ICL. Staining cryosections with Sudan IV for the cuticle on Z. mays leaves showed similar results as seen with that of T. aestivum (Fig 2.4). The cuticle was stained in red to pink colour and it was observed as a continuous layer on the epidermal cells (Fig 2.4 a, b and c). Cuticular over hangings on stomatal guard cells were consistent with that of T. aestivum (Fig 2.4 d). Auramine O staining was also consistent with both Sudan IV staining on Z. mays and earlier observations.
on *T. aestivum* Auramine O staining (Fig 2.5). The cuticle on *Z. mays* leaves could be resolved into CP, ECL and ICL, where ECL was sandwiched between brightly fluorescent layers of CP and ICL (Fig 2.5 a, b, c and d). Brightly stained cuticular overhangings on stomata could also be seen on *Z. mays* cryosections (Fig 2.5 e and f). The calcofluor staining on *Z. mays* leaf cryosections showed a different pattern of staining when compared to that of *T. aestivum* (Fig 2.6). Consistent with earlier staining techniques, the cuticle could be clearly resolved into CP, ECL and ICL (Fig 2.6 a). The conspicuous blue colour uniformly spread throughout the periclinal and anticlinal cell walls of the epidermis indicated the strong presence of cellulosic material (Fig 2.6 a). In the anticlinal epidermal cell walls a green to yellow colour originating from Auramine O staining appeared to be diffused along with the blue colour from Calcofluor staining (Fig 2.6 a and c). Confocal microscopy could also clearly resolve different layers of the cuticle (Fig 2.6 b, d and f). Calcofluor staining was however not observed in leaf edges and stomata of *Z. mays* sections and was consistent with the same on *T. aestivum* (Fig 2.6 c, d, e and f).

Staining for the visualisation of the cuticle on *L. angustifolius* was also done using Sudan IV, Auramine O and Calcofluor. Consistent with the observations made on *T. aestivum* and *Z. mays*, Sudan IV staining for the cuticle on the leaf surface of *L. angustifolius* showed cuticle as a continuous thin layer on epidermis (Fig 2.7 a and b). Unlike *T. aestivum* and *Z. mays* cuticular thickenings could be seen on cuticle at the anticlinal cell junctions of the epidermal cells (Fig 2.7 c). Cuticular overhanging on the guard cells of stomata were also confirmed on *L. angustifolius* leaf cross sections (Fig 2.7 d). Auramine O staining also confirmed all the observations made with Sudan IV for *L. angustifolius* leaf cryosections (Fig 2.8). The cuticular thickenings earlier observed with the Sudan IV staining could be further confirmed with

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Figure 2.1 Arrangement of the cuticle on *T. aestivum* leaf sections. For the visualisation of the cuticle Sudan IV stained cryosections were observed using bright field microscopy. Cuticle was observed as a bright red to pink layer on the epidermal cells as indicated by arrows in images (a) and (b). (c) Arrangement of cuticle on leaf edge is shown by the arrow. (d) Cuticular arrangement on stomatal guard cells. The black arrow 1 points the cuticular overhanging on guard cells. Arrow 2 points the stomatal chamber and arrow 3 shows the chloroplasts in parenchymal cells. Scale bar is 5 μm.
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Figure 2.2 The arrangement of the cuticle visualised with Auramine O staining. The cross sections of *T. aestivum* were stained with Auramine O and observed under fluorescence microscope (a, c and e) and confocal microscope (b, d and f). (a) The arrow indicates the cuticular proper (CP) that could be seen a bright layer on the epidermal cells. (b) Leaf edge showing CP is indicated with white arrow. (c and d) Along with cell wall (CW) the layers of the cuticle could be seen with differential intensity in brightness. (e) Cuticular over hangings on the stomatal guard cells is indicated with white arrows. (f) External cuticular layer (ECL) could be seen as a dark layer above internal cuticular layer (ICL). ICL could be seen as a bright layer continuous with the CW. The boundaries of all the layers of the cuticle (CP, ECL and ICL) were seen to be diffused with adjacent layers. As indicated by the curly brackets the thickness of the cuticle (C) is also variable along the cell periphery, where it is minimal on the periclinal cell wall (top curly bracket) and maximum on the anticlinal cell walls (bottom curly bracket). Scale bar is 5 μm.
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Figure 2.3 Distribution of cuticular material on *T. aestivum* phylloplane. Dichromatic staining was performed by staining cryosections with Auramine O followed by Calcofluor. Calcofluor has affinity towards cellulosic material while Auramine O stains wax and lipid regions. (a, c and e) are fluorescence microscopy images while (b, d and f) are confocal microscopy images. (a) Yellow to red colour fluorescence was observed in epidermal cell layers of *T. aestivum* is indicated by white arrows. The insight clearly differentiates the cuticular region stained green to cell wall region stained yellow to red is indicated with a white arrow. (b) Confocal image clearly differentiates the cuticle proper (CP) stained in pink to red from other layers of cuticle. (c and d) Leaf edge showing a dense deposition of cuticular material, that is considerably different to that of epidermal cells (a and b). Auramine O stain seen as green in image (c) and red in image (d) is indicated with the arrows. The insight on image (c) shows the presence of only green to yellow colour staining originating from Auramine O. (e) The guard cells of stomata showed a similar pattern earlier observed with that of epidermal cells. Clear differentiation between cell wall region and cuticular region can be observed in the insight. Arrows indicate the cell wall region. (f) Cuticle was resolved into three different layers namely cuticular proper (CP), external cuticular layer (ECL) and internal cuticular layer (ICL). ECL is observed to be a dull fluorescing layer sandwiched between brightly fluorescing layers of CP and ICL. Note, the bright field channel is eliminated from the confocal image (f) to show the different layers of cuticle. Scale bar is 10 μm.
Figure 2.4 Sudan IV staining for the cuticle on *Z. mays* leaf cryosections. (a and b) Cuticle stained pink to red could be seen (indicated with arrows) as a continuous layer on the epidermal cells of leaf. (c) Cuticular arrangement on leaf edge. (d) Intense red to pink colour on stomatal guard cells indicate cuticular over hangings is shown with arrow. Scale bar equals to 10 μm. 

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Figure 2.5 Auramine O staining on *Z. mays* leaf cross sections. (a, c and e) After staining the sections with Auramine O, epifluorescence was collected using fluorescence microscopy (a, c, and e) and (b, d and f) confocal microscopy. (a and b) cuticular proper (CP) can be seen as a bright layer above the dark external cuticular layer (ECL). (c and d) ECL is sandwiched between bright layers of CP and internal cuticular layer (ICL). (e and f) Cuticular over hangings are very evident on the stomatal guard cells were shown by arrows. Scale bar equals to 5 μm.
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Figure 2.6 Dichromatic staining for cuticle distribution. (a, c and e) Fluorescence microscopy and (b, d and f) confocal microscopy on the Z. mays leaves after double staining with Auramine O and Calcofluor. (a) The distribution of Calcofluor in the epidermal cells in blue colour clearly demarcates the cell wall regions from cuticle stained in green to yellow colour. In the insight, cuticle could be resolved into a bright outermost layer called cuticular proper (CP). External cuticular layer (ECL) is stacked upon a bright layer of internal cuticular layer (ICL) which in-turn is continuous with the cell wall (CW) (b) CP can be observed as a bright pink layer in the confocal image was indicated by an arrow.(c) Leaf edge does not show intense blue colour as seen in epidermal cells. In the insight the CP appear to be thick over the leaf edge when compared to that of epidermal cells and different layers of cuticle are further evident. (d) Similar to image (c) the intense cuticular material deposition could be seen in leaf edge in bright red to pink colours. (e and f) Cuticular over hangings are indicated with arrows. Similar to leaf edges stomatal guard cells did not show any Calcofluor staining indicating absence of cellulosic material. Scale bar approximately equals to 10 μm.
Auramine O staining. Dichromatic staining performed using Auramine O and Calcofluor stains could clearly resolved different layers of cuticle and confirms the observations made for Sudan IV and Auramine O staining (Fig 2.9). To the best of my knowledge the arrangement of cuticle on the leaves of *L. angustifolius*, was reported for the first time.

### 2.3.2 Three-dimensional micromorphology of the cuticle

To understand the structural arrangement of the cuticle respective to the underlying cell wall 2D imaging was performed on the dichromatically stained epidermal cells of *T. aestivum*, *Z. mays* and *L. angustifolius*. Confocal microscopy allows to separate the fluorescence obtained from individual stains into separate channels. This not only helps to understand the distribution of cuticular and cellulosic material on the cell but also shows their spatial arrangement by eliminating the interference caused by other channels (Fig 2.10). Z-stacks were then performed by taking optical sections through the sample which were then reconstructed to obtain 3D images (Fig 2.11). From the 2D and 3D images clear differences between different plant species for the cuticular deposition were evident. *T. aestivum* and *Z. mays* had similar extended deposition of cuticular material in their anticlinal cell walls (Fig 2.11 a, b, d, e, g and h). The same was observed to be diminished and was limited to cell junctions for *L. angustifolius* (Fig 2.11 c, f and i). The spatial distribution of the cellulosic material was also found to be uniform among the plant species of the study (Fig 2.10 d, e and f). For all the Z-stacks there was a significant decrease in the intensity of fluorescence deeper into the tissue (Fig 2.11 e, f, h and...
Figure 2.7 *L. angustifolius* cuticle staining with Sudan IV. (a) and (b) The cuticle on *L. angustifolius* could also be seen as continuous pink to red layer over the epidermal cells of phylloplane is indicated with arrows. (c) Cuticular arrangement over the leaf edge. Arrow 1 shows the chloroplast. Arrow 2 indicates the cuticular thickenings at the anticlinal cell junctions of the epidermal cells. (d) Cuticular over hangings are evident over the guard cells of the stomata and is pointed by an arrow. Scale bar is 10 μm.
Figure 2.8 Staining *L. angustifolius* leaf sections for the cuticle with Auramine O. Auramine O staining helped to visualise the cuticle over the epidermis in fluorescence microscopy. (a, c and e) Epifluorescence was collected using a fluorescence microscope. For images (b, d and f) epifluorescence was collected in combination with bright-field settings. Cuticular thickenings at anticlinal cell junctions (a, b, c and d) and overhanging on stomata (e and f) are indicated with arrows. Scale bar is 10 μm.

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Figure 2.9 Double staining *L. angustifolius* cross sections for the cuticle. Auramine O and Calcofluor were used to stain cuticle and cellulosic material respectively. Epifluorescence was collected using a fluorescence microscope (a, c and d) and confocal microscope (b, d and f) shows the cuticle resolved into different layers namely cuticle proper (CP), external cuticular layer (ECL) and internal cuticular layer (ICL) from cell wall (CW). (a) Clearly resolved layers of CP and CW were shown with the arrows on epidermal cells. Insight shows the same at anticlinal cell junctions. (b) The distribution of the cuticular material on the leaf edge. CP (indicated with arrow) could be seen as a bright pink layer resolved from underlying layer of CW layers stained in blue. (c and d) CP, ECL, ICL and CW arrangement on epidermal cell. (e and f) The cuticular overhangs on stomatal guard cells are indicated with the arrows.
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Figure 2.10 The ultra-structure of the cuticle. Confocal microscopy images presented here show the arrangement of cuticle on epidermal cells in four channels for three plant species. (a, b and c) The red channels represent the cuticular arrangement after staining with Auramine O. (d, e and f) The blue colour originating from Calcofluor staining represents the cellulosic cell wall material. (g, h and f) Brightfield images of epidermal cell wall showing both cuticle and cell walls. (j, k and l) Overlay of all the channels show the precise positioning of cuticle and cell wall on epidermal cells. Tailed arrows show the cuticle and blunt-end arrows indicate the cell wall. Scale bar is 5 μm.
Figure 2.11 3D reconstruction of the cuticle. The z-stacks generated from the confocal are reconstructed to show three different surfaces of cuticle. The arrangement of cuticle is similar for *T. aestivum* and *Z. mays*, where the cuticular material was extended to anticlinal cell wall regions. The intensity of cuticular extensions were observed to be diminished for *L. angustifolius*. Tailed arrows indicate the nucleus and blunt-end arrows represent the chloroplasts.
micromorphology on the phylloplane. It was found that A. thaliana does not have any epicuticular waxes on its leaf surface, instead it was covered with an epicuticular wax sheath which is also referred to as amorphous wax sheath (Jeffree, 2007). T. aestivum and L. angustifolius had single type of epicuticular wax plates on their leaf surfaces the fine structure of the wax crystals was remarkably different between the species. By contrast, Z. mays exhibited four different types of epicuticular wax crystals on its phylloplane. The description and classification of epicuticular waxes is based on the epicuticular wax classification system proposed by Barthlott et al., (1998). Broadly, Barthlott et al., (1998) classified epicuticular waxes into films, layers or crusts and crystalloids. These groups are further divided into sub-groups and sub-sub-groups. Barthlott et al., (1998) identified that, the most common type of epicuticular waxes found are crystalloids. In accordance with earlier observations this study identified the wax crystals on T. aestivum, Z. mays and L. angustifolius as crystalloids. The crystalloids of T. aestivum, Z. mays and L. angustifolius were sub-grouped to, “plates and platelets.” “Plates and platelets,” were divided into four groups namely, “entire platelets, irregular platelets, membranous platelets and plates (Fig 2.12).” The platelets of T. aestivum and L. angustifolius were recognised to be irregular crenate platelets (ICP) (Fig 2.12 c and d) and membranous platelets (MP) (Fig 2.12 e and f) respectively. Along with ICP and MP the phylloplane of Z. mays has an additional types of plates known as entire platelets (EP) (Fig 2.12 a and b). The presence of different types of wax crystals on a single plane was described as “syntopism.”

The arrangement of the epicuticular waxes for T. aestivum appear to be very dense under lower magnification and gaps between the wax crystals could only be resolved under higher magnification (Fig 2.13). These gaps represent the underlying wax layer that is smooth.
Sometimes these gaps were much extended giving rise to an area clear of any wax structures. Individual wax platelets appear to be fused with adjacent wax platelets forming complex continuous and random structures (Fig 2.13). The arrangement of these fused waxes is consistently irregular (Fig 2.13 d, e and f). Most of the fused platelets are perpendicular to the surface of leaf while some were positioned at an angle (Fig 2.13 f). The epicuticular waxes on wheat leaf were identified as, “crystalloids,” in the Barthlott et al., (1998) classification of epicuticular waxes. These wax crystals can further be assigned to sub-group named, “irregular platelets.” As suggested by the Barthlott et al., (1998) classification, based on the appearance of the margin, the irregular platelets can be grouped as gnawed, crenate, sinuate and laciniate platelets. *T. aestivum* wax platelets can therefore be identified as, “irregular crenate platelets (ICP).” Though the shape of wax platelet is consistent with Barthlott classification the size is contrastingly different. The reported size for irregular platelets in literature was 1-10 μm in height but the current average size recorded was less than 170 nm (or 0.17 μm) in height. The thickness of the platelet recorded in the current study is approximately 25 nm ± 2.2.

*Zea mays* also had a dense covering of epicuticular wax crystals on its phylloplane (Fig 2.14 b). Following Barthlott’s epicuticular wax crystal classification the randomly arranged wax crystals were identified as, “crystalloids”. The presence of entire platelets (EP), membraneous platelets (MP), irregular crenated platelets (ICP) and granules (G) were confirmed using SEM (Fig 2.14). EP are with continuous margin, have irregular shape and are big in comparison to other crystalloids (Fig 2.14 c). ICP are smaller entire platelets but with crenation and are much similar to the same found on *T. aestivum*. Crenated platelets are not always fused with other crystalloids (Fig 2.14 f). MP are entire platelets or crenated platelets with a
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Figure 2.12 Schematic drawings of epicuticular waxes. The left side column are scanning electron microscopy (SEM) images of different epicuticular wax crystalloids and right side column represent the respective schematic drawings. (a) Entire plateltes (EP) and (b) its schematic drawing. (c) Irregular crenate platelets (ICP). (d) Schematic drawing for ICP. (e and f) Membranous platelets (MP) and respective schematic drawing. EP, ICP and MP fall under a group called plates, which is further a group in crystalloid family.
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Figure 2.13 Epicuticular waxes on *T. aestivum* leaf. The wax plates on the phylloplane of *T. aestivum* show an irregular arrangement of wax crystals. As proceeded from (a) low magnification to (f) high magnification individual wax plates can be resolved. (a) The eclipse on the top shows dense cover of epicuticular waxes and bottom eclipse shows a zone devoid of epicuticular waxes. (b) Stomata can be clearly seen even under low magnification. (a, b, c and d) Epicuticular wax crystals appear to be densely covered at low magnifications. (e and f) Individual wax platelets could be clearly resolved and identified as irregular crenate platelets (ICP) under high magnification and were indicated with arrows. Scale bar at the bottom is 40 μm on (a); 10 μm on (b); 1 μm on (c); 500 nm on (d); 400 nm on (e) and 150 nm on (f).
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Figure 2.14 Epicuticular wax micromorphology on *Z. mays* leaf. The wax micromorphology at increasing magnification from (a to f). (b) The eclipse on the top shows region on phylloplane covered with epicuticular waxes. The eclipse on the bottom shows region clear of any epicuticular waxes. The wax crystals could be identified as (c) entire platelets (EP), (e) membraneous platelets (MP), (f) irregular crenate platelets (ICP) and granules (G). Epicuticular wax arrangement on *Z. mays* falls under a class described as syntopism. Scale bar approximately (a) 40 µm; (b) 10 µm; (c) 1 µm; (d) 500 nm; (e) 400 nm and (f) 200 nm.
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Figure 2.15 Epicuticular waxes on *L. angustifolius*. Wax crystals are presented at an increasing magnification from (a) to (f). (a and b) The arrangement of wax platelets at low magnification. (c) The area around the stomatal wax chimney is clear of any wax crystals. (d) Hair like projections appear to be randomly place on phylloplane. (e and f) The wax crystalloids could be identified as membranous platelets (MP) where a fine thread like extension is evident on most of the wax platelets. Scale bar approximately (a) 50 μm; (b) 10 μm; (c) 5 μm; (d) 1 μm; (e) 800 nm and (f) 200 nm.
Figure 2.16 Surface architecture of *A. thaliana*. Scanning electron microscopy (SEM) images shows the presence of wax sheath over the leaf surface of *A. thaliana* at various magnifications. (a and b) Stomatal wax chimneys covering the stomatal chamber are indicated with arrows. (c, d, e and f) show the fine structure of epicuticular wax sheath of *A. thaliana*. The folds of wax sheath are shown with arrows. Scale bar approximately (a) 20 μm; (b) 10 μm; (c) 6 μm; (d) 2 μm; (e) 2 μm and (f) 1 μm.
membranous extension (Fig 2.14 e). The extensions of MP can sometimes run over other crystalloids giving rise to a fused appearance. Granules (G) appear as small globular structures (Fig 2.14 f). Due to the presence of different forms of wax crystals arranged in random array on same plane, the arrangement of the crystalloids is complex when compared to that of *T. aestivum*. Most of the crystalloids are arranged perpendicular to the underlying wax layer while some are arranged at an angle. The occurrence of different forms of crystalloids on a single phylloplane is earlier described as syntopism and *Z. mays* forms a typical example for syntopic arrangement of epicuticular wax crystals (Barthlott et al., 1998; Beattie and Marcell, 2002). The size of crystalloids is much varied ranging from few nanometers (for granules) to several hundred nanometers (for entire platelets). The thickness is similar to that of *T. aestivum* wax crystals which is approximately 25 nm.

The arrangement of the epicuticular wax crystals on *L. angustifolius* is very similar to that of *T. aestivum* and *Z. mays*, with dense cover of crystalloids (Fig 2.15). Only membranous platelets (MP) could be identified on the phylloplane of *L. angustifolius*. The height and thickness of the platelets is approximately 225 nm and 41 nm. The extension of the *L. angustifolius* MP is oriented straight (Fig 2.15 f). The arrangement of the crystalloids thus gives rise to a hairy and bushy appearance under lower magnification (Fig 2.15) arrangement of MP around stomata is unique for *L. angustifolius* (Fig 2.15 b and c) where the region around stomatal wax chimney is clear of any wax crystalloids (Fig 2.15 b).

As stated above *A. thaliana* does not have any epicuticular wax crystals on its leaf surface (Fig 2.16). But a conspicuous wrinkled wax sheath with regular folds could be visualised under SEM and is consistent with the literature (Fig 2.16) (Luo et al., 2007). The wax sheath on *A.*
*thaliana* can be grouped under smooth layer of Barthlott et al (1998) classification system of epicuticular waxes.

2.3.4 Image analysis

2.3.4.1 Relative wax density

For image analysis ten images per species were randomly selected from collected SEM images (Table 2.1). Relative wax density of wax structures (RD) was calculated in a two-step process. In the first step the number of white pixels per image was calculated separately using a plugin in ImageJ software and a median number of white pixels per species (per ten images that were selected earlier for each species) was thus calculated. The number dark objects that represent the spaces between the wax crystals, were calculated per each image using a software called, “Pixcavator”. In the second step, a measure for the structural complexity of wax micromorphology was computed separately for each image in a species group. This was achieved by multiplying the number of dark objects obtained for each image with the number of white pixels per median number of white pixels of the same species group. The final number thus obtained describes the complexity of wax micromorphology and was termed as, relative density (RD) of wax structures. It was interesting to find that the RD values are in decreasing order from *T. aestivum*, *Z. mays*, *L. angustifolius* to *A. thaliana*. One way ANOVA on RD between groups with Tukey HSD and LSD post hoc testing was performed to find that *T. aestivum* and *A. thaliana* are statistically significant with each other as well as with *Z. mays* and *L. angustifolius*. There was no statistical significance between *Z. mays* and *L. angustifolius* (Table 2.1).
2.3.4.2 Measuring area of epicuticular waxes using ImageJ

Similar to image analysis for RD, the perimeter and percentage cover of waxes was calculated on ten images that were randomly selected for each species. The images were then converted to 8 bit image to adjust the threshold in imageJ software. Noise was despeckled before analysing particles for the area of epicuticular waxes (AE) and perimeter of wax crystals per image of size 1024 × 768 pixels. It was very interesting to find that AE of *T. aestivum* and *L. angustifolius* are very similar and are statistically insignificant with each other after one way ANOVA between the groups. For the same tests, AE on *Z. mays* and *A. thaliana* are statistically significant with each other and also with *T. aestivum* and *L. angustifolius*. The perimeter was however insignificant with in the groups and between groups except for *A. thaliana*, after testing with one way ANOVA (Table 2.2).

2.3.5 The chemical composition of plant cuticular waxes

Chloroform was used as a solvent for extracting wax from the leaves of *T. aestivum*, *Z. mays*, *L. angustifolius* and *A. thaliana*. Four time points 15, 30, 45 and 60 seconds were used to check for best yields of wax from the leaf tissue (Fig 2.17). There were no observed differences in the number of peaks resolved for each of the extraction time points under the conditions mentioned for section 2.8.8 and therefore, for all the further experimentation a 15 second extraction was performed. For *T. aestivum* long chain even saturated fatty acids ranging between C16 to C30 and long chain even alcohols ranging from C24 to C30 were consistently observed in the wax extracts (Fig 2.18 a). Except for hentriacontane (C31 alkane) and hexatriacontane (C36 alkane), both even and odd numbered long chain alkanes ranging between C23 to C37 were also detected. Further, it was interesting to find that the FID detector response representing that of octacosanol (C28 alcohol) was observed to be significantly
different from other peaks and it was consistent with earlier report (Koch et al., 2006). Similarly for *Z. mays*, except for eicosanoic acid (C20 fatty acid) long chain even numbered saturated fatty acids ranging between C16 to C30 were detected along with the long chain even numbered alcohols ranging between C24 to C30 (Fig 2.18 b). Like *T. aestivum* detection of alkanes in *Z. mays* followed the same trend where all long chain alkanes between C23 to C37 were detected excluding hentriacontane (C31 alkane) and hexatracontane (C36 alkane). But for *Z. mays*, the flame ionisation detector response was contrastingly high for triacontanoic acid (C30 fatty acid). For *L. angustifolious* saturated long chain fatty acids having even numbered carbons namely C16, C18, C22, C24, C28 and C30, even numbered long chain primary alcohols C24, C26, C28 and C30 could be identified (Fig 2.18 c). Alkanes in the epicuticular waxes of *L. angustifolious* followed the trend earlier observed with *T. aestivum* and *Z. mays*. *Arabidopsis thaliana*, on the other hand is very well described in the literature (Rashotte et al., 1997; Millar et al., 1999) (Fig 2.18 d). For fatty acids though all the even numbered saturated fatty acids between C16 and C30 are earlier reported, this study could only detect C16, C18, C24, C26 and C30 in the epicuticular wax extracts of *A. thaliana* leaves. Detection of C24 to C30 even numbered long chain primary alcohols was consistent with literature. Though all the alkanes with carbon numbers ranging between C25 to C31 were detected in this study, only odd numbered alkanes C25, C27, C29 and C31 were reported in literature (Rashotte et al., 2001; Rowland et al., 2006). 15-Nonacosanone (C29 ketone) detection was consistent with the literature and all the results are summarised in table 2.3.
Table 2.1 Relative density (RD) of wax crystals. The table describes the RD values obtained for each species after the image analysis. Image analysis was performed using an algorithm that combines the data from two software namely Pixcavator and ImageJ. RD values were normalised against the average RD value for each plant species separately. Averages followed by same letter are statistically similar after Duncan’s posthoc testing.

<table>
<thead>
<tr>
<th>Species</th>
<th>RD</th>
<th>RD Normalised</th>
<th>Total no. of images collected</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. aestivum</em></td>
<td>148.74 ± 11.3 a</td>
<td>1 ± 0.1</td>
<td>64</td>
</tr>
<tr>
<td><em>Z. mays</em></td>
<td>76.41 ± 5.5 b</td>
<td>1 ± 0.1</td>
<td>37</td>
</tr>
<tr>
<td><em>L. angustifolius</em></td>
<td>55.30 ± 4.6 b</td>
<td>1 ± 0.1</td>
<td>61</td>
</tr>
<tr>
<td><em>A. thaliana</em></td>
<td>2.38 ± 0.5 c</td>
<td>1 ± 0.2</td>
<td>70</td>
</tr>
</tbody>
</table>
Table 2.2 Area for the epicuticular waxes (AE). AE was calculated after performing image analysis on SEM images. Perimeter of wax crystals per image and total number of images collected was also described. Averages followed by same letter are statistically similar after Duncan’s posthoc testing.

<table>
<thead>
<tr>
<th>Species</th>
<th>Area of epicuticular waxes (AE) (nm)</th>
<th>Range of Area percentage</th>
<th>Perimeter of wax crystals / image (nm)</th>
<th>Total no. of images collected</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. aestivum</td>
<td>5751615.49 ± 502229.20 b</td>
<td>17.98 – 29.15</td>
<td>794.21 ± 61.50 a</td>
<td>64</td>
</tr>
<tr>
<td>Z. mays</td>
<td>7651255.82 ± 778638.7 a</td>
<td>20.27 – 39.13</td>
<td>807.62 ± 49.90 a</td>
<td>37</td>
</tr>
<tr>
<td>L. angustifolius</td>
<td>4753368.59 ± 258034.00 b</td>
<td>20.57 – 27.40</td>
<td>709.21 ± 48.10 a</td>
<td>61</td>
</tr>
<tr>
<td>A. thaliana</td>
<td>16.81 ± 1.20 c</td>
<td>1.87 – 3.26</td>
<td>1.65 ± 0.10 b</td>
<td>70</td>
</tr>
</tbody>
</table>
Figure 2.17 Overlay of chromatograms of the epicuticular wax extracts of *A. thaliana*. Wax extraction was performed for four different time points, 15 (black), 30 (red), 45 (green) and 60 (pink) seconds separately on fresh *A. thaliana* leaf tissue. All the extracted waxes were analysed separately as mentioned in section 2.2.8. The overlay of chromatograms show that there were no differences in the number of peaks resolved after different extraction time points.
Chapter 2 Elucidating the micromorphology of the cuticle and epicuticular waxes
Chapter 2 Elucidating the micromorphology of the cuticle and epicuticular waxes
Figure 2.18 Cuticular wax profile of *T. aestivum*, *Z. mays*, *L. angustifolius* and *A. thaliana*. A sample of chromatogram of cuticular wax extract for retention time between 20 to 40 minutes shown for (a) *T. aestivum*, (b) *Z. mays*, (c) *L. angustifolius* and (d) *A. thaliana*. Peaks were labelled with fa for fatty acids, alk for alkanes, alc for alcohols and k for ketones. The number prefix represents the carbon number. For example fa 16 indicates 16 carbon chain fatty acid. Alkane 24 is an internal standard (Int std).
Table 2.3 Summary of the chemical constituents detected. All the chemical constituents detected were compared to the retention time of commercial standards. Carbon number, chemical name and retention times for each of the constituent is described below along with the plant species it was detected in. T stands for *T. aestivum*, Z for *Z. mays*, L for *L. angustifolius* and A stands for *A. thaliana*.

<table>
<thead>
<tr>
<th>Carbon number</th>
<th>Chemical name</th>
<th>Retention time (min)</th>
<th>Plant species detected in</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fatty acids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Hexadecanoic acid</td>
<td>14.12</td>
<td>T, Z, L, A</td>
</tr>
<tr>
<td>18</td>
<td>Octadecanoic acid</td>
<td>18.08</td>
<td>T, Z, L, A</td>
</tr>
<tr>
<td>20</td>
<td>Eicosanoic acid</td>
<td>21.96</td>
<td>T</td>
</tr>
<tr>
<td>22</td>
<td>Docosanoic acid</td>
<td>25.65</td>
<td>T, Z, L</td>
</tr>
<tr>
<td>24</td>
<td>Tetracosanoic acid</td>
<td>29.07</td>
<td>T, Z, L, A</td>
</tr>
<tr>
<td>26</td>
<td>Hexacosanoic acid</td>
<td>32.30</td>
<td>T, Z, A</td>
</tr>
<tr>
<td>28</td>
<td>Octacosanoic acid</td>
<td>35.03</td>
<td>T, Z, L, A</td>
</tr>
<tr>
<td>30</td>
<td>Triacontanoic acid</td>
<td>38.18</td>
<td>T, Z, L, A</td>
</tr>
<tr>
<td><strong>Alcohols</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>Tetracosanol</td>
<td>27.48</td>
<td>T, Z, L, A</td>
</tr>
<tr>
<td>26</td>
<td>Hexacosanol</td>
<td>30.78</td>
<td>T, Z, L, A</td>
</tr>
<tr>
<td>28</td>
<td>Octacosanol</td>
<td>33.89</td>
<td>T, Z, L, A</td>
</tr>
<tr>
<td>30</td>
<td>Triacontanol</td>
<td>36.82</td>
<td>T, Z, L, A</td>
</tr>
<tr>
<td><strong>Ketones</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>15-Nonacosanone</td>
<td>32.56</td>
<td>A</td>
</tr>
<tr>
<td><strong>Alkanes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>Tricosane</td>
<td>19.02</td>
<td>T, Z, L</td>
</tr>
<tr>
<td>24</td>
<td>Tetracosane</td>
<td>20.98</td>
<td>Internal standard</td>
</tr>
<tr>
<td>25</td>
<td>Hexacosane</td>
<td>22.89</td>
<td>T, Z, L, A</td>
</tr>
<tr>
<td>26</td>
<td>Heptacosane</td>
<td>24.74</td>
<td>T, Z, L, A</td>
</tr>
<tr>
<td>27</td>
<td>Octacosane</td>
<td>26.52</td>
<td>T, Z, L, A</td>
</tr>
<tr>
<td>28</td>
<td>Nonacosane</td>
<td>28.26</td>
<td>T, Z, L, A</td>
</tr>
<tr>
<td>30</td>
<td>Triacontane</td>
<td>29.93</td>
<td>T, Z, L, A</td>
</tr>
<tr>
<td>31</td>
<td>Hentriacontane</td>
<td>31.56</td>
<td>T, Z, L, A</td>
</tr>
<tr>
<td>32</td>
<td>Dotriacontane</td>
<td>33.13</td>
<td>A</td>
</tr>
<tr>
<td>33</td>
<td>Tritriacontane</td>
<td>34.66</td>
<td>T, Z, L, A</td>
</tr>
<tr>
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<td>Tetratriacontane</td>
<td>36.15</td>
<td>T, Z, L</td>
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<td>35</td>
<td>Pentatriacontane</td>
<td>37.58</td>
<td>T, Z, L</td>
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<td>37</td>
<td>Heptatriacontane</td>
<td>39.00</td>
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<td>38</td>
<td>Octatriacontane</td>
<td>41.68</td>
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</table>

*Chapter 2 Elucidating the micromorphology of the cuticle and epicuticular waxes*
2.4 Discussion

The cuticle is a selectively permeable layer present on the plant surface that comes in direct contact with the atmosphere and protects the plant from various environmental stress like temperature, light and microbes (Carver and Gurr, 2007; Pfundel et al., 2007; Riederer, 2007; Domínguez et al., 2011). Microscopic imaging of plant cuticles at high resolution is the effective way to understand the structural organisation of the cuticle. For example the cuticle on the fruit surface of *Solanum lycopersicum* was imaged using bright field and fluorescence microscopies using stains like Sudan IV, Nile blue, Auramine O and Calcofluor (Buda et al., 2009). However, to date such detailed structural analysis of cuticle using microscopy techniques (excluding electron microscopy) is limited only to *Solanum lycopersicum*. Similarly, scanning electron microscopy (SEM) has also been an invaluable tool in micrographing and understanding the micromorphology of the cuticles, especially epicuticular waxes on the surface of cuticle (Barthlott and Neinhuis, 1997; Barthlott et al., 1998; Beattie and Marcell, 2002). SEM has also been extensively used in reporting the epicuticular wax damage after use of surfactant based agrochemical spray formulation (Falk et al., 1994; Tamura et al., 2001; Gonthier, 2010). But, the damage to the epicuticular waxes was never quantified, which predominantly may be due to of lack of tools. Therefore, to understand the structural organisation of the cuticle, the cross sections of the leaves of *T. aestivum*, *Z. mays* and *L. angustifolius* were stained and imaged at high resolution using different staining and microscopy techniques respectively. Unprecedented high resolution was achieved for imaging the cuticle on the phylloplane of *T. aestivum*, *Z. mays* and *L. angustifolius*. To study the epicuticular wax micromorphology, epicuticular waxes were imaged at a high resolution by using (SEM). Further, novel methods for image analysis have been developed and employed to quantify the epicuticular waxes from the SEM images.
Sudan IV staining allowed the visualisation of the cuticle on the epidermal cells of *T. aestivum*, *Z. mays* and *L. angustifolius*. The cuticle was observed as a continuous thin layer both on adaxial and abaxial leaf surfaces. Cuticular overhanging on stomatal guard cells were consistently observed on all the plants species of the study. These overhanging form a stomatal chimney in a three dimensional array that covers and protects the stomatal apparatus (Barthlott et al., 1998). The presence of stomatal chimneys was further confirmed in 3D reconstruction of cuticle and in SEM images. Differences in cuticular organisation on leaf edge and epidermal cells of phylloplane observed on all the plants of the study, can be explained as a consequence of the anatomical organisation of the cells on the leaf surface. Dichromatic staining was performed using Auramine O and Calcofluor to resolve different layers of cuticle from the cell wall. *T. aestivum*, *Z. mays* and *L. angustifolius* responded differently to dichromatic staining. The observation of yellow to red colour for dichromatic staining on *T. aestivum* cell walls could be explained as a consequence of cross talk between the fluorophores of Auramine O and Calcofluor (Carlsson and Mossberg, 1992). Wide knowledge for Poaceae members (grasses) to have a cellulosic cell walls supports the prospect of crosstalk between the fluorophores (Carpita and Gibeaut, 1993) and rules out the possibility of thin cell walls. The cell wall layer could be distinctly resolved from cuticular layers with the 3D models generated after the confocal microscopy. Dichromatic staining on *Z. mays* leaf cross sections was successfully achieved with an unprecedented resolution for different layers of cuticle and cell wall. From the 3D reconstructions of *T. aestivum* and *Z. mays* it was clear that the deposition of the cuticular material was extended into the anticlinal cell walls. For *L. angustifolius* the presence of cuticular thickenings at anticlinal cell junctions was consistently observed with all the employed staining procedures. However, from the 3D reconstructions it was clear that, such thickenings are limited to the anticlinal cell junctions. The amount of cuticular material in

*Chapter 2 Elucidating the micromorphology of the cuticle and epicuticular waxes*
anticlinal cell walls of *L. angustifolius* was much reduced when compared to that of *T. aestivum* and *Z. mays*. The reason for such variations could again be attributed to the anatomical differences where *L. angustifolius* is a dicot and *T. aestivum* and *Z. mays* are monocots. Optical opacity being the reason, there was a contrasting decrease in the fluorescence intensity on the images as proceeded into deeper layers of tissue along the z-stack (Buda et al., 2009). However, spatial distribution and minimal availability of fluorophore at the deeper layers tissue cannot be ruled out. To the best of my knowledge, cuticle imaging on the leaf surfaces of *T. aestivum*, *Z. mays* and *L. angustifolius* at a high resolution was reported here for the first time.

The latest and most successful report on classification of the epicuticular waxes was written by Barthlott et al (1998). This method approached epicuticular wax classification based on characterisation of the morphological features. Some reports argue for a better model of classification that not only considers the morphology but also chemical composition of wax crystals (Jeffree, 2007). In this context, it is essential to understand that, developing such comprehensive system of classification for epicuticular waxes may be a reality in future but not possible with present day technology and tools (Stark and Tian, 2007).

The epicuticular waxes on the surface of *T. aestivum* were identified according to the Barthlott et al (1998) classification system as ICP (irregular crenate platelets). For the crenate platelets there were contrasting differences in size, between the current report and report from Barthlott et al (1998). For describing crenate platelets Barthlott et al (1998) used *Odosicyo* sp. and *Grevillea bipinnatifida* whereas the current research used *T. aestivum*. This may explain the observed size differences. There were four different types of epicuticular waxes observed on the leaf surface of *Z. mays*. The occurrence of different types of wax crystals was earlier described as “Syntopism” in the literature (Barthlott et al., 1998). Though there was another
report on epicuticular waxes of *Z. mays* following Barthlott et al., classification of epicuticular waxes, it overlooked the arrangement of sytopism (Beattie and Marcell, 2002). Further, the terminology and description elaborated for different wax crystals was misleading (Beattie and Marcell, 2002). For example rods and rodlets described appear to be projections of platelets or deformed wax crystals. *L. angustifolius* on the other hand covered with a dense array of membranous platelets. The leading edges of these membranous plates overlap the adjacent platelets and contribute towards the fused appearance of the platelets. *L. angustifolius* was not described earlier for its epicuticular waxes. Consistent with literature it was observed in this study that the leaf surface of *A. thaliana* is devoid of any epicuticular wax crystals, instead it is covered by a wax film or sheath (Jeffree, 2007).

As there were no methods described to quantify epicuticular waxes, two procedures namely, “Relative density of wax crystals” (RD) and “Area of epicuticular waxes” (AE) were developed. RD is based on direct proportionality of the wax crystals to the number of gaps or spaces separating them. Large number of gaps define dense cover of epicuticular waxes, thus leading to high RD value and vice versa. The epicuticular waxes on *T. aestivum* appear to be fused with adjacent wax crystals giving rise to a network of wax structures and thus leading to an increased number of dark objects, hence a statistically significant RD value. Unlike *T. aestivum*, wax platelets on *Z. mays* and *L. angustifolius* were not fused with adjacent wax platelets, thus could be resolved into separate structures at high magnification. This in-turn leads to less number of isolated spaces and a smaller RD value. The presence of an epicuticular wax sheath on *A. thaliana* explains the low RD value for it. These variations with RD directed the investigation to the development of AE, which is a robust straightforward analysis method that involves the precise calculation of epicuticular wax surface area. From the range of AE
values (excluding *A. thaliana*), it is evident that, this method of image analysis is sensitive to pick the subtle differences between the morphological arrangement of epicuticular waxes. The AE value on *Z. mays* is significantly different from that of *T. aestivum* and *L. angustifolius*, which is a possible outcome of the syntopic arrangement of epicuticular waxes on *Z. mays* phylloplane. As described earlier, the presence of different types of wax crystals on a single phylloplane is described as syntopic arrangement of epicuticular waxes. The absence of epicuticular waxes on *A. thaliana* contributed towards the significant AE value in comparison to that of *T. aestivum*, *Z. mays* and *L. angustifolius*.

The epicuticular wax plates of *T. aestivum*, *Z. mays* and *L. angustifolius* were also described as primary alcohol plates in pervious literature because of the alcohol content in waxes (Koch et al., 2006; Jeffree, 2007), which is evident from GC data in this research. Further, octacosanol that was found to be most abundant in wax extracts of *T. aestivum* predominantly contributes towards the formation of ICP (irregular crenate platelets) on its phylloplane (Koch et al., 2006). Similarly, the composition of epicuticular waxes on the surface of *Z. mays*, *L. angustifolious* and *A. thaliana* is responsible for the specific type of wax micromorphology (Beattie and Marcell, 2002; Koch et al., 2006; Jeffree, 2007). For *A. thaliana*, though the cuticular wax profile was extensively reported in literature (Jetter et al., 2007; Li et al., 2008) even chained alkanes were not reported. The identification of long chained even numbered alkanes could therefore be explained as a consequence of silylation of long chain even numbered alkyl esters (Hannoufa et al., 1993). With further development of techniques to isolate epicuticular waxes it would be possible to predict the structure of the epicuticular waxes basing on their chemical composition (Koch et al., 2006). However, it is to be noted that this research focuses to
characterise the leaf surface of selected plants species for their interaction with agronomic surfactants and nanostructured liquid crystalline particles (NLCP).

In this study the cuticle on the leaves of *T. aestivum*, *Z. mays* and *L. angustifolius* were visualised using various staining procedures and microscopy tools. Novel approaches of image analysis were performed to quantify the data obtained from SEM on epicuticular waxes of *T. aestivum*, *Z. mays*, *L. angustifolius* and *A. thaliana*. The standardised protocols for staining cuticle and methods developed for image analysis in this research would be pivotal studying and understanding the interactions of surfactant based agrochemical spray formulations and nanostructured liquid crystalline particles (NLCP) with plant surfaces for the subsequent chapters in this thesis. The techniques employed in this research may also be applied to study the self-assembly structures formed after recrystallization of waxes on polar and non-polar surfaces (Koch et al., 2006). Further, the study of the epicuticular waxes may also lead to potential industrial applications. For example the concept of *lotus effect* has given rise to development of superhydrophobic, self-cleaning surfaces and self-assembling materials (Barthlott and Neinhuis, 1997; Koch et al., 2006; Samaha et al., 2012).
Chapter 3: NANOSTRUCTURED LIQUID CRYSTALLINE PARTICLES AS AN ALTERNATIVE DELIVERY VEHICLE FOR PLANT AGROCHEMICALS

This chapter was written basing on the publication; Nadiminti, P.P., Dong, Y.D., Sayer, C., Hay, P., Rookes, J.E., Boyd, B.J., and Cahill, D.M. (2013). Nanostructured liquid crystalline particles as an alternative delivery vehicle for plant agrochemicals. *ACS Applied Materials & Interfaces* 5, 1818-1826. Impact factor 5.9. Cahill, D.M. and Boyd, B.J., conceived the project; Rookes, J.E., contributed to drafting the manuscript; Dong, Y.D., provided the nanostructured liquid crystalline particles (NLCP) and Fluoro-NLCP; Sayer, C., and Hay, P., provided technical expertise and comments on early drafts of the manuscript; Nadiminti, P. P., designed and carried out all the experiments, analysed the results, prepared all the figures and tables, drafted and submitted the manuscript.

3.1 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 solubilisation of leaf cuticular waxes, reducing the surface tension on droplets of water-based agrochemical formulations and by enhancing subsequent hydration of the cuticle (Rutter et al., 1990). A reduction in the contact angle of spray droplets on initially hydrophobic leaf surfaces (Sanyal et al., 2008) enables dispersion of the chemical solution across the leaf that leads to the enhanced uptake of the active ingredient by both stomatal penetration (Currier et al., 1964; Stevens et al., 1991) and uptake through the cell walls of the epidermis (Riederer and Schönherr, 1990; Hess and Foy, 2000). Furthermore, surfactants increase the solubility of poorly water soluble actives, effectively increasing the active ingredient concentration which reduces the volume of formulation required (Stock and Holloway, 1993). 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 (Knoche et al., 1992) of the fruits and leaves of many plant species (Neinhuis et al., 1992; Tamura et al., 2001; Keese and Camper, 2006). Surfactants can also alter soil properties (Kuhnt, 1993; Ying, 2006) and have been shown to be toxic to terrestrial invertebrates, microbes and to aquatic organisms (Cserháti et al., 2002; Singh et al., 2002; Liwarska-Bizukojc et al., 2005).

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 (Kunst and Samuels, 2003; Samuels et al., 2008). Cuticular waxes that come in direct contact with the atmosphere polymerise 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, esters and ketones (Kunst and Samuels, 2003) and they may be mixed with secondary metabolites such as pentacyclic triterpenoids (Jenks et al., 1995; Buschhaus et al., 2007), tocopherols (Griffiths et al., 2000), butanoids and propanoids (Wen et al., 2006). The composition of cutin is very different from that of cuticular waxes by having both \( \omega \)-hydroxy fatty acids and mid chain hydroxy fatty acids (Kolattukudy, 1981; Samuels et al., 2008) and glycerol along with unsubstituted, epoxy and polyhydroxy fatty acids, \( \alpha, \omega \)- and polyhydroxy \( \alpha, \omega \)-dicarboxylic acids, fatty alcohols and phenolics (Pollard et al., 2008; Samuels et al., 2008). 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 (Neinhuis et al., 1992; Barthlott and Neinhuis, 1997). Following the use of various commercial ionic and non-ionic surfactants, phytotoxicity has previously been reported on cucumber (*Cucumis sativus*) (Temple and Hilton, 1963), barley (*Hordeum vulgare*) (Krogh et al., 2003), soy bean (*Glycine max*), mung bean (*Phaseolus aureus*), sorghum (*Sorghum bicolor*), oat (*Avena sativa*), turnip (*Brassica rapa*), mustard (*Sinapis alba*) and sunflower (*Helianthus annuus*) (Ying, 2006).

Clearly 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 bio-relevant surfaces (Dong et al., 2011) including those that are hydrophobic (Chang et al., 2012) 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 (Müller et al., 2002; Boyd, 2003; Rizwan et al., 2010). 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 (Angelov et al., 2003; Rizwan et al., 2007; Rittman et al., 2010). 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’ (Fig 3. 1). (Rizwan et al., 2007; Boyd et al., 2009). 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 150-300nm in diameter (Dong et al., 2006). 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.

This study examines the effect on plant leaf surfaces of nanostructured liquid-crystalline particles (NLCP) derived from phytantriol (3,7,11,15-tetramethyl-1,2,3-hexadecanetriol) 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.

3.2 Materials and Methods

3.2.1 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 mins, rinsed three times in sterile dH2O and transferred to 90 mm in diameter 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 plates 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 (Rookes et al., 2008).

Figure 3.1 Graphical representation of the synthesis of NLCP, the internal nanostructures and Cryo-TEM image of liquid crystalline particles (displayed in order left to right). Briefly, the process of synthesis involves dispersing phytantriol in water along with F127, followed by a high energy shearing to obtain NLCP. NLCP can either have bicontinuous cubic (cubosomes) or inverted hexagonal (hexosomes) nanostructure. Both cubosomes and hexosomes have non-intersecting hydrophilic and lipophilic regions. Cryo-TEM image to extreme right shows the internal nanosturcutre of bicontinuous cubic structures, hence the name cubosomes.
After 14 days of growth the seedlings were transplanted to 100 mm in diameter 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 sterilised by immersion in 80% (v/v) ethanol for 30 sec, followed by washing in 2% (v/v) sodium hypochlorite for two min. The seeds were then rinsed with sterile dH2O five times and transferred to a plastic tray containing a thin layer of absorbent cotton wool saturated with sterile distilled water. 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., Aus.), respectively. The seeds were sterilised according to the procedures described above for *L. angustifolius* but were sown directly into 100 mm in diameter plastic pots filled with sterile potting mix. Seedlings were grown and maintained under the same conditions as described for *L. angustifolius*.

### 3.2.2 Preparation of surfactants and liquid crystalline nanoparticles

The surfactants, empimin, and empigen (Fig 3.2) were provided by Nufarm Australia Limited. Surfactant solutions were prepared by dilution in dH2O to concentrations of 0.1%, 1% and 5% (v/v) immediately prior to use.
Figure 3.2 Chemical structure of surfactants. (a) Empimin - anionic sodium di(2-ethylhexyl) sulfosuccinate (b) Empigen - cationic and amphoteric betaine C_{12-14} alkyl dimethyl.
NLCP were prepared as previously described (Dong et al., 2006) with minor variations. Briefly, 900 mg of phytantriol (Roche Products Pty Limited, Dee Why NSW 2099 Australia) was dispersed in 4.1 g of F127 (BASF Australia Ltd, Southbank VIC 3006 Australia) solution (2.4% w/w) by ultrasonication (Misonix XL2000, Misonix Incorporated, Farmingdale, NY, USA) for 30 min in pulse mode (0.5 sec pulses interrupted by 0.5 sec breaks) at 40% of maximum power, resulting in a milky dispersion of NLCP. Particle size was characterized using Malvern NanoS nanosizer (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 contain 18% w/w phytantriol) were diluted to the equivalent phytantriol concentrations of 0.1, 1 and 5% v/v with sterile distilled water. Sterile distilled water was used as the control for all the surfactant and NLCP treatment. 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 labelled cubosomes 0.05 mg of 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-carboxyfluorescein (Avanti Polar Lipids, Inc, 700 Industrial Park Drive Alabaster, Alabama 35007-9105, USA) was added per gram of phytantriol.

3.2.3 Treatment of leaves with surfactants and NLCP and macroscopic analysis

Using a micropipette 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. 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).

3.2.4 Cuticle examination by light, epifluorescence and confocal microscopy

For examination of the adaxial leaf cuticle of each species leaves were excised and then washed briefly in dH₂O followed by rinsing in embedding medium, (Tissue-tek® OCT compound, ProSciTech Pty Ltd, Thuringowa, QLD, Australia). The leaves were then cut using a scalpel blade into smaller pieces (approximately 5 mm × 5 mm) 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 microtome (Microm HM550 OMP, Thermo Scientific, Scoresby, VIC, Australia), and sections were placed on gelatin-coated slides (Cahill et al., 2002) then rinsed in dH₂O. Sections were subsequently stained with 0.1% w/v Sudan IV or 0.1% w/v Auramine O (Sigma-Aldrich) to stain the cuticle as previously described (Buda et al., 2009). 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 - 490nm 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 labelled NLCP leaves were treated as described above and were then excised 1 h after treatment and visualized using confocal microscopy.

3.2.5 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 (Pathan et al., 2008) 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 mm² section of leaf was excised and mounted onto an aluminium stub. Each sample was coated with gold palladium for 120 sec at 40mA (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 concentrations 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 (Schneider et al., 2012) 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. Area of epicuticular waxes (AE) was calculated as described earlier in section 2.3.4.2. Before performing image analysis, to improve the resolution, brightness, contrast and midtones on all
the microscopy images were adjusted using Microsoft ® office picture manager 2010 (Microsoft Pty. Limited, North Ryde, NSW, Australia). Adobe ® Phtoshop ® CS6 version 13.0 ×64 was used to present the images for this research (Adobe Systems Pty. Ltd., Sydney, NSW, Australia). The data obtained for RD and AE was statistically analysed using analysis of variance (ANOVA) with Tukey’s HSD post-hoc test, IBM SPSS Statistics 21 (IBM Australia Ltd, St Leonards NSW, Australia).

3.3 Results

3.3.1 Effect of treatment of leaves with surfactants and NLCP

Three concentrations (0.1%, 1% and 5%) of each of the surfactants and NLCP were tested separately on each plant species. Droplet behaviour 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, empimemin and NLCP droplets spread out across the leaf surface after application to cover a larger area. It was also observed that the response to surfactant and NLCP treatments was concentration dependent and irreversible. The responses of leaves to surfactant or NLCP application are summarised in table 3.1.

3.3.1.1 Effect of NLCP spray formulations on *T. aestivum* and *Z. mays*

With application of the surfactant empigen at a concentration of 0.1%, ‘ring-like’ necrotic spots, limited to the site of application were observed on the monocot, *T. aestivum* (Fig 3.3 a and c). Necrotic spots with uniform damage were further observed after increasing the concentration of the application to 1% and 5% (Fig 3.3 e and g). In comparison to empigen, the leaves of *Z. mays* were equivalent to the observations made on *T. aestivum*. However, the necrotic spots formed showed uniform damage after 0.1% empigen treatment. Under SEM, the
Table 3.1 Phytotoxicity caused by surfactants or NLCP on leaves of *T. aestivum*, *Z. mays*, *L. angustifolius* and *A. thaliana*. Leaves were individually treated either with surfactant or NLCP and photographed. Damage to leaves was given a rating on a scale of 0-5, where 0 – no observed phytotoxicity, 1- slightly phytotoxic, 2 – greater than slight phytotoxicity, 3 – moderate phytotoxicity, 4 – greater than moderate phytotoxicity and 5 - severe phytotoxicity. * indicate statistical significance after one-way ANOVA.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plant species</th>
<th>0.10%</th>
<th>1%</th>
<th>5%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phytotoxicity rating</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Empigen</td>
<td><em>T. aestivum</em></td>
<td>1.0 ± 0.0</td>
<td>4.2 ± 0.4</td>
<td>5.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td><em>Z. mays</em></td>
<td>1.0 ± 0.0</td>
<td>2.7 ± 0.4</td>
<td>5.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td><em>L. angustifolius</em></td>
<td>1.5 ± 0.2</td>
<td>5.0 ± 0.0</td>
<td>5.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td><em>A. thaliana</em></td>
<td>2.0 ± 0.0</td>
<td>4.0 ± 0.0</td>
<td>5.0 ± 0.0</td>
</tr>
<tr>
<td>Empimín</td>
<td><em>T. aestivum</em></td>
<td>1.0 ± 0.0</td>
<td>4.0 ± 0.6</td>
<td>5.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td><em>Z. mays</em></td>
<td>1.3 ± 0.3</td>
<td>3.0 ± 0.0</td>
<td>4.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td><em>L. angustifolius</em></td>
<td>1.5 ± 0.2</td>
<td>4.3 ± 0.5</td>
<td>4.9 ± 0.1</td>
</tr>
<tr>
<td></td>
<td><em>A. thaliana</em></td>
<td>2.5 ± 0.4</td>
<td>4.6 ± 0.5</td>
<td>4.7 ± 0.4</td>
</tr>
<tr>
<td>NLCPs</td>
<td><em>T. aestivum</em></td>
<td>0.1 ± 0.2*</td>
<td>0.2 ± 0.2*</td>
<td>2.0 ± 0.4</td>
</tr>
<tr>
<td></td>
<td><em>Z. mays</em></td>
<td>0.0 ± 0.0*</td>
<td>0.0 ± 0.0*</td>
<td>0.3 ± 0.2*</td>
</tr>
<tr>
<td></td>
<td><em>L. angustifolius</em></td>
<td>1.0 ± 0.0</td>
<td>2.9 ± 0.1</td>
<td>4.8 ± 0.3</td>
</tr>
<tr>
<td></td>
<td><em>A. thaliana</em></td>
<td>1.0 ± 0.0*</td>
<td>1.7 ± 0.2*</td>
<td>3.7 ± 0.9</td>
</tr>
</tbody>
</table>

Chapter 3 NLCP as next generation agrochemical delivery vehicles
Figure 3.3 The effect of empigen treatments on the leaves of *T. aestivum*. Whole leaf and SEM images of *T. aestivum* (left column) following treatment with empigen. (a) Water treated control; (c) 0.1% empigen; (e) 1% empigen; (g) 5% empigen. The intensity of phytotoxicity increases from 0.1% to 5% empigen treatments and is indicated with black arrows. Corresponding SEM images (right column) show the progressive damage of epicuticular wax crystals with increase in concentration of empigen. The structure of plate like wax crystals on the adaxial surface of the leaves is evident in control image (b). (d) Shows the damage to the wax crystals where the integrity of wax structure is disturbed followed by 0.1% empigen treatment. Complete solubilisation of wax crystals can be visualised in images after (f) 1% and (h) 5% empigen treatments respectively. Scale bar on a, c, e, g = 1 cm; b = 1000 nm, d = 740 nm, f = 1020 nm, h = 920 nm.
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 adaxial surface of untreated control leaves of *T. aestivum* (Fig 3. 3 b) and *Z. mays* displayed vertically oriented, plate-like epicuticular wax crystals. Treating leaves of *T. aestivum* (Fig 3. 3 d) and *Z. mays* with either of the surfactants, even at a low concentration of 0.1% resulted in a remarkable solubilisation 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 solubilisation on the leaves of both *T. aestivum* (Fig 3. 3 f and h) and *Z. mays* (data not shown).

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% (Fig 3. 4 a, c and e). Phytotoxicity symptoms were, however, observed on the *T. aestivum* leaves when the concentration of applied NLCP was increased to 5%, and further the intensity of necrosis was low when compared to that of surfactant treatments (Fig 3. 4 g). The phytotoxicity effects of NLCP on *Z. mays* are very similar to that of *T. aestivum*. There was little epicuticular wax solubilisation following the NLCP treatments at 0.1% and 1% on the leaves of both *T. aestivum* (Fig 3. 4 b, d and f) and *Z. mays*. Solubilisation 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% (Fig 3. 4 h). 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 3.4 NLCP treatment on the leaves of *T. aestivum*. Whole leaf images in left column after NLCP treatment at following concentrations (a) Water treated control; (c) 0.1%, (e) 1% (g) 5%. Phytotoxicity symptoms on whole leaves could be seen only after 5% phytantriol application is indicated with black arrows. SEM images (right column) show the intact structure of the epicuticular waxes after NLCP treatments at various concentrations in following images (b) control, (d) 0.1%, (f) 1%. Further increase in concentration of NLCP to (h) 5% resulted in intense solubilisation of epicuticular wax structure. Scale bar on a, c, e, g = 1 cm; b = 595 nm, d = 732 nm, f = 520 nm, h = 1462 nm.
3.3.1.2 *L. angustifolius* was sensitive to NLCP spray formulations

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* (Fig 3.5 a, c, e and g). The wax micromorphology on the leaves of *L. angustifolius* was quite different to *T. aestivum* and *Z. mays* and was composed of fine thread-like structures along with plate-like wax crystals (Fig 3.5 b). A similar trend of epicuticular wax solubilisation 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* (Fig 3.5 d, f and 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 severe phytotoxicity after 1% and 5% NLCP applications (Fig 3.6 a, c, e and g). With wax solubilisation, NLCP treatments on *L. angustifolius* leaves, at a concentration of 0.1% was sufficient to solubilise the fine structures of the wax layer leaving behind highly altered wax micromorphology (Fig 3.6 b and d). The intensity of solubilisation also increased with increasing concentration of NLCP (0.1% to 5%) leading to complete solubilisation of epicuticular waxes at 5% (Fig 3.6 f and g).

3.3.1.3 *A. thaliana* does not display epicuticular waxes

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 (Fig 3.7 a, c, e and g). The epicuticular wax structure did not have any distinct wax crystals and appeared to be composed of an undifferentiated amorphous wax film
Consistent with the visual symptoms the damage to the amorphous wax film caused by surfactant and NLCP treatments was concentration dependent (Fig 3. 7 b, d, f and h).

### 3.3.1.4 Comparison of the effect of NLCP and F127 on the phylloplane

To determine the reason for phytotoxicity and wax solubilisation on the leaves of various plants after NLCP treatments, the effect of stabiliser F127 alone was tested. 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 solubilisation was observed following 0.1% F127 treatment on all the species tested (Fig 3. 8 c, f, i and 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 (Fig 3. 8 a-f). These results may indicate that F127 is responsible for solubilisation of epicuticular waxes. For *L. angustifolius* and *A. thaliana* there were no observed differences in the intensity of wax solubilisation following F127 and NLCP treatments (Fig 3.8 g–l).

### 3.3.2 Wax solubilisation following treatment of leaves with surfactants and NLCP

To describe the structural complexity of epicuticular waxes on the leaf surface an arbitrary unit, the relative density (RD) of wax cover was developed. RD values equal to 1 correlate to intact and unaltered epicuticular wax cover and lower values of RD indicate a solubilisation 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* (Fig 3. 9 a and b) showed a dose dependent solubilisation of waxes that strongly correlate with our former observations on phytotoxicity and wax solubilisation. Additionally, the RD values after 0.1% and 1% NLCP treatments on *T. aestivum*...
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Figure 3.5 Impact of empigen on whole leaf and wax micromorphology of *L. angustifolius*. Empigen treatment on whole leaves of *L. angustifolius* (left column) at different concentrations. The treatments are as follows (a) Water treated control; (c) 0.1%; (e) 1%; (g) 5%. The intensity of phytotoxicity increased as with increase in concentration of surfactant empigen from 0.1% to 5% and is indicated with black arrows. Corresponding SEM images (right column) show the epicuticular wax solubilisation after the same treatment. (b) Shows the arrangement of epicuticular waxes on lupin leaves where thread like structures are closely packed throughout the adaxial surface. The fine structure is greatly reduced to small stub like structures after 0.1% empigen treatment. Further, increment in the concentration of empigen to 1% and 5% resulted in complete solubilisation of waxes in images (f) and (h) respectively. Scale bar on a, c, e, g = 1cm; b = 16 μm, d = 8 μm, f = 7 μm, h = 74 μm.
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Figure 3.6 Effect of NLCP on wax micromorphology of *L. angustifolius*. Whole leaf images of the NLCP treatment on *L. angustifolius* leaves (left column). (a) Water treated control (c) 0.1% NLCP treatment show slight discolouration of leaves which further increased to intense discolouration after (e) 1% LNP treatment, is indicated with black arrows. Complete wilting of leaf could be seen after treating leaves with (g) 5% NLCP. SEM images (right column) show (b) intact structure of epicuticular wax which is disrupted with after (d) 0.1% NLCP treatment. Complete solubilisation of the epicuticular waxes could be observed after (f) 1% NLCP treatment and (h) 5% NLCP treatment. 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.
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Figure 3.7 Empimin treatment on the leaves of *A. thaliana*. Whole leaf (left column) and SEM (right column) images of *A. thaliana* leaves following empimin treatment. The treatments are as follows, (a) and (b) Water treated control; (c) and (d) 0.1%; (e) and (f) 1%; (g) and (h) 5%. Phytotoxicity observed on whole leaves of *A. thaliana* after treating them with empimin at different concentrations is indicated with black arrows. SEM image (b) show the structure of epicuticular wax film which is characteristic of *A. thaliana* leaves. Increased phytotoxicity and damage to wax film after increasing 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.
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Figure 3.8 Response of epicuticular waxes to treatment with NLCP and F127. SEM images of adaxial surface of (a, b and c) *T. aestivum* (wheat), (d, e and f) *Z. mays* (maize), (g, h and i) *L. angustifolius* (lupin) and (j, k and l) *A. thaliana* (arabidopsis) compare the damage to epicuticular waxes after 1% v/v NLCP treatment which has 0.1% of F127 to 0.1% v/v F127 alone. Though both the treatments had similar amounts of F127 the damage to epicuticular waxes was very different. SEM images also show that the dicots *T. aestivum* and *Z. mays* showed minimal effect of 1% NLCP but the monocots *L. angustifolius* and *A. thaliana* failed to tolerate the same. 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.
and *Z. mays*, were equivalent to that of controls indicating structural integrity of the wax micromorphology (Fig 3.9 a and b). For *L. angustifolius*, the RD values decreased with increase in concentration of surfactants (Fig 3.9 c) and are also consistent with observations made for phytotoxicity and wax solubilisation. 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 solubilisation RD values for Arabidopsis showed a concentration dependent decrease for both NLCP and surfactant treatments at all tested concentrations (Fig 3.9 d). With 0.1% F127 treatments (Fig 3.9 e) the RD values and wax solubilisation correspond to each other.

The development of RD involved usage of an algorithm that equates the data from two image analysis software, “Pixcavator” and “ImageJ”. Though the development of RD involved the use of a coherent rational for extracting data from SEM images, loss of information was a common problem encountered. Therefore, a robust method, area of epicuticular waxes (AE) was developed to evaluate the subtle differences in the wax micromorphology. The new method AE employs widely accepted ImageJ software alone to obtain the area of epicuticular waxes. Additionally, AE eliminates the costs associated with the use of commercial software, Pixcavator. AE values also strongly correlated with that of RD values and phytotoxicity observations (Fig 3.10). A high AE value signifies an intact micromorphology and a lower AE indicates high degree of wax solubilisation or less wax integrity. A dose dependent wax solubilisation of epicuticular waxes along with the subtle differences among the water treated controls was evident (Fig 3.10). Similar to the RD values, the AE values for *T. aestivum* and
Z. mays, after 0.1% and 1% NLCP treatments were corresponding to that of controls (Fig 3.10 a and b). Consistent, with phytotoxicity and RD observations, AE values on L. angustifolius (Fig 3.10 c) were also dependent on the concentration of surfactant or NLCs used. Additionally, 0.1% and 1% NLCP treatments on L. angustifolius appeared to be better than that of surfactant treatments. Though, all the treatments on A. thaliana (Fig 3.10 d) appeared to be similar it should be noted that A. thaliana does not contain any epicuticular waxes.

3.3.3 Interaction of fluorescent NLCP with leaf surfaces

The fluorescently tagged NLCP were used to enable us to examine 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 localised fluorescence on the adaxial surface (Fig 3.11 and 3.12). No such localisation of fluorescence could be observed with the controls that were treated with unlabelled NLCP (Fig 3.11 and 3.12). Leaves sampled at different time points of 5 min and 1 h showed a similar pattern of fluorescence, with maximum intensity along anticlinal walls of the epidermal cells. 5% NLCP were not tested as they were eliciting phytotoxicity effects on all the plants.
Figure 3.9 Structural integrity of epicuticular wax micromorphology. Graphs show differences in custom developed unit, “Relative Density of wax cover” (RD) after empigen (white bars), empimin (black bars) and NLCP (grey bars) treatments at various concentrations (water treated control or 0%, 0.1%, 1% and 5%) on leaves of (a) *T. aestivum* (b) *Z. mays* (c) *L. angustifolius* and (d) *A. thaliana*. (e) A comparison of NLCP and F127 treatments between *Z. mays* (white bars), *T. aestivum* (black bars), *L. angustifolius* (grey bars) and *A. thaliana* (striped bars) show that F127 alone is responsible for wax solubilisation.
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Figure 3.10 Quantification of the area of epicuticular waxes after different treatments. In addition to RD, an arbitrary unit, “Area of epicuticular waxes” (AE) was developed to quantify the change in micromorphology of the waxes after different surfactant and NLCP treatments. On graphs (a) *T. aestivum* (b) *Z. mays* (c) *L. angustifolius* and (d) *A. thaliana*, black bars and white bars represent empigen, and empimin treatments at 0.1%, 1% and 5% respectively. For similar concentrations tested NLCP treatments were indicated with grey bars. All the controls were tested with distilled water. On graphs (a) and (b) the NLCP treatments at 0.1% and 1% were statistically similar to water treated controls ($p > 0.05$) whereas surfactant treatments at similar concentrations were statistically different ($p < 0.05$) from each other in comparison to the controls. Both the surfactant and NLCP treatments were statistically different from controls and other treatments when tested at a concentration of 5%. For (c) None of the treatments on *L. angustifolius* were statistically similar to water treated controls ($p > 0.05$). (d) All the treatments along with untreated controls were similar to each other for *A. thaliana* ($p < 0.05$). (e) A comparison of NLCP and F127 treatments between *Z. mays* (white bars), *T. aestivum* (black bars) and *L. angustifolius* (grey bars) show that F127 alone is responsible for wax solubilisation. Note *A. thaliana* was not represented in the graphs as it does not have epicuticular waxes on the surface.
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Figure 3.11 Fluoro-NLCP adsorbed to the leaf surfaces. (a) Adaxial surface of untreated *T. aestivum* (wheat) leaf showing auto-fluorescence. The red colour has originated from the chloroplasts of the parenchymal layers. (b) fluoro- NLCP (FNLCP) adsorbed to the surface of *T. aestivum*. The cell walls of the epidermal cells and stomatal guard cells could be clearly seen as fluoro – NLCP localised in to the anticlinal cell junctions of epidermal cells on leaf surface. Note no such fluorescence could be observed on the surface of untreated leaf (a). (c) Cross section (CS) of *T. aestivum* showing the fluorescence from fluoro- NLCP is indicated with white arrow. The fluorescence appeared to be limited to the epidermal cell region. (d) Untreated *Z. mays* (maize) showing auto-fluorescence after UV light excitation. (e) Fluoro – NLCP absorbed on adaxial leaf surface. The streaks represent the fluoro – NLCP localised in the anticlinal cell junctions of epidermal cells. Brightly fluorescing stomata could also be observed. (f) The presence of fluoro - NLCP is indicated by a white arrow on the cross section of *Z. mays* leaf surface. Similar to *T. aestivum*, the fluoro – NLCP on *Z. mays* leaves were also limited to epidermal cell layer. (g) Auto - fluorescence from *L. angustifolius* (lupin) control leaf. (h) Fluoro – NLCP adsorbed on the edge of *L. angustifolius* leaf surface. Streaks represent the anticlinal cell junctions and stomata are represented by bright fluorescent spots. (i) *L. angustifolius* cross section showing the adsorption of fluore fluoro – NLCP is indicated with a white arrow. (j) Adaxial surface of untreated *A. thaliana* (arabidopsis) leaf. (k) Fluoro – NLCP adsorbed on *A. thaliana* leaf surface. (l) Fluoro – NLCP on *A. thaliana* leaf cross section is shown with a white arrow. Scale bar on a = 124μm, b = 98μm.
Figure 3.12 Confocal images of fluoro- NLCP adsorbed to the surface of *A. thaliana* and *L. angustifolius* leaves. Untreated control leaf surfaces of (a) *A. thaliana* and (b) *L. angustifolius* leaves showing the chloroplasts of parenchymal layers. (c) All the fluorescent streaks represent the anticlinal cell walls of various cells on the adaxial epidermis of *A. thaliana*. Fluoro- NLCP adhered on the epidermal layer of the leaf. Fluoresce is well defined along the anticlinal cell wall of the epidermal layer. (d) Adsorption along the periclinal cell wall of *L. angustifolius* can also be seen as a smear in the image. Scale bar on c = 124μm, d = 98μm.
3.4 Discussion

It has recently been shown that the NLCP can efficiently adsorb to model hydrophobic surfaces made up of either tristearin or silicon (Dong et al., 2011; Chang et al., 2012). 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 it was shown that application of NLCP to leaf surfaces caused significantly less disruption to the cuticle and reduced phytotoxicity compared with two commonly used surfactants. Further, application of fluorescently labelled NLCP showed their adsorption to leaf surfaces that was most prominent at anticlinal cell walls. Together the 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 in controlled or under field conditions has not yet been reported.

All species selected for this study showed dose–dependent phytotoxicity symptoms after surfactant (empigen and empimin) treatments, and are consistent with earlier reports (Noga et al., 1987; Wolter et al., 1988; Neinhuis et al., 1992). There were clear differences between the two surfactant treatments; empigen caused distinct necrotic spotting of leaves 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 different physicochemical properties of surfactants empigen (a cationic, alkyl dimethyl betaine) (Maza and Parra, 1995) and empimin (an anionic, sodium di(2-ethylhexyl) sulphosuccinate). It also appears that empimin is slightly more phytotoxic towards the species tested. Additionally, the observation of ring like 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 as a consequence of evaporation and surface tension of the solute (water in this case) (Deegan et al., 1997). 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 is greatly reduced in comparison to surfactant treatments. A further increase in concentration of NLCP application to 5% caused phytotoxicity symptoms to *T. aestivum*, however, the leaves of *Z. mays* displayed no obvious effect. The absence of any phytotoxic symptoms in *Z. mays* can be due to the strong tensile strength of its leaves which enables them to resist greater amounts of NLCP (Balsamo and Orkwiszewski, 2008). 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 (Dominy et al., 2008). This difference may also have contributed to the better tolerance towards NLCP application by monocots than dicots. With *A. thaliana*, the phytotoxicity of NLCP treatments followed a similar trend to that which was observed with surfactant treatments, but is 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 (Domínguez et al., 2008). In the current study it was observed that, the presence of plate like waxes on the adaxial surface of *T. aestivum* and *Z. mays* leaves. *L. angustifolius* displayed similar plate like waxes but also had fine ‘thread-like’ structures on its surface while *A. thaliana* was observed to have an amorphous wax film on its leaf surface (Jeffree, 2007).
Epicuticular waxes are solubilised after the application of surfactants (Knoche et al., 1992; Bussotti et al., 1997; Tamura et al., 2001) 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 solubilisation of epicuticular wax structures. I have developed two methods namely, relative density (RD) of wax cover and area of epicuticular waxes (AE). The loss of subtle information after the use of RD lead to the development of AE. Using these methods it was found that *T. aestivum*, *Z. mays* and *L. angustifolius* showed significant differences in their response to treatment with NLCP versus surfactants while for *A. thaliana* no such differences were observed.

The block copolymer F127 ensures the colloidal stability of the submicron-sized NLCP in water and as such, is an essential component in the NLCP dispersion. One percent phytantriol NLCP contains the same amount of F127 as a 0.1% F127 solution alone and considering that it would not be possible to obtain NLCP without F127, phytantriol alone was not tested for analysis of phytotoxicity and wax solubilisation. However, the presence of phytotoxicity symptoms (not wax solubilisation) 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. For the monocots *Z. mays* and *T. aestivum* the solubilisation values for 1% NLCP indicated no wax solubilisation, whereas for the equivalent F127 treatments there was severe disruption. This comparison indicates that F127 alone is likely responsible for wax solubilisation on monocots. On the dicots *L. angustifolius* and *A. thaliana* both NLCP and F127 treatments elicited wax solubilisation. This differential behaviour of F127 to induce wax solubilisation 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 (Dong et al., 2011) 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 solubilisation.

In this study on the plants grown under defined conditions in plant growth chambers, the development of the cuticle on plant surfaces is more pronounced under natural conditions. Therefore it was 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 (Dong et al., 2011), the use of less water in their formulation and their potential for use with both lipid- and water soluble actives. Furthermore, the constituents of the cubosome dispersion (phytantriol and F127) are commonly used in cosmetic and pharmaceutical formulations and unlike many surfactants, are not toxic to the non-target organisms of soil and water ecosystems (Singh et al., 2002).
Chapter 4: RESPONSES OF PLANTS TO ABIOTIC AND BIOTIC STRESS AFTER APPLICATION OF NANOSTRUCTURED LIQUID CRYSTALLINE PARTICLES AND SURFACTANTS

4.1 Introduction

Plants are continually exposed to a number of abiotic stresses within their environment including high temperatures, UV or high intensity light, high salinity in soils, increased ozone and mechanical wounding. Losses to plant-based industries due to environmental stress are significant, for example, a loss of $200 billion to agricultural productivity was estimated after heat and drought in USA for the years 1980 to 2012 (Suzuki et al., 2014). Pathogen, insect and nematode attack on the other hand create biotic stress on the plants. Globally, biotic stress through plant diseases may account for as much as 10% loss in agricultural production (Strange and Scott, 2005). Under field conditions, abiotic and biotic factors together present crop plants with life threatening challenges, which they need to overcome by eliciting an intricate array of physiological, biochemical and molecular mechanisms that help them to overcome the stress imposed. Spatial or temporal stress responses are activated depending on the specific stress factor or combination of stress factors acting on the plant. For example, a spatial stress response is one, shown by chloroplasts that are reported to be the first organelles to be damaged in A. thaliana following exposure to UV radiation (Wituszynska et al., 2014). The damage caused to the chloroplasts further leads to production of a variety of signalling molecules that result in programmed cell death. Simultaneously in the same study, a vascular bundle-dependent temporal signal propagation was demonstrated after UV stress (Wituszynska et al., 2014). Anything that alters the leaf surface and the protective cuticular components, especially the epicuticular waxes, is likely to lead to enhanced sensitivity of the leaf to a variety of stresses.

Chapter 4 Responses of plants to abiotic and biotic stress after application of NLCP
This chapter presents an investigation into the comparative effects of treatment of leaves with either NLCPs or surfactants and their subsequent response to either a single abiotic treatment, UV-C light, or a single biotic treatment, infection by a pathogen. Two sensitive markers of stress responses in leaves, the β 1,3 glucan, callose and the reactive oxygen species (ROS), hydrogen peroxide were used to compare treatments.

Sessile autotrophs are invariably exposed to UV radiation, which is one the most important abiotic stress factors. UV radiation can be classified into three groups, UV-A, UV-B and UV-C depending on their respective wavelengths of 315–400 nm, 280–315 nm and 100–280 nm (Williamson et al., 2014). UV-A is most abundantly found at the earth’s surface as it is not filtered by the stratosphere and is non-toxic. Most of the UV-B radiation that reaches the earth is absorbed by ozone, but UV-B radiation with a wavelength greater than 290 nm can cross the stratospheric barrier and is the most damaging to life on earth (Jansen et al., 1998; Williamson et al., 2014). UV-C is absorbed by the earth’s ozone layer and therefore does not reach the earth’s surface. Exposure to UV-B radiation results in the production of thymine dimers in DNA, tissue necrosis, photo-oxidation of hormones and damage to photosynthetic machinery (Schmelzer et al., 1988; Jansen et al., 1998; Jenkins, 2009). To minimise the damaging effects of UV irradiation plants carefully choreograph developmental changes such as maintaining a stunted growth habit and alterations in leaf morphology and anatomy (Jansen et al., 1998). It has been widely found for a number of species that UV radiation also enhances the expression of wound responsive and pathogenesis related (PR) genes and chalcone synthase (CHS) genes (A.H.Mackerness et al., 1999; Brosche and Strid, 2003; Fragniere et al., 2011). ROS was shown to be important for the accumulation of PR proteins in Nicotiana tabacum leaves after UV-B irradiation (Green and Fluhr, 1995). These stress responsive genes are also linked to the
production of jasmonic acid (JA), salicylic acid (SA), ROS and ethylene (ET) for downstream signal processing (Jenkins, 2009). Abiotic stress responses in plants stimulate the production of ROS which in turn stimulates different gene products that are responsible for protecting the plant (Vinocur and Altman, 2005).

Biotic stress is caused by an invading organism whether that be a pathogen, insect or a nematode. The stress response following pathogen attack is initiated either by recognising pathogen associated molecular patterns (PAMP) or by effector triggered immunity (Spoel and Dong, 2012). For a biotrophic plant pathogen interaction, the recognition of pathogens $avr$ (avirulence) genes by the plant $R$ (resistance) genes leads to resistance and plants become susceptible to the pathogen when it fails to recognise $avr$ genes of the pathogen (Dangl and Jones, 2001). Once the $avr$ gene products are recognised by the $R$ genes of the plant, ROS production is stimulated. Systemically propagating ROS signals further stimulates the phytohormones JA, SA and ET, leading to spatial and temporal biotic stress responses that involve cell wall fortification (eg callose production) and production of antimicrobial compounds (Dangl and Jones, 2001; Glazebrook, 2005). Thus hydrogen peroxide production was shown to induce resistance in $A. thaliana$ to a virulent $Hyaloperonospora arabidopsidis$ by activating OXIDATIVE SIGNAL-INDUCIBLE1 (OXI1) protein kinase (Rentel et al., 2004).

In order to cope with the abiotic or biotic stress a cascade of hormonal and physiological processes are initiated via ROS (Baxter et al., 2013). The stress response in the plants is elicited by the stimulation of ROS that may further leads to gene activation via phytohormones induction (Fujita et al., 2006). For example heat stress (Suzuki et al., 2013) or root herbivory (Soler et al., 2013; Wondafrash et al., 2013) was shown to stimulate abscisic acid production.
in shoots leading to stomatal closure and reduction in the rate of transpiration. Long term effects of ROS mediated stress response would include control on gene expression that would regulate growth and development, production of primary and secondary metabolites for the defence against stress (Gilroy et al., 2014). It was reported that a metabolic reprogramming occurs in local tissue *A. thaliana* few seconds after the application of high light stress (Suzuki et al., 2013) and further, the systemic propagation rate for ROS signal was proposed to be 8.4 cm min$^{-1}$ (Miller et al., 2009) Once produced ROS acts via different phytohormones like jasmonic acid, salicylic acid, abscisic acid and ethylene, which further trigger various cell functions like plant growth, development (Carol and Dolan, 2006) and death (Van Breusegem and Dat, 2006). ROS-induced phytohormone production also stimulates the production of plant defence molecules like callose that play a role in various biotic and abiotic stress responses (Vellosillo et al., 2010; Yi et al., 2014). A number of reviews have concluded that callose is produced around a wound site after biotic or abiotic stress (Hématy et al., 2009; Galatis and Apostolakos, 2010; Piršelová and Matušíková, 2013).

Callose is a glucose polymer linked via β-1,3 glycosidic bonds with branching via β-1,6 glycosidic bonds (Chen and Kim, 2009). Callose synthesis is the result of expression of *GLUCAN SYNTHASE-LIKE (GSL)* genes that encode transmembrane domains localised on the plasma membrane (Ellinger and Voigt, 2014). GSL5, GSL7 and GSL12 expression were identified to be required for callose deposition in response to fungal pathogens and wounding, deposition in phloem and deposition in plasmodesmata respectively (Ellinger and Voigt, 2014). Callose production was shown to be stimulated in various stress conditions during plant development. Callose was reported to be produced in 90 min after treating *A. thaliana* leaves with flg22 which is a bacterial microbial associated molecular pattern (MAMP) (Daudi et al.,
Further, callose was shown to be involved in the resistance towards powdery mildew Golovinomyces cichoracearum infection on A. thaliana (Ellinger et al., 2013). Callose production via JA-dependent signalling was also shown to control downy mildew caused by Plasmopara viticola in Vitis vinifera (grape) after external application of β-aminobutyric acid (Hamiduzzaman et al., 2005). Similarly abiotic stress caused by metal ion toxicity was extensively reported to induce callose production in a variety of plants. Treating Lemna minor (duckweed) with lead (Samardakiewicz et al., 1996) and T. aestivum with aluminium (Sivaguru et al., 2000) increased the production of callose in their roots, indicating an abiotic stress response in plants towards mechanically applied metal ions.

In this chapter plants were tested for their response to UV-C after spray-application of surfactants and nanostructured liquid crystalline particles (NLCP). NLCP as described in chapter 3 were tested as an alternative delivery vehicle that can deliver the agrochemical active without damaging the epicuticular waxes. On the other hand, surfactants are traditionally applied in various agricultural practices for the delivery of agrochemical actives. The surfactant-based spray formulations are known to irreversibly solubilise epicuticular waxes that may lead plant stress by making it more susceptible to deleterious abiotic and biotic factors (Tamura et al., 2001; Keese and Camper, 2006). Therefore, in this chapter, NLCP were tested on different plants to investigate the NLCP-induced stress response against surfactant-induced stress response after exposing the plants to UV. In the presence of UV the amount of callose produced in the leaves of A. thaliana and L. angustifolius was shown to be lower in comparison to that in leaves treated with surfactants. Novel image analysis was further used to confirm the results found using a callose biochemical assay. Hydrogen peroxide was also shown to slightly decrease after NLCP treatments on A. thaliana and L. angustifolius. Zea mays did not show
either increased callose production or hydrogen peroxide formation after UV, NLCP and surfactants treatments. Together, these results support the use of NLCP rather than surfactants as an agrochemical delivery system.

4.2 Materials and Methods

4.2.1 Growth conditions for plants and treatments with surfactants and NLCPs

*A. thaliana, L. angustifolius* and *Z. mays* were grown as described in section 3.2.1 but the plants were potted in 5 cm diameter × 7 cm high black plastic pots. All the solutions were prepared and applied onto the plants according to procedures described in section 3.2.2.

4.2.2 Treatment of plants with UV-C

NLCP and surfactant treatments were made as described in section 3.2.2. Briefly, 0.1% (v/v) solutions of NLCP, empimin and empigen were separately spray-applied to obtain a uniform coverage of leaves. Six plants for *A. thaliana*, three plants each for *L. angustifolius* and *Z. mays* were spray-applied with either NLCP or surfactants, followed by UV-C irradiation. Only the third or fourth pair of leaves on each *L. angustifolius* plant were spray treated while the spray formulation landing on rest of the plant was not controlled. The second leaf on *Z. mays* plants was similarly treated with either NLCP or surfactants. For drying the spray applied on the plants, they were transferred to the growth chambers. After one hour the plants were exposed to UV-C radiation as described below.

*A. thaliana, L. angustifolius* and *Z. mays* were irradiated under UV-C radiation provided by a 80 cm germicidal (non-ozone producing) UV-C lamp with an emission peak at 254 nm (Australian Ultra Violet, Vic, Australia) and fitted in a light proof chamber (120 length × 60 breadth × 50 cm high). UV-C radiation was measured using a UVX digital radiometer with a UVX-25 sensor (Ultra-Violet Products Ltd, Cambridge, UK) to provide dosages of 250, 500,
750 and 1000 J m\(^{-2}\) s\(^{-1}\) to plants of *A. thaliana*, *L. angustifolius* and *Z. mays*. For *A. thaliana* the six plants (three plants per pot) were exposed at a height of 7 cm from the base of the chamber. On three *L. angustifolius* plants (one plant per pot) third or fourth pair of leaves were marked and exposed at a height of 10 cm from the base of the chamber. Though all the plant is exposed to the UV radiation tissue from only the third or fourth pair of leaves was collected for callose or hydrogen peroxide studies. For *Z. mays*, as the leaves are quite long (up to 20 cm), UV-C intensities were measured at the base of the chamber and at four different positions spaced ten centimetres apart in a straight line. Dosages of 250, 500, 750 and 1000 J m\(^{-2}\) s\(^{-1}\) were simultaneously calculated basing on the average intensity calculated earlier. Similar to *L. angustifolius* plants, although all of the *Z. mays* plant was exposed to radiation, only tissue from the second leaf was collected for further experimentation. After exposure the plants were transferred back to the growth chambers and were maintained for five days under controlled conditions before collecting the leaf tissue. Appropriate controls for UV-C radiations were simultaneously prepared by spraying equal number of plants with water and not exposing them to radiation. NLCP or surfactant controls on the other hand were prepared by spraying the plants with respective formulation at a concentration of 0.1% but were not exposed to UV-C radiation. For obtaining leaf area measurements, individual plants were photographed separately three and five days after the UV treatments and the images analysed using imageJ software as previously described (section 2.3.4.2).

### 4.2.3 Detection of callose and hydrogen peroxide in whole leaves

For eliminating any chlorophyll-induced autofluorescence, leaf tissue excised after five days from each species was immediately transferred to 80% (v/v) ethanol. After complete removal of chlorophyll leaf tissue was placed in 0.5% (w/v) aniline blue (Sigma-Aldrich, Sydney, NSW, NSW,
Australia) in 0.15M K$_2$HPO$_4$ (Sigma-Aldrich) overnight. The tissue was then rinsed in dH$_2$O and finally mounted in 50% glycerol on glass slides. The slides were observed using epifluorescence microscopy (Axioskop 2 mot plus microscope, Zeiss, Göttingen, Germany) with an ultraviolet (365nm excitation, 420nm emission) filter.

To examine the hydrogen peroxide accumulation freshly prepared one mg ml$^{-1}$ 3,3-diaminobenzidine (DAB) in water (Sigma-Aldrich) at a pH 3.8 was used. Excised leaf tissue was floated on the DAB solution in a Petri plate in a growth cabinet for one hour. The tissue was subsequently transferred to 80% (v/v) ethanol to remove any chlorophyll. The tissue was mounted as above and observed under the microscope as described for callose, but viewed under bright-field settings. Visualisation of callose and hydrogen peroxide by staining was repeated in two independent experiments.

4.2.4 Measurement of leaf area and area of callose within leaves

Image analysis to determine the extent of callose production within leaves five days after treatments, was performed as described in section 2.3.4.2. For determining total leaf area per plant after UV treatments, images obtained for both third and fifth day after treatments were analysed. Briefly, ImageJ version 1.46r software (Schneider et al., 2012) was used to convert a .jpeg image into an 8 bit image. Noise was despeckled before adjusting the threshold of the image. The area of either leaf or callose was subsequently calculated depending on the scale provided for each image. Analysis of variance (ANOVA) with Duncan’s post-hoc test was performed on the collected data using a commercial software package (IBM SPSS Statistics 21, IBM Australia Ltd, St Leonards NSW, Australia).
4.2.5 Quantitation of callose and hydrogen peroxide in leaves

Callose was quantified as described by Mintoff, (2014) for *A. thaliana*. For *L. angustifolius* and *Z. mays* for each treatment 150 mg leaf tissue was harvested from three plants (50 mg per plant) and quantified as described earlier. All the experiments were repeated three times.

For *A. thaliana* five days after the treatments, 50 mg of tissue was harvested and snap frozen using liquid nitrogen. The tissue was subsequently homogenised in 20 mM potassium phosphate buffer at pH 6.5 and centrifuged at 13000 g for 15 min at 4 °C (Xing et al., 2007; Estavillo et al., 2011; Mintoff, 2014). Determination of hydrogen peroxide was performed using an Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Mulgrave, Vic, Australia). Briefly 50 μl of the supernatant collected earlier was added to a 96 well fluorescence plate (Greiner Bio-One, Frickenhausen, Germany) followed by addition of 50 μl of reaction mixture containing the Amplex® Red reagent and horseradish peroxidase. After 30 minutes incubation in the dark, fluorescence intensities of the samples were determined by excitation at 544 nm and then emission at 590 nm (FLUOstar Omega, BMG labtech Pty. Ltd. Mornington, Vic, Australia). For *L. angustifolius* and *Z. mays* for each treatment 150 mg leaf tissue was harvested from three plants (50 mg per plant) and quantified as described for *A. thaliana*. Experiments were repeated thrice and Duncans post-hoc test was performed as above.

4.2.6 Growth and maintenance of *Hyaloperonospora arabidopsidis*

*H. arabidopsidis* Noks1 conidiospore inoculum was kindly provided by Dr Sharl Mintoff (Deakin University) and was applied onto the leaves of two to four week old *A. thaliana* Ws-eds1 maintained as described in section 4.2.1. *H. arabidopsidis* was maintained by infecting new plants every week. For testing the impact of NLCP and surfactants on the ability of Noks1 to infect leaves, leaves of *A. thaliana* Col-0 were sprayed with a 0.1% (v/v) spray solution to
obtain uniform coverage. Once the spray solution was dried under normal growth conditions, 2 μl of freshly prepared conidiospores inoculum containing $1 \times 10^5$ spores was applied by micropipette to the adaxial surface of leaves. For counting conidophores, the plants incubated for seven days after inoculation were removed from the pot and all the conidiophores on each plant were carefully counted using a stereo microscope (Zeiss 2000-C Stemi, Zeiss, NSW, Australia). After incubating the plants for seven days, leaf tissue that was infected with \textit{H. arabidopsisidis} was harvested and transferred to 80% ethanol to remove chlorophyll. Leaves were then stained for the presence of callose as described above. For callose quantification 50 mg leaf tissue was harvested and transferred to 80% ethanol. After complete removal of chlorophyll callose was extracted as described above. Experiments were repeated thrice and Duncans post-hoc test was performed as previously described.

4.3 Results

4.3.1 Impact of surfactant or NLCP application on response of leaves of \textit{A. thaliana} to UV-C irradiation

\textit{A. thaliana} plants were exposed to 0, 250 and 500 J m$^{-2}$ s$^{-1}$ UV-C radiation after treating the plants with 0.1% (v/v) NLCP, or one of the surfactants empimin and empigen. Plants that were treated with water only formed an untreated control group. There were no difference between treatments three days after exposing the plants to UV-C (Fig 4.1). Five days after UV-C exposure at 250 and 500 J m$^{-2}$ s$^{-1}$ the water-treated control group was found to have the largest leaf area when compared to all the other treatment groups (Fig 4.1). A UV-C dose-dependent decrease in leaf area was clearly observed for the controls five days after the UV exposure. For \textit{A. thaliana} sprayed with NLCP and surfactants, no clear differences between treatments were observed after five days (Fig 4.1).
4.3.2 UV-C-induced callose production in plants pre-treated with NLCP or surfactants

4.3.2.1 UV-C induced stress response in *A. thaliana*

Callose production on the leaves of *A. thaliana* following the UV-C treatments was compared with that found after the application of NLCP, empimin and empigen followed by an exposure to UV-C radiation. Observations on callose formation were made using fluorescence microscopy on untreated controls (0 J m\(^{-2}\) s\(^{-1}\)) did not show any callose production five days after mock UV exposure (Fig 4.2 a). For NLCP controls (treated with 0.1% NLCP and 0 J m\(^{-2}\) s\(^{-1}\) UV) segregated callose fluorescence could be detected on the leaves of *A. thaliana* (Fig 4.2 b). Compared with the NLCP treatments the spray application of empimin (Fig 4.2 c) and empigen (0 J m\(^{-2}\) s\(^{-1}\) UV) (Fig 4.2 d) resulted in intense callose production five days after incubation. UV-C exposure of 250 J m\(^{-2}\) s\(^{-1}\) (Fig 4.2 e) stimulated a low level callose production while all the other treatment groups elicited a strong callose response (Fig 4.2 f - h). Callose production was observed as tiny particles across the leaf tissue and was reported to increase in the order of treatment from NLCP (Fig 4.2 f), empigen and empimin (Fig 4.2 g and h). After the exposure to 500 J m\(^{-2}\) s\(^{-1}\) of UV callose production in the controls (UV alone) (Fig 4.2 i) and NLCP (Fig 4.2 j) treatment group appeared to be similar (Fig 4.2 k and l). The callose production on the plants subjected to 750 and 1000 J m\(^{-2}\) s\(^{-1}\) of UV, was found to be very intense irrespective of the treatments, but the callose intensities after 1000 J m\(^{-2}\) s\(^{-1}\) appeared to be higher compared against 750 J m\(^{-2}\) s\(^{-1}\) (Fig 4.2 m - t). For all of the treatments callose production was observed to be localised on the cell walls of epidermal and mesophyll cells (Fig 4.3). Callose production was initially observed as a tiny speckle on the cell periphery (Fig 4.3 b) which progressively accumulated along the cell walls of epidermal and mesophyll cells (Fig 4.3 c and d).
Figure 4.1 Leaf area of *A. thaliana* plants after spray application of NLCP or surfactant treatments followed by an exposure to UV-C radiation. Mean leaf area measured (for nine plants from three replicates) after three days was shown with white bars and that of five days was shown in black. Same letters above bars represent statistically similar groups. Though controls were statistically different from the treatment groups and no clear differences between the treatment groups was observed. All the bars indicated by same letters are statistically similar after Duncan’s posthoc testing. SE of the mean is shown for each treatment. UTC = untreated control, CTRL = control.
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Figure 4.2 Callose production in *A. thaliana* after spray application of NLCP or surfactants followed by UV-C exposure. A clear dose-dependent increase in callose production was observed on six plants tested per treatment over two replicates five days after spray applications. The tailed arrows in (a) and (b) show the brightly fluorescent trichomes on the leaf surface and arrow heads on (c) and (k) point to the callose aggregations formed after various treatments. (a) The controls (0 J m\(^{-2}\) s\(^{-1}\)) did not show any callose response after a water treatment. (b) NLCP spray application (0 J m\(^{-2}\) s\(^{-1}\)) was similar to untreated control by having no callose accumulation. (c and d) Surfactants empimine and empigen were shown to induce larger amounts of callose in comparison to that of NLCP and untreated controls. Callose was observed as tiny speckles ranging in different sizes on the images, these speckles coalesced to form large aggregates of callose. (e) The UV exposure at 250 J m\(^{-2}\) s\(^{-1}\) alone was observed to have the lowest callose accumulation when compared with that of (f) NLCP, (g) empimine and (h) empigen-treated plants. (i) 500 J m\(^{-2}\) s\(^{-1}\) of UV and (j) NLCP treatments (followed by 500 J m\(^{-2}\) s\(^{-1}\) of UV exposure) were found to have similar amounts of callose. (k) and (l) empimine and empigen treatments at 500 J m\(^{-2}\) s\(^{-1}\) UV however were shown to elicit an intense callose response. All the treatments after 750 J m\(^{-2}\) s\(^{-1}\), (m) UV control, (n) 750 J m\(^{-2}\) s\(^{-1}\) NLCP, (o) 750 J m\(^{-2}\) s\(^{-1}\) empimine and (p) 750 J m\(^{-2}\) s\(^{-1}\) empigen appeared to have equal amounts of callose produced. For control plants treated with (q) UV 1000 J m\(^{-2}\) s\(^{-1}\) heavy callose production was observed. A similar trend for callose production was observed to be extended over (r) NLCP, (s) empimine and (t) empigen treatment groups. Scale bar is equal to 200 \(\mu\)m.
Figure 4.3 Detail of callose accumulation in cells and cell walls of *A. thaliana*. The callose accumulation five days after NLCP and surfactant treatments followed by UV irradiation, was observed predominantly on the cells walls of epidermal and underlying parenchymal cells. (a) A water treated control (0 J m$^{-2}$ s$^{-1}$) *A. thaliana* leaf showing mesophyll cells. No callose production was observed. (b) White arrow points to callose accumulated on the cell walls of the mesophyll cells after NLCP spray application (0 J m$^{-2}$ s$^{-1}$). (c) Epidermal cells of leaf showing distinct fluorescence from callose on the cell walls is indicated with the arrow (after exposure to UV-C at 500 J m$^{-2}$ s$^{-1}$). (d) Extensive callose production on the edges of mesophyll cells of *A. thaliana* leaves after empimfin treatment (0 J m$^{-2}$ s$^{-1}$). Scale bar equals to 275 μm.
4.3.2.2 Biochemical quantification and image analysis of callose on A. thaliana

For the control group treated with UV at 0, 250, 500, 750 and 1000 J m\(^{-2}\) s\(^{-1}\) a dose-dependent increase in callose was clearly observed, where untreated controls had the least amount of callose and UV 1000 J m\(^{-2}\) s\(^{-1}\) treatments accumulated higher amounts of callose compared to untreated controls (Fig 4.4 a). The amount of callose extracted for UV controls and NLCP treatments was very similar. A trend similar to controls (treated with UV alone) was observed for the NLCP treatment group (Fig 4.4 a) and NLCP spray applications after 750 and 1000 J m\(^{-2}\) s\(^{-1}\) were shown to induce similar quantities of callose. Callose extracted for the empimin treatment did not follow any trend for callose production after UV treatments (Fig 4.4 a), but UV exposure at 750 J m\(^{-2}\) s\(^{-1}\) had the highest callose produced compared with any other treatment (Fig 4.4 a). Empigen treatment on the other hand did not show any significant difference in the callose production at the tested UV doses of 0, 250 and 500 J m\(^{-2}\) s\(^{-1}\). A dose-dependent increase in callose was observed for UV treatments at 750 and 1000 J m\(^{-2}\) s\(^{-1}\) following empigen spray application (Fig 4.4 a).

The callose produced after different treatments was visualised using epifluorescence microscopy and to quantify the visual data novel image analysis was performed on the micrographs. To calculate the area of callose, the micrographs were processed using imageJ software. For all the treatments the data computed for area of callose after image analysis was in good agreement with that of callose biochemical quantification. A UV dose-dependent increase in callose area was evident while individual group controls recorded the lowest area of cover and 1000 J m\(^{-2}\) s\(^{-1}\) exposure produced the highest callose area (Fig 4.4 b). NLCP-induced callose accumulation followed a similar trend to that which was earlier observed for the control group, but the UV doses 0 and 250 J m\(^{-2}\) s\(^{-1}\) were observed to have higher amounts.
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Figure 4.4 In-vitro and in-silico callose quantification for NLCP, surfactant and UV treatments.

(a) Biochemical quantification of total callose in *A. thaliana* leaf for each treatment, is presented as percentage average above untreated control. The callose production was lowest for untreated control and highest for plants treated with empimin followed by UV exposure of 750 J m\(^{-2}\) s\(^{-1}\). A UV dose-dependent increase in callose production was consistently observed for the UV control (treated with UV alone) and NCLP treatment groups. At all the tested concentrations callose extracted for the empimin treatment group was higher when compared to that of other treatment groups. Empigen spray application followed by UV treatments at 0, 250 and 500 J m\(^{-2}\) s\(^{-1}\) resulted in almost equal amount of callose being extracted. 750 and 1000 J m\(^{-2}\) s\(^{-1}\) treatments were however similar to control and NLCP groups. Please note that UTC is taken as 100% and values for other treatments are relative to the UTC. (b) Image analysis for callose stained in the leaves of *A. thaliana*. For all the treatment groups, a UV dose-dependent increase in callose production was evident, where group controls had the least amount of callose and plants treated with UV 1000 J m\(^{-2}\) s\(^{-1}\) had the highest amount of callose measured. Empimin treatment followed by UV 1000 J m\(^{-2}\) s\(^{-1}\) and UV exposure of 250 J m\(^{-2}\) s\(^{-1}\) after empigen treatment, however, were out of the trend and computed to have less callose area. All the bars indicated by same letters are statistically similar after Duncan’s posthoc testing. SE of the means are shown. UTC = untreated control, ctrl = control.
of callose produced (Fig 4.4 b). The area of callose found following empimin treatments was much higher in comparison to that of all the other treatment groups (Fig 4.4 b). For empimin treatments at lower doses of 0, 250 and 500 J m\(^{-2}\) s\(^{-1}\), though there was a dose-dependent increase in the calculated callose area, higher doses 750 and 1000 J m\(^{-2}\) s\(^{-1}\) did not show a proportionate increase. A UV dose-dependent response for the callose area quantification was also observed for empigen spray treatments (Fig 4.4 b).

4.3.2.3 Callose visualisation in \textit{L. angustifolius} leaves

On the leaves of \textit{L. angustifolius} aniline blue staining did not show any callose formation for the untreated controls (0 J m\(^{-2}\) s\(^{-1}\)) but for the NLCP spray application (0 J m\(^{-2}\) s\(^{-1}\)) a slight increase in the callose accumulation was recorded (Fig 4.5 a and b). In contrast to UV controls and NLCP treatments, empimin and empigen spray applications (0 J m\(^{-2}\) s\(^{-1}\)) stimulated an intense callose production (Fig 4.5 c and d). A similar trend earlier observed for the callose production on the controls (UV 0 J m\(^{-2}\) s\(^{-1}\)) was evident for 250 and 500 J m\(^{-2}\) s\(^{-1}\) UV treatment following all the spray applications (Fig 4.5 e to i). For UV 750 J m\(^{-2}\) s\(^{-1}\) and 1000 J m\(^{-2}\) s\(^{-1}\) treatments the water treated controls, and NLCP spray treatments, very negligible amounts of callose were produced while that of surfactant treatments, was very intense. Additionally, it is very interesting to observe that for most of the empimin and empigen treatments callose production was elicited along the surfactant drying patterns (Fig 4.5 d, h, k and s).

4.3.2.4 Quantification of callose in \textit{L. angustifolius}

The biochemical quantification of callose in the leaves of \textit{L. angustifolius} did not show any significant differences in the amounts of callose extracted, for plants irradiated with increasing doses of UV of 0, 250, 500, 750 and 1000 J m\(^{-2}\) s\(^{-1}\) (Fig 4.6 a). Similar observations were recorded for UV treatments after NLCP spray applications but with few differences. The
callose extracted for 500 and 1000 J m\(^{-2}\) s\(^{-1}\) treatments was shown to be a little higher compared to other treatments of the NLCP group (Fig 4.6 a). The amounts of callose extracted for UV exposure after empimin spray application was significantly higher from that of all the other groups and there was a consistent dose-dependent increase in the callose quantity. However, callose produced after a UV exposure at 1000 J m\(^{-2}\) s\(^{-1}\) after emipimin spray application was not as high as that of 750 J m\(^{-2}\) s\(^{-1}\) treatments (Fig 4.6 a). The amount of callose quantified following empimin 500, 750 and 1000 J m\(^{-2}\) s\(^{-1}\) UV-C treatments was observed to be the highest among all the other treatments. Callose accumulation stimulated by the surfactant empigen also appeared to be higher when compared against UV control and NLCP groups, but remained low against the empimin treatment. Treatment with empigen at 1000 J m\(^{-2}\) s\(^{-1}\) produced the highest amount of callose found within the group (Fig 4.6 a).

Image analysis on the fluorescence microscopy images for determination of the area of callose found on the leaves of *L. angustifolius* was found to be similar to that found using biochemical quantification (Fig 4.6 b). The callose area found for untreated controls and treatment with 250 and 500 J m\(^{-2}\) s\(^{-1}\) was negligible but the same area for controls subjected to UV treatments at 750 and 1000 J m\(^{-2}\) s\(^{-1}\) was higher than that of low dose treatments (Fig 4.6 b). For NLCP spray applications though the callose area calculated was higher than the UV controls, no significant trend was established (Fig 4.6 b). For empimin spray applications followed by UV treatment the image analysis recorded highest amounts of callose and was consistent with the results earlier obtained for callose microscopy and the biochemical assays (Fig 4.6 b). Treatment with 1000 J m\(^{-2}\) s\(^{-1}\) UV irradiation following empimin application, however, elicited callose that was less in amount than that found for the 250, 500 and 750 J m\(^{-2}\) s\(^{-1}\) UV treatments. The callose area analysed for empigen was greater when compared to that of UV controls and NLCP.
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Figure 4.5 UV, NLCP and surfactant-induced callose production in *L. angustifolius*. Five days after spray application of NLCP or surfactants (a) water treated control (0 J m\(^{-2}\) s\(^{-1}\)) did not elicit any callose production. (b) Unlike the water treated control the NLCP treatment elicited a slight increase in the production of the callose. (c) Spray application of empimin on *L. angustifolius* (0 J m\(^{-2}\) s\(^{-1}\)) induced intense callose production compared against other treatments. (d) Empigen (0 J m\(^{-2}\) s\(^{-1}\)) treatment also induced callose production but to a low level compared with the empigen treatment but much more than the untreated controls and NLCP treatments. (e) Controls after UV 250 J m\(^{-2}\) s\(^{-1}\) exposure did not induce callose production but (f) NLCP comparatively showed a slight increase in callose production. (g) UV 250 J m\(^{-2}\) s\(^{-1}\) exposure after plants treated with empimin spray application was shown to induce callose production in large amounts while (h) empigen stimulated callose production was less when compared to that of empimin. (i to t) A dose dependent increase in fluorescence intensities of callose was clearly evident for all the treatments but with few differences. For empimin treatments (k, o and s) the callose production tested across all the UV doses was intense and similar when compared to UV (i, m and q) and NLCP (j, n and r) treatments. (d, h, k and s) Callose production along the surfactant drying patterns was observed. Scale bar equals to 200 μm.
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Figure 4.6 Quantification of callose after NLCP, surfactant and UV treatments on the leaves of *L. angustifolius*. (a) The biochemical quantification of callose after the NLCP or surfactant spray applications followed by UV treatments on the leaves of *L. angustifolius*. For the control group treated with UV doses of 0, 250, 500, 750 and 1000 J m\(^{-2}\) s\(^{-1}\) no significant differences between the treatments could be observed. Similarly, UV exposure followed by NLCP application appeared to be similar to that of UV controls. For the empimin treatments, callose produced for 500, 750 and 1000 J m\(^{-2}\) s\(^{-1}\) UV doses, was recorded to be higher than any other treatments. The empigen spray application followed by UV treatments on the *L. angustifolius* leaves showed a slight increase in the amount of callose extracted, when compared to the control and NLCP groups, but was significantly less than that of empimin. Please note that UTC is taken as 100% and values for other treatments are relative to the UTC. (b) *In-silico* quantification of callose production. Image analysis to quantify the area of callose did not followed any trend but was similar to results from the callose biochemical assay on the leaves of *L. angustifolius*. The UV control group elicited the least amount of callose production. Though the callose area after NLCP spray treatments appeared to be higher than the UV controls, no significant trend could be established. Empimin-induced callose production after the UV treatments was found to be higher when compared to all the other treatment groups. Within the empimmin group a slight decrease in the area of callose calculated was found after 1000 J m\(^{-2}\) s\(^{-1}\) UV treatment. Empigen-induced area of callose was higher than that of the NLCP and UV spray treatments but less than the empimin spray application. All the bars indicated by the same letters are statistically similar after Duncan’s posthoc testing. SE of the means are also shown. UTC = untreated control, ctrl = control.
treatments and was less compared with the empinin treatments (Fig 4.6 b). Empigen treatment followed by UV exposure seemed to produce higher amounts of callose when compared against UV controls and the NLCP treatment group although no dose-dependent trend was observed (Fig 4.6 b).

### 4.3.2.5 Stress response in *Zea mays* after UV, NLCP and surfactant treatments

There were no observable differences in the amount of callose obtained on the leaves of *Z. mays* following the various treatments. Untreated controls did not show any production of callose and all the treatments had similar levels of callose accumulation (Fig 4.7). Similar to the observations made on *A. thaliana* and *L. angustifolius*, the callose produced after the various treatments on the leaves of *Z. mays* was observed as tiny speckles that appeared to be produced in association with the cells walls. For *Z. mays* autofluorescence from the veins of the leaf could be clearly observed after staining with aniline blue (Fig 4.7).

There was no observable trend of increased callose quantified in the *Z. mays* control leaves treated with 0, 250 and 500 J m$^{-2}$ s$^{-1}$ UV (Fig 4.8 a) however callose extracted after high doses UV 750 and UV 1000 J m$^{-2}$ s$^{-1}$ was observed to be higher although not significantly so within the control treatment group. Similarly there was no trend of increased callose production for callose extracted after NLCP spray application. Empimin treatments in combination with UV irradiation were shown to have the highest amount of callose extracted in comparison to control, NLCP and empigen treatment groups. With increasing doses of UV from 0, 250, 500, 750 to 1000 J m$^{-2}$ s$^{-1}$ callose production also increased for empimin treatments following UV irradiation. For callose extracted after empigen spray application, a UV dose-dependent callose production could not be observed and all the treatments appeared to produce similar amounts of callose after extraction. It was, however, surprising to note that 500 J m$^{-2}$ s$^{-1}$ treatment was
shown to have the least amount of callose produced. The results obtained for *Z. mays* biochemical callose extraction from the treated leaves were consistent with that found for the fluorescence microscopy observations and there were no statistically significant differences. Similarly, the image analysis on *Z. mays* for the callose area did not show any prominent differences for the UV doses tested at 0, 250, 500 and 750 J m$^{-2}$ s$^{-1}$ (Fig 4.8 b). For all the tested UV doses at 1000 J m$^{-2}$ s$^{-1}$ a steep increase in the callose area was evident. But such a difference for 1000 J m$^{-2}$ s$^{-1}$ was not observed for empigen spray application (Fig 4.8 b).

4.3.3 UV-C-induced hydrogen peroxide production following NLCP and surfactant spray application

In addition to callose, hydrogen peroxide (H$_2$O$_2$) produced five days after the UV, NLCP and surfactant treatments was measured to examine the H$_2$O$_2$ stress response of *A. thaliana*, *L. angustifolius* and *Z. mays*. For *A. thaliana* unlike the callose quantification a dose-dependent trend was not observed for the H$_2$O$_2$ quantified after the UV treatments ranging from 0, 250, 500, 750 and 1000 J m$^{-2}$ s$^{-1}$ (Fig 4.9 a). The highest and lowest amount of H$_2$O$_2$ quantified for the experiment was between 0.37 μM to 0.83 μM respectively. For *A. thaliana* controls UV dosage at 0 and 250 J m$^{-2}$ s$^{-1}$ yielded similar amounts of H$_2$O$_2$. It was surprising to observe a decrease in H$_2$O$_2$ for the UV treatments at 500, 750 and 1000 J m$^{-2}$ s$^{-1}$ (Fig 4.9 a). Similar to the controls, UV treatments after NLCP spray application did not significantly alter the amount of H$_2$O$_2$ found for the doses 0, 250 and 500 J m$^{-2}$ s$^{-1}$. With further increase in UV exposure to 750 and 1000 J m$^{-2}$ s$^{-1}$, the total H$_2$O$_2$ quantified increased (Fig 4.9 a). Empimin spray application followed by UV however appeared to produce greater amounts of H$_2$O$_2$ when
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Figure 4.7 Callose visualisation on the leaves of *Z. mays*. Callose was observed under the fluorescence microscope after staining with aniline blue. On all the images autofluorescence could be observed as bright streaks (indicated by blunt end arrows) and also along wavey cell walls (tailed arrows with a dark centre). Smaller tailed arrows point to the callose that can be seen as speckles of various sizes. (a-i) Random distribution of callose on the leaves of *Z. mays*. No significant differences were observed for callose produced after 0, 250 and 500 J m$^{-2}$ s$^{-1}$ UV doses. (m-t) For the UV treatments of 750 and 1000 J m$^{-2}$ s$^{-1}$ a slight increase in the callose was observed compared to other UV treatments. Scale bar equals to 200 μm.
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Figure 4.8 Quantification of callose in *Z. mays* leaves after various treatments. (a) Quantification of callose following chemical extraction. Callose amounts after 750 and 1000 J m\(^{-2}\) s\(^{-1}\) treatments were higher when compared to those after 0, 250 and 500 J m\(^{-2}\) s\(^{-1}\) UV treatments. Similar to UV-treated controls no trend could be established for UV treatments at 0, 250 and 500 J m\(^{-2}\) s\(^{-1}\) on plants sprayed with NLCP, but UV treatments of 750 and 1000 J m\(^{-2}\) s\(^{-1}\) induced greater amounts of callose production. For UV treatments after empimin spray application, a dose dependent increase in callose production was observed where 0 J m\(^{-2}\) s\(^{-1}\) elicited the least amounts and 1000 J m\(^{-2}\) s\(^{-1}\) resulted in production of higher amount of callose. UV exposure of 1000 J m\(^{-2}\) s\(^{-1}\) on *Z. mays* treated with empimin showed the maximum amount of callose production when compared to all other treatments. Empigen treatment followed by UV treatment were very similar to that of NLCP treatments where there was no apparent trend for callose production. No statistical difference between the treatments was found. Please note that UTC is taken as 100% and values for other treatments are relative to the UTC. (b) Callose area calculated on the micrographs was in good agreement with the biochemical callose quantification. The callose area calculated for 0, 250, 500 and 750 J m\(^{-2}\) s\(^{-1}\) UV treatments for all the treatment groups including controls was similar, except for the empimin treatment which had high amount of callose area computed after 750 J m\(^{-2}\) s\(^{-1}\) UV treatment. Excluding empigen, all the spray applications followed by 1000 J m\(^{-2}\) s\(^{-1}\) UV dose induced large amounts of callose accumulation. All the bars indicated by the same letters are statistically similar after Duncan’s posthoc testing. SE of the means are also shown. UTC = untreated control, ctrl = control.
compared to all the other treatments. Similar to the other treatments no definite trend could be established for the empimin sprayed prior to UV irradiation (Fig 4.9 a). H2O2 extracted for UV treatments followed by empigen spray application were very similar to that of NLCP treatments. But surprisingly UV treatment at 500 J m⁻² s⁻¹ after empigen spray application was observed to have a less amounts of H2O2 yielded (Fig 4.9 a). Unlike *A. thaliana* the H2O2 quantification in leaves of *L. angustifolius* followed a trend where all the group controls untreated, NLCP, empigen and empimin controls (0 J m⁻² s⁻¹), consistently yielded greater amounts of H2O2 when compared to the other treatments within the group (Fig 4.9 b). For the UV-treated control group, a decrease in the amount of H2O2 measured was evident from 0 to 250 J m⁻² s⁻¹. For all the other UV doses no significant differences could be observed in the amount of H2O2 found. A very similar trend was observed for NLCP treatments followed by UV irradiation, but the amount of H2O2 found was less when compared to that of the control group (UV alone). UV treatments after empimin spray application were shown to accumulate higher amounts of H2O2 when compared to all the other treatments. An apparent decrease in H2O2 produced from empimin control UV 0 J m⁻² s⁻¹ to UV 250 J m⁻² s⁻¹ was observed and all the other UV treatments following empimin application were similar to that found for treatment with UV at 250 J m⁻² s⁻¹. The amount of H2O2 found following UV treatments of plants treated with empigen was similar to that of NLCP but slight increase in the amount of H2O2 was recorded. It was surprising to observe a steep decrease in amount of H2O2 found for empimin treatments followed by UV at 1000 J m⁻² s⁻¹. On the other hand H2O2 quantification for *Z. mays* did not show any differences for any tested treatment or UV exposure. These results are in strong agreement with that for callose production in *Z. mays*. 

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Figure 4.9 Quantification of hydrogen peroxide in *A. thaliana* and *L. angustifolius*. Following the NLCP or surfactant spray treatment the plants were exposed to UV radiation. Five days after the UV exposure H\textsubscript{2}O\textsubscript{2} was quantified. (a) Tissue collected from nine plants was biochemical screened for H\textsubscript{2}O\textsubscript{2} on *A. thaliana* five days after NLCP and surfactant treatments followed by UV irradiation. The H\textsubscript{2}O\textsubscript{2} extracted for UV treatments (no NLCP or surfactants) 500, 750 and 1000 J m\textsuperscript{-2} s\textsuperscript{-1} was observed to be greater than that of 0 and 250 J m\textsuperscript{-2} s\textsuperscript{-1}. Exposing the *A. thaliana* plants to UV after NLCP treatment also followed the trend observed for controls but with few differences. UV doses of 250 J m\textsuperscript{-2} s\textsuperscript{-1} recorded higher amounts of H\textsubscript{2}O\textsubscript{2} when compared to 0 and 500 J m\textsuperscript{-2} s\textsuperscript{-1}. The hydrogen peroxide produced for emipimin spray application followed by UV exposure was observed to be greater when compared to all the other treatments. UV dosage 250 J m\textsuperscript{-2} s\textsuperscript{-1} on empiminit treated plants produced maximum amounts of H\textsubscript{2}O\textsubscript{2} than any other treatment. Empigen UV treatments were very similar to that of NLCP spray applications. (b) The extraction of H\textsubscript{2}O\textsubscript{2} in the leaves of *L. angustifolius* after various treatments was different to that of *A. thaliana*. For all the treatment groups the H\textsubscript{2}O\textsubscript{2} yields were greater for untreated control (0 J m\textsuperscript{-2} s\textsuperscript{-1}) when compared to UV 250 J m\textsuperscript{-2} s\textsuperscript{-1}. All the other treatments 500, 750 and 1000 J m\textsuperscript{-2} s\textsuperscript{-1}, had similar amounts of H\textsubscript{2}O\textsubscript{2} yields. UV irradiation after NLCP spray application followed a similar trend earlier observed for UV controls. Empimin yields for H\textsubscript{2}O\textsubscript{2} were greater than all the treatments and that of empigen were similar to NLCP treatments. All the bars indicated by the same letters are statistically similar after Duncan’s posthoc testing. SE of means are also shown. UTC = untreated control, ctrl = control.
4.3.4 Biotic stress treatment of *A. thaliana*

To investigate if NLCP or surfactant treatments would alter the interaction of plants to infective pathogens, *Hyaloperonospora arabidopsidis* Noks1 was inoculated onto *A. thaliana* after treating the plants with either NLCP or surfactants at a concentration of 0.1% (v/v) (Fig 4.10 a and b). The number conidiophores counted per plant after various treatments decreased in the order of controls, empigen, NLCP and empimim treatments (Fig 4.10 c). To visualise the plant stress response callose production was observed under the microscope in response to the Noks1 infection after NLCP and surfactant treatments. After Noks1 infection callose could be observed as continuous beaded strands coincident with hyphae within leaf tissue (Fig 4.10 b). On each hypha callose was produced in association with each haustorium of the rapidly growing Noks1 hyphae. Following NLCP or surfactant treatments callose was observed as tiny specks of varying sizes randomly distributed across the leaf tissue. These callose specks coalesced to form aggregates of varying sizes and shapes (Fig 4.10 c). For *A. thaliana* leaves treated with both Noks1 and NLCP or surfactant sprays, distinct patterns associated with *H. arabidopsidis* penetration and NLCP (or surfactants) callose formation were clearly observed (Fig 4.10 d to f). The amount of callose observed after both *H. arabidopsidis* and surfactant treatments (on the same leaf) was higher than that found after separate treatment with the pathogen or surfactant alone. Callose was also quantified in the plants that were infected with *H. arabidopsidis* seven days after NLCP or surfactant spray applications. For all the controls that were treated with water, NLCP, empimim or empigen separately (not infected with *H. arabidopsidis*), the callose extracted was similar in amount to that of water-treated controls (Fig 4.11). All the plants infected with *H. arabidopsidis* after NLCP or surfactant spray were observed to produce considerable amount of callose to that of respective controls (Fig 4.11).
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Figure 4.10 Conidiophore counts following *H. arabidopsidis* infection of *A. thaliana* leaves seven days after inoculation. (a) White fluffy growth of *H. arabidopsidis* causing downy mildew (shown by white arrow head) seven days after infection can be seen on the adaxial surface of the leaves and petioles of *A. thaliana*. Scale bar equals to 1 cm. (b) The asexual reproduction of the pathogen proceeds through the conidiospores that are produced on the tree like structures called conidiophores. Heavy growth of conidiophores on the adaxial surface of *A. thaliana* leaves can be seen. The arrow head in the insert shows the tree like conidiophores observed under stereo microscope. (c) The *A. thaliana* plants were spray applied with 0.1% (v/v) NLCP or surfactant formulations. After the sprays dried, four leaves per plant were inoculated with $10^5$ conidiospores and total number of the conidiophores per plant were counted on 30 plants (of three replicates) after seven days of incubation. All the bars indicated by the same letters are statistically similar after Duncan’s posthoc testing. SE of the means are shown.
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Figure 4.11 The pattern of callose deposition after biotic and abiotic stresses. Different patterns of callose deposition were observed for plants exposed to biotic and abiotic stress seven days after *H. arabidopsidis* Noks1 infection after spray application of NLCP or surfactants. (a) Untreated control showing no callose formation after aniline blue staining. The arrow indicates the autofluorescing veins of the leaf. (b) *A. thaliana* treated with *H. arabidopsidis* Noks1, showing callose deposition along hyphae around each haustorium (indicated by black centre arrow). (c) Callose produced in response to empimim treatment. The callose could be observed as spots of various sizes. The small spots of callose sometimes coalesced to form large random sized aggregations (small white arrow). (d) Callose visualised after NLCP spray treatment followed by *H. arabidopsidis* Noks1 infection. Both hyphal strands with abundant haustorial callose (dark centred arrow) and callose spots of different sizes (small white arrow) could be observed. (e) Intense deposition of callose around hyphae (dark centred arrow) and callose spots (white arrow) could be observed after spray application of empimim followed by *H. arabidopsidis* Noks1 application. (f) Hyphal strands with callose and callose spots could be observed following empigen treatments but with reduced intensity when compared to (e). Scale bar equals to 260 μm.
Figure 4.12 Quantification of callose after abiotic and biotic stress. All the controls were treated with either water, NLCP or surfactants separately, while treatments groups were treated with water, NLCP or surfactants followed by *H. arabidopsis* Noks1 infection. Callose was extracted seven days after incubation and determined following extraction and quantitation. Untreated, NLCP and surfactant controls had similar amounts of callose while all the treatment groups sprayed with NLCP or surfactants and infected with *H. arabidopsis* Noks1 had similar amounts of callose. UTC = untreated control, ctrl = control, treat = treatment.
No significant differences could be established for callose production after various treatments.

4.4 Discussion

Traditionally agrochemical spray formulations that include surfactants have been extensively used in various agricultural practices to deliver systemically active ingredients into plants (Castro et al., 2013). However, intact epicuticular waxes that are solubilised by surfactants are critical for protecting the plant from abiotic (Riederer and Schreiber, 2001; Pfündel et al., 2007) and biotic stress (Neinhuis et al., 1992; Metraux et al., 2014). To test the effects of pre-treatment of leaves with either surfactants or NLCP, plants were exposed to an abiotic stress, UV-C light, and a biotic stress, infection by the biotrophic oomycete pathogen *H. arabidopsidis*. The impact of disruption of the cuticle by the surfactants or NLCP was then assessed by two sensitive measures, the production of callose and of the reactive oxygen species, hydrogen peroxide, within the epidermis and sub epidermal layers of the leaf. It was found that under the tested conditions NLCP sprays at 0.1% (v/v) induced stress responses in the dicot *L. angustifolius* which was much reduced when compared to that of surfactants after UV-C exposure. Novel image analysis techniques were simultaneously employed to quantify the visual data obtained for callose and validated the low impact of NLCP on leaves. The pathogenicity of *H. arabidopsidis* remained unaltered after NLCP and surfactant treatments.

Together with the results obtained for chapter 3 and chapter 4 for NLCP - plant interactions in the presence of UV it can be concluded that NLCP can deliver the agrochemical actives without subjecting the plant to heightened damage from either biotic or abiotic stresses in their environment.

For the UV treatments on *A. thaliana* at exposure levels of 250 and 500 J m⁻² s⁻¹, a sharp decrease in total leaf surface area of the plants was evident for NLCP and surfactant spray...
applications. It was likely that the UV treatments on *A. thaliana* triggered development changes that altered growth leading to a decrease in leaf area (Nogués and Baker, 2000; Kunz et al., 2006). Additionally, the controls treated with UV alone were exposed to a single abiotic stress whereas plants treated with UV followed by NLCP or surfactant sprays were exposed to two different abiotic stress factors. Thus care should be taken in interpreting the results for plants treated with both UV and NLCP or surfactants against plants treated with UV alone as plants respond differently to a combination of different stresses (Kunz et al., 2006; Mittler, 2006). It is also likely that for the combination of UV treatment plus spray applications that plants were primed for systemic acquired acclimation which negatively regulates the plant growth.

The results obtained for callose quantification in the plants treated with NLCP or surfactants and UV are in good agreement with the results obtained for leaf area measurements. For all the NLCP and surfactant treatments after 750 and 1000 J m\(^{-2}\) s\(^{-1}\), there was a sudden increase in amount of callose produced because UV alone induced severe stress response. Additionally, these plants were also stressed because of the cuticular damage induced by NLCP or surfactant spray applications leading to a sudden increase in the callose production. It was earlier shown for *A. thaliana* that UV treatments after 1000 J m\(^{-2}\) s\(^{-1}\), induced leaf curling, lesion formation and enhanced production of callose and lignin on the leaf surface five days after UV exposure (Mintoff et al., 2014). The trends obtained for callose image analysis were in good agreement with that of biochemical quantification. For empimin treatment after 1000 J m\(^{-2}\) s\(^{-1}\) a sudden decrease in the amount of callose was a consequence of surfactant induced cuticular damage and phytotoxicity leading to increased number of dead cells on the leaf of *A. thaliana*. Further, the results obtained for callose production in *A. thaliana* after NLCP and surfactant spray applications were coherent with phytotoxicity ratings after NLCP and surfactant treatments.
(Table 3.1 of chapter 3). It was shown that, NLCP applications at 0.1% were less phytotoxic when compared to that of surfactants. In the presence of UV these results clearly show that, NLCP unlike surfactants do not severely damage the cuticle and thus lead to a reduced NLCP induced callose production.

It was interesting to observe that UV, NLCP and surfactant treatments on *L. angustifolius* resulted in different stress response when weighed against *A. thaliana*. Unlike *A. thaliana*, *L. angustifolius* was resistant to all the UV doses and NLCP spray treatments and was further confirmed by biochemical assays and image analysis for callose production. Callose extracted after empigen treatments followed by UV irradiation at 500, 750 and 1000 J m\(^{-2}\) s\(^{-1}\), was shown to be greater than that of NLCP treatments. Similarly, all the tested doses of UV after empimin treatments were shown to elicit large amounts of callose than any other treatments on *L. angustifolius*. For *L. angustifolius* it was earlier shown (in chapter 3 of this thesis) that epicuticular wax solubilisation following NLCP spray applications of 0.1% (v/v) was less when compared to surfactant induced wax solubilisation (Nadiminti et al., 2013). Surfactants when tested at similar concentrations to that of NLCP resulted in total epicuticular wax solubilisation. The presence of comparatively intact epicuticular waxes on the surface of *L. angustifolius*, could have contributed for better surface reflection of UV radiation (termed as leaf specular reflectance) (Pfündel et al., 2007) on NLCP treated leaves leading to a reduction in callose stress response. The same explanation remains coherent for the absence of callose in the *L. angustifolius* leaves exposed solely to UV radiation (no NLCP or surfactant treatments).

It was further suggested that leaf specular reflectance is strongly dependent on the plant species (Grant, 1987; Pfündel et al., 2007) and it may hold true for the enhanced production of callose in the leaves of *A. thaliana*. It is widely accepted that *A. thaliana* leaf surface is devoid of

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epicuticular wax crystals (Jeffree, 2007) and was further shown in chapter 3 of this thesis (Nadiminti et al., 2013). However, for _A. thaliana_ callose production induced by leaf diffuse reflection originating by the diffusion of specular reflectance into deeper leaf tissue layers cannot be ruled out (Grant, 1987). In chapter 2, it was shown, that the epicuticular waxes on the phylloplane of _Z. mays_, show a special arrangement known syntopism (Barthlott et al., 1998). Syntopism and specular reflectance of _Z. mays_ leaves together with anatomical toughness (Dominy et al., 2008) should have contributed for UV reflectance and corresponding reduced callose production following NLCP and surfactant spray applications. Further, UV induced tolerance to different stress factors cannot be ruled out for _Z. mays_ (Nogués and Baker, 2000; Kunz et al., 2006). These observation were consistent with the hypothesis made earlier that, NLCP do not plasticise or change the physicochemical properties of the cuticle, rather adsorb on to hydrophobic plant surface (Dong et al., 2011; Chang et al., 2012). Whereas agrochemical surfactants were known to irreversibly damage the hydrophobic cuticle (Stock and Holloway, 1993) and change the physiochemical properties of plant surfaces (Castro et al., 2014).

Callose development was observed to be highly site specific and engineered spatial response toward abiotic and biotic stress factors was observed (Piršelová and Matušíková, 2013). A leading step towards quantifying the callose production from microscopy data, after the biotic stress was made by the development of software called “CalloseMeasurer” (Zhou et al., 2012). This software however needs proficiency in computational skills to run the algorithm developed in C/C++ within the Acapella™ framework. To overcome this problem a more robust and user friendly method using imageJ (Schneider et al., 2012) was employed to quantify callose from microscopy images after UV, NLCP and surfactant treatments. The
results obtained for image analysis were consistent and sometimes even sensitive than the existing biochemical quantification of callose. For *Z. mays*, the biochemical quantification of callose did not exhibit any definite trend but the results computed using image analysis for callose area exhibited a dose dependent response for UV 750 and 1000 J m\(^{-2}\) s\(^{-1}\) after NLCP and surfactant treatments.

The biochemical quantification of H\(_2\)O\(_2\) produced five days after UV, NLCP and surfactant treatments were not conclusive for observations made *A. thaliana, L. angustifolius* and *Z. mays*. For *A. thaliana* it may be possible to infer that, greater phytotoxicity and cuticular damage may have led to the production of higher amounts of H\(_2\)O\(_2\) compared to NLCP and empigen spray applications. Screening H\(_2\)O\(_2\) production from 0 to 48 hours post treatment may provide conclusive answers.

*H. arabidopsidis* Noks1 conidiospores were then inoculated on the leaves of *A. thaliana*, after drying the NLCP and surfactant sprays. Though there were differences in the number of conidophores per plant these differences failed to be consistent with callose assays (biochemical and image analysis). It was therefore clear that, none of the treatments interfered with the infection and growth of the pathogen. *H. arabidopsidis* conidiophores usually emerge through stomata and as the spray application of NLCP and surfactants damaged the stomatal apertures, there was a physical decrease in the number of conidiophores and the pathogen remained viable within the leaf tissue of *A. thaliana*. Extending, this study to investigate the interactions of necrotrophic fungi that interact with the phylloplane may be helpful.

Investigating the physiological stress response through the biochemical quantification and image analysis showed that NLCP do not stress the plant as commercial agrochemical surfactants. Clearly the abiotic stress response on the leaves of *L. angustifolius* and *Z. mays*
was limited after the use of NLCP. These results were coherent with earlier observations made in this thesis for epicuticular wax micromorphology. Biochemical and image analysis on callose production after NLCP and surfactant treatments was shown here for the first time. Together, NLCP unlike the agrochemical surfactants interact with the plant surface by adsorption and do not irreversibly damage the hydrophobic plant cuticle. Therefore, to further investigate the efficacy of NLCP to deliver agronomic actives, auxin herbicide 2,4-dichlorophenoxy acetic acid (2,4-D) may be tested.
Chapter 5: EFFICACY OF THE NANOSTRUCTURED LIQUID CRYSTALLINE PARTICLES FOR THE DELIVERY OF THE AUXIN HERBICIDE 2, 4-DICHLOOROPHENOXYACETIC ACID

As a part of Australian Research Council Linkage Project (Project LP0991494) field trials were conducted at Brookton (WA, Australia) by Kalyx Australia Pty Ltd (WA, Australia), at East Greenmount (QLD, Australia) by Andrew Somerville (QLD, Australia) and at Devonport (TAS, Australia) by Peracto Pty Ltd (TAS, Australia) under the supervision of Nufarm Australia Ltd (Laverton North, VIC, Australia). Although, similar results were obtained from all the three field sites, the data obtained from Kalyx Australia was the most complete and is drawn upon in this chapter. Data from Kalyx Australia was transformed to match this thesis format and is summarised in the Tables 5.2 and 5.3. For the data presented in figures 5.6, 5.7 and 5.8 sampling was personally made at the trial site located in Devonport (TAS, Australia) and further investigated as described in the methods section. Nadiminti designed and carried out all the other experiments, wrote the thesis chapter and prepared all the figures and tables.

5.1 Introduction

Herbicides are one of the most-used agrochemicals that are traditionally employed at various plant developmental stages for protecting the crop from weeds. It has been estimated that herbicide sales are $US 17 billion out of a total agrochemical sale of $85 billion (Green, 2014). There is increased demand for the use of herbicides globally with a 39% increase in demand over the last decade (Gianessi, 2013; Green, 2014). Herbicides are often applied as a spray formulation that usually contains an active ingredient amalgamated with surfactants, adjuvants and stabilisers. Examples of surfactants are alcohol ethoxylates and organo silicones and that of adjuvants are vegetable oils and humectants. The effects of surfactants, their mode of action,
off-target phytotoxicity and potential environmental pollution have been discussed in section 1.2 of this thesis. The active ingredient forms the functional part of the agrochemical that elicits the corresponding herbicidal effect. Some of the most commonly used active ingredients for the control of weeds are acetolactate synthase (ALS) inhibitors, Photosystem II inhibitors and synthetic auxins (Heap, 2014). Glyphosate is one of the popularly used examples of ALS inhibitors that act on the acetolactate synthase enzyme to inhibit the synthesis of branched chain amino acids (Zhou et al., 2007). Photosystem II inhibitors such as atrazine interfere with the fundamental processes of photosynthesis which are vital for the survival of plants (Heap, 2014). Synthetic auxin herbicides induce growth inhibition leading to senescence and tissue decay (Grossmann, 2010). One of the most commonly used auxin herbicides is 2,4-dichlorophenoxy acetic acid (2,4-D).

2,4-dichlorophenoxy acetic acid (2,4-D) closely resembles one of the naturally occurring phytohormones indole-3-acetic acid (IAA) (Fig 5.1). IAA controls various plant processes like cell division and elongation, embryo development, floral meristem differentiation and root development (Friml and Palme, 2002). Being structurally similar to IAA, 2,4-D also stimulates cellular and developmental processes making it a potential candidate for killing dicot weeds. 2,4-D when applied at the correct dose, disrupts the balance between the phytohormones ethylene and abscisic acid (ABA) leading plant to death (Grossmann, 2010). Unlike other active ingredients that target and block certain biochemical pathways, 2,4-D stimulates the plant systemically at both gene and cellular levels to disrupt the balance between cellular processes that quite rapidly kill the plant. Briefly, auxin response factors (ARFs), DNA binding transcriptional activator proteins, mediate the transcription of auxin-responsive genes. The ARFs bind to Aux/IAA transcriptional repressor proteins under conditions of low
concentrations of auxin, in-turn blocking transcription. Under high auxin concentrations Aux/IAA repressor proteins along with ARFs, bind to the F-box protein transport inhibition response 1 (TIR1). The binding of Aux/IAA proteins to F-box proteins is mediated by auxin that serves as a ‘molecular glue.’ The degradation of the Aux/IAA which is bound to F-box proteins then proceeds via the ubiquitin-proteasome pathway, leaving ARFs to activate auxin-response genes (Song, 2014). The continuous activation of auxin-responsive genes leads to stimulation of ethylene leading to lateral cell expansion, leaf abscission, growth abnormalities and senescence. Simultaneously, ABA largely accumulates in the plant leading to growth inhibition and stomatal closure. Stomatal closure further leads to inhibition of CO₂ assimilation and transpiration ultimately killing the plants that are sensitive to auxin herbicides (Grossmann, 2010; Song, 2014).

2,4-D was one of the first herbicides to be commercialised for the control of weeds (Song, 2014). The selective toxicity of 2,4-D against dicot weeds makes it an invaluable tool for the control of weeds in cereal crops. For enhancing the activity of 2,4-D against dicot weeds surfactants were demonstrated to be important. The use of 2,4-D in combination with an organosilicone surfactant was shown to increase the efficacy of 2,4-D from 84% to 90% (Sharma and Singh, 2001). Similary ethylene oxide surfactants containing a C₁₃/C₁₅ linear alkane and ethoxylated n-alcohols were shown to increase the penetration of 2,4-D into leaves of various plants (Liu, 2004) and isolated cuticle (Schönherr, 1993). Addition of surfactants for enhancing 2,4-D efficiency is a common agricultural practice. However, addition of a surfactants may not always enhance the activity of agrochemical active. For example addition of non-ionic surfactant (eoxylated oil) to 2,4-D spray formulation could not enhance the control of weeds 

Cyperus rotundus (Coco-grass) and Oxalis latifolia (Oxalis) (Devendra et al.,
As described in section 1.2 of this thesis it is important to note that the use of surfactants is often strongly associated with ecological effects like off-target phytotoxicity, contamination of water bodies and soil. Additionally, surfactant based agrochemicals often lead to phytotoxicity of crop plants, making them susceptible to secondary infections and biotic stress ultimately leading to yield loss. There is therefore, an urgent need for developing an alternate delivery mechanisms for 2,4-D that can enhance the efficacy of the auxin herbicide and prevent or reduce the use of surfactants.

In this chapter nanostructured liquid crystalline particle (NLCP) were used as an alternative to surfactant-based delivery of agrochemical actives. 2,4-D was loaded, at different concentrations onto the NLCP and its ability to kill the dicot weeds was tested against that of two surfactants in both laboratory-based studies and in field trials. *In-vitro* experiments were conducted on *A. thaliana* and *L. angustifolius* while field studies were performed to test the control of the weed *Raphanus raphanistrum* in the barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*) crops. In the field studies 2,4-D loaded NLCP yielded promising results, when tested against Estercide 800, a commercially available 2,4-D herbicidal formulation. When tested at low concentration, NLCP-mediated 2,4-D delivery resulted in an increase in crop yield of up to 15%. Interestingly, the crop phytotoxicity appeared to be directly proportional to yield losses. Estercide 800 appeared to be more phytotoxic and resulted in a yield loss of up to 16%. A direct correlation between crop phytotoxicity and yield loss was established supporting the use of NLCP as a potential agrochemical delivery vehicle.
Figure 5.1 Chemical structure and physical properties of 2,4-D. The chemical structure of 2,4-D is very similar to that of the natural auxin phytohormone indole-3-acetic acid (IAA). With a positively charged aromatic ring associated with a strong negatively charged carboxyl group 2,4-D is analogous to IAA. The physical properties of 2,4-D are also tabulated. The figure was prepared from data presented by Song (2014) and Liang et al., (2014).
5.2 Material and methods

5.2.1 In-vitro 2,4-D studies

As only dicot plant species are sensitive to 2,4-D Arabidopsis thaliana and L. angustifolius (lupin) were selected for the study. The growth and maintenance of the plants was performed as described in section 2.2.1.

5.2.1.1 Preparation of surfactants and NLCP for 2,4-D studies

NLCP were prepared as previously described in section 3.2.2 of this thesis. Briefly, 900 mg of phytantriol (Roche Products Pty Limited, Dee Why NSW 2099 Australia) was dispersed in 4.1 g of F127 (BASF Australia Ltd, Southbank VIC 3006 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 NLCPs. Particle size was characterized using a nanosizer Malvern NanoS (Malvern Instruments, Malvern, UK) at 25 °C. For preparation of herbicide-encapsulated cubosomes, 30 mg of 2,4 D ethylhexyl ester was added to 1 g of phytantriol and mixed using a roller mixer for at least one week prior to dispersion preparation. All dispersions were stored at ambient temperature for a minimum of two days prior to use.

The stock of phytantriol NLCP containing 3% 2,4-D was diluted to 0.01% (v/v) and 0.1% (v/v) in distilled water. The concentration of phytantriol for 0.01% (v/v), 0.1% (v/v) 2,4-D loaded NLCP was then calculated to be 0.059% (v/v) and 0.59% (v/v) respectively. 2,4-D ethyl hexyl ester, empimin and empigen provided by Nufarm Australia Limited were then diluted separately and mixed to match the concentrations of 2,4-D and phytantriol to obtain the following solutions; 0.01% (v/v) 2,4-D in 0.059% (v/v) empigen, 0.1% (v/v) 2,4-D in 0.59% (v/v) empigen, 0.01% (v/v) 2,4-D in 0.059% (v/v) empimin and 0.1% (v/v) 2,4-D in 0.59%
(v/v) empimin. These solutions were sprayed individually on to six, three-week-old arabidopsis plants and three, three-week-old *L. angustifolius* plants to obtain uniform coverage. Appropriate controls were setup for all the treatments and the experiment was repeated.

**5.1.1.2 Assessing phytotoxicity, and water content for various treatments**

For assessing phytotoxicity following treatment, plants were photographed at one and seven days after spraying. The photographs were then used to assess the phytotoxicity for each of the treatments as described in section 3.2.3 of this thesis with some modifications as described. Images were assessed for each treatment on a scale of 0 to 5, where a score of 0 = no observed phytotoxicity (healthy, undamaged leaves), 1 = trace phytotoxicity (wilting/leaf curling), 2 = minor phytotoxicity (minor tissue damage/reduction in plant size/intense leaf and stem curling), 3 = moderate phytotoxicity (coalesced patches of necrosis/wilting/heavy leaf and stem curling), 4 = high level phytotoxicity (extensive or intense tissue damage/pronounced wilting/leaf senescence) and 5 = severe phytotoxicity (complete necrosis/ death). For scoring phytotoxicity following treatments three blind assessments were conducted by independent assessors. Data obtained was subjected to statistical analysis separately for each plant species, using commercial software (IBM SPSS Version 21, IBM Australia Ltd., St Leonards NSW, Australia). As the data was normally distributed, Duncan’s test was used to test for significant differences between treatment groups.

To measure the water content, shoots of the treated plants were weighed immediately after harvesting and subsequently maintained at 70° C in an incubator for 5 days. After measuring the dry weight the water content of the samples was calculated using the formula: water content = (fresh weight – dry weight)/fresh weight × 100 (Maheswari et al., 2010).
5.2.2 Field trials

5.2.2.1 Field trials on *Triticum aestivum*

Field trials were performed during September 2011 to assess the efficacy of 2,4-D loaded NLCP to kill the invasive weed *Raphanus raphanistrum* (wild radish) in *Triticum aestivum* cv. STL (wheat) crop fields located close to Brookton (WA, Australia). Plots of 12 m × 2.5 m, were marked on the farmer sown crop. A randomized complete block design (RCB) with three replicates for each treatment was adopted for testing the formulations. Using a hand held boom sprayer 2,4-D NLCP were delivered to the crop at early booting stage (Zadoks growth stage 4.1 to 4.3) at concentrations of 0.03% (v/v), 0.06%(v/v), 0.13%(v/v), 0.19%(v/v) and 0.27%(v/v). The corresponding concentration of NLCP would respectively be 0.27%(v/v), 0.58%(v/v), 0.88%(v/v) and 1.30%(v/v). Commercially available 2,4-D (Estercide 800, Nufarm Australia Limited, VIC Australia) was applied as a control at equivalent concentrations to that of 2,4-D NLCP. The concentration of surfactant mixed was, however, unknown for the commercial formulation. Crop phytotoxicity and weed phytotoxicity was recorded 7 days, 19 days and 41 days after application. Crop and weed phytotoxicity was rated on a scale of 0 to 100 where 0 = No visible crop damage and 100 = Total necrosis or death. With respect to 7 days after application, the crop stage was subsequently recorded to be 50% emergence of inflorescence (Z55). For 19 days and 41 days after application of 2,4-D crop was observed to be at start of grain fill (Z71) and grain maturity (Z91) respectively. Though, all the stages of *R. raphanistrum* were observed at the time of 2,4-D application, most of the plants were observed to be at flowering and podding stage. The crop was harvested 82 days after application and the yield was expressed in tonnes/hectares (t/ha). All the data was subjected to Fisher’s least significant difference test (LSD).
5.2.2.2 Field trials on *Hordeum vulgare*

For field trials on a *Hordeum vulgare* crop all the parameters were kept constant except for the size of the plot was adjusted to 10 m × 2.5 m. The trial site was located near to Devonport (TAS, Australia) and the trial was performed between November 2011 to March 2012. On 20th December 2011, five *H. vulgare* plants were harvested, from each test plot 27 days after spray application, labelled and packed in zip lock bags. Packed plant material was then placed in cold bags that maintained a temperature between 4° C – 8° C during transportation to Deakin University (VIC, Australia).

5.2.2.3 Scanning electron microscopy of leaves of *H. vulgare*

The plant material collected from the field site was further processed for scanning electron microscopy as described in section 2.2.6 of this thesis. Image analysis was performed according to the protocol described in sections 2.2.7 and 2.3.4.2 of this thesis. Briefly, three images per treatment were randomly analysed using ImageJ version 1.46r (Schneider et al., 2012). The images were converted to 8 bit images before adjusting the threshold and despeckling noise. The total area of epicuticular waxes was subsequently calculated and statistically analysed with Duncan’s posthoc test (IBM SPSS Statistics 21, IBM Australia Ltd, St Leonards NSW, Australia).

5.3 Results

5.3.1 NLCP versus surfactant based 2,4-D delivery

For testing the herbicidal efficiency of 2,4-D NLCP on *A. thaliana* and *L. aungustifolius*, 2,4-D NLCP spray formulations were tested against 2,4-D alone mixed in water, 2,4-D and empimin mixed with water and 2,4-D and empigen mixed with water, at concentrations of 0.01%(v/v) and 0.1%(v/v). In each of the formulations the concentration of either NLCP or
surfactants was kept constant at 0.059% or 0.59%. 2,4-D diluted in water alone was used as a control. There was a significant difference in the effect of 2,4-D when sprayed in combination with either the NLCPs or surfactants. For *A. thaliana*, the effect of 2,4-D was evident 24 hours after application (HAA), with curled petioles and folded phylloplanes observed across all the tested formulations and concentrations. The petioles of *A. thaliana* were extended and leaf lamina folded to hide the abaxial surface. Leaf burning was an additional effect when 2,4-D was applied at a high concentration of 0.1% 2,4-D in combination with both surfactants while this was not exhibited by 2,4-D (in water) controls and 2,4-D NLCP treated plants.

Phytotoxicity symptoms were observed for all the spray applications with prominent differences seven days after application (DAA) (Fig 5.2). For the plants treated with 2,4-D alone at 0.01% there was a significant decrease in the size of the plant along with intense leaf and petiole curling effects (Fig 5.2 a and b). For application of 2,4-D at the higher concentration of 0.1%, it was noted that the plants were extremely wilted and symptoms like leaf ‘burning,’ and petiole and lamina curling were evident (Fig 5.2 c). 2,4-D NLCP treatments seven DAA were comparable with that of 2,4-D (in water) controls but the plant size appeared to be bigger after 0.01% 2,4-D NLCP application (Fig 5.2 e). Weighed against controls (2,4-D in water) at same concentration 0.1% 2,4-D NLCP applications on *A. thaliana* appeared to induce a greater amount of phytotoxicity (Fig 5.2 f). Among all the tested formulations, 0.01% 2,4-D when sprayed in combination with empimin was most phytotoxic to *A. thaliana* (Fig 5.2 h). Leaf senescence was distinguishable along with leaf burning, wilting and petiole/leaf curling. These effects intensified with an increased concentration of 2,4-D to 0.1% leading to plant death seven DAA (Fig 5.2 i). The impact of 2,4-D empigen seven DAA was comparable to that of empimin at 0.01% and 0.1% 2,4-D (Fig 5.2 k and l).
For *L. angustifolius* all the treatments at 0.01% had stem curling 24 HAA which remained persistent even after seven DAA but with the additional feature of stubbing of shoot tips (Fig 5.3). Leaf burning could be visualised on plants treated with 2,4-D in combination with surfactants (Fig 5.3 h and k). Seven DAA 2,4-D treatments stem curling, stubbing of shoot tip remained persistent on all the treated plants (Fig 5.3). However, the 2,4-D surfactant treated plants elicited leaf burning along with earlier phytotoxicity symptoms (Fig 5.3 g to h).

The phytotoxicity ratings were consistent with the observations earlier made for *A.thaliana* and *L. angustifolius* spray applications (Table 5.1). For 0.01% 2,4-D spray treatment all the blind scores were statatistically similar to each other on *A. thaliana* 24 HAA. But after an incubation for seven days, 0.01% 2,4-D delivery using surfactants were rated to be most phytotoxic and were significantly different from that of 2,4-D NLCP delivery and controls. Blind scores for 0.01% 2,4-D NLCP were rated to be less phytotoxic weighed against surfactants and are followed by 0.01% 2,4-D (in water) controls (Table 5.1). However, for higher concentrations of 0.1% 2,4-D empimin delivery alone appeared to be significantly different when juxtaposed against other treatments 24 HAA (Table 5.1). For 0.1% 2,4-D, it is very interesting to note that no differences between surfactant and NLCP based treatments seven DAA were observed and further the data was statistically similar. This indicates that the efficiency of NLCP for the delivery of 2,4-D was comparable to that of surfactants. Among all the tested formulations on both *A. thaliana* and *L. angustifolius* 0.1% 2,4-D empimin was rated to be most phytotoxic seven DAA, (Table 5.1).

**5.3.2 Physiological effects of 2,4-D delivery using NLCP and surfactants**

Seven days after spray application of 2,4-D NLCP and surfactants the plants were harvested to record fresh weight and dry weight and to determine the water content of the plants. Water
content can be used as a direct measure of the physiological status of the plants under investigation (Jolly et al., 2014; Yang et al., 2014). For *A. thaliana* a direct correlation for the fresh weight measured and amount of 2,4-D applied was observed (Fig 5.4). All the controls had the highest fresh weight which progressively decreased with the increase in the concentration of the 2,4-D. Plants treated with 2,4-D NLCP at 0.01% had a fresh weight which is similar to the controls treated with 0.01% 2,4-D but are different from 0.01% 2,4-D surfactant treatments. Conversely, the fresh weights of the plants treated with 0.1% 2,4-D NLCP and 0.1% 2,4-D surfactants were statistically similar (Fig 5.4 a). The results scaled for dry weights of *A. thaliana* plants correlated with that of fresh weights, where controls recorded the highest weight, which progressively decreased with increase in concentration of applied 2,4-D (Fig 5.4 c). All the other treatments had similar dry weights except for 0.1% 2,4-D. Water content percentage also followed the similar trend observed for fresh and dry weights. However, 0.01% of 2,4-D NLCP treated plants were observed to have a water content not statistically different to that of untreated controls. Surprisingly 0.1% 2,4-D NLCP treated plants were observed to have a significantly higher amount of water content in comparison to similar surfactant treatments (Fig 5.4 e).

The fresh and dry weights of *L. angustifolius* recorded for untreated control and group controls (NLCP control, empimin control and empigen control) were measured to have significantly higher weights compared with the other treatments. All the other fresh and dry weights scaled for *L. angustifolius* did not show any significant differences for the various treatments (Fig 5.4 b and d). It was however, interesting to observe that the percentage of water content for all the treatments was similar to that of controls, except for 0.1% empimin 2,4-D treatment (Fig 5.4 f).
Chapter 5 Efficacy of nanostructured liquid crystalline particles for the delivery of 2,4-D
Figure 5.2 The impact of auxin herbicide 2,4-D on *A. thaliana* seven days after application. (a) Untreated control. (b) Juxtaposed against controls, plants sprayed with 0.01% 2,4-D (in water) showed a drastic change in morphological features characterised by curled petioles and lamina. (c) Severe wilting and reduction in plant size could be observed after 0.1% 2,4-D treatment (d) Plants were sprayed with 0.1% NLCP spray solution that did not contain 2,4-D. (e) Though 0.01% 2,4-D NLCP treatment induced severe morphological effect, the plants were bigger and less affected when compared to (b), petiole curling and lamina folding was consistent. (f) 0.1% 2,4-D NLCP has almost killed the plants and were more effective in comparison to that of (e). (g) Empimin control, 0.1% empimin (diluted in water) was sprayed to plants (h) 0.01% 2,4-D in empimin was more effective compared to that of (b and e) and plants showed extreme phytotoxicity symptoms and appeared to be dead. (i) Plants were almost dead after the use of 0.1% 2,4-D in empimin. (j) For empigen control, empigen was diluted in water and sprayed at a final concentration of 0.1% (k) The effect of 0.01% 2,4-D in empigen was very comparable to that of empimin (h). (l) 0.1% 2,4-D in empigen elicited severe phytotoxicity that appeared similar to (i). Scale bar is 1 cm.
Chapter 5 Efficacy of nanostructured liquid crystalline particles for the delivery of 2,4-D
Figure 5.3 Herbicidal effect of 2,4-D on *L. angustifolius*. 2,4-D was delivered to the plant surface in combination with NLCP, empimin and empigen and photographed seven days after spray application. (a) Untreated control of *L. angustifolius*. (b) 2,4-D diluted in water sprayed at a concentration of 0.01% resulted in stem curling. (c) 0.1% of water diluted 2,4-D spray, lead to sever curling of the stem and stubbing of the shoot tip. (d) NLCP diluted to 0.1% with water sprayed to plant. (e and f) 0.01% and 0.1% 2,4-D NLCP did not kill the plants, phytotoxicity appeared to be very similar to that of (b and c). Stem curling and stubbing of shoot tip are evident. (g) Empimin control sprayed with 0.1% empimin. (h) 0.01% 2,4-D sprayed with empimin showed sever phytotoxicity effect on the leaves, stem curling and stubbing of the shoot tip is evident. (i) 0.1% 2,4-D showed intense phytotoxicity effects on the plants that included leaf burning and stem curling. This treatment appeared to be most phytotoxic when compared to all the other treatments. (j) Empigen control treated with 0.1% empigen in water. (k) 0.01% is less phytotoxic to that of empimin and phytotoxicity effects were similar to (b and e). (l) Though 0.1% 2,4-D shows leaf burning and stem curling the intensity is much reduced when juxtaposed against (i). Scale bar is 4 cm.

Table 5.1 Phytotoxicity scores on *A. thaliana* and *L. angustifolius* ranging between 0 to 5 were given to plants treated with 2,4-D using NLCP and traditional surfactants. After the analysis of variance (ANOVA) significant differences were determined through Duncan’s test. Means followed by the same letters are not statistically significant. For 0.01% 2,4-D treatments, all the day 1 observations were similar. Day 7 observations after 0.01% surfactant based 2,4-D delivery were significantly different from all the other groups. It was observed that NLCP, empimin and empigen 0.1% 2,4-D 7 DAA were suffixed by same letter indicating significant similarity between the groups. 2,4-D control, NLCP and empigen 0.1% 2,4-D day one
treatments on *A. thaliana* were similar. *L. angustifolius* was however resistant to 2,4-D formulations. DAA = days after application.

<table>
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<th>Plant species</th>
<th>DAA</th>
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<th>0.01% 2,4-D</th>
<th>0.10% 2,4-D</th>
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<td>1.42 ± 0.31</td>
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<td>3.33 ± 0.28</td>
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<tr>
<td></td>
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<td>1.83 ± 0.31</td>
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<td>7</td>
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<td></td>
<td>7</td>
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</table>
5.3.3 Evaluation of crop phytotoxicity after the use of 2,4-D NLCP

In *T. aestivum* crop fields NLCP 2,4-D formulation was tested against the commercially available auxin herbicide Estercide 800 at similar concentrations for the control of the weed *R. raphanistrum*. Following the application of 2,4-D at different concentrations for both 2,4-D NCLP and Estercide 800 separately, the crop phytotoxicity was recorded 7, 19, 41 and 82 DAA (Table 5.2 a). For Estercide 800, the crop phytotoxicity was observed to be independent of concentration of the applied formulation seven DAA (Table 5.2 b) and ranged from 1.3 to 5.3. Except for 0.27% estercide treatment, crop phytotoxicity symptoms were not observed after 19 and 41 DAA (of 2,4-D in Estercide 800) (Table 5.2 b) and additionally the crop height also appeared to be decreased when compared to other concentrations of Estercide treatments (Table 5.2 b). The crop phytotoxicity observations remained consistent even after 41 DAA and were statistically significant. Weighed against untreated controls, the crop yield after Estercide 800 treatments was observed to be in the range of 3.3% to 10.1% for the tested concentration range of 0.03% - 0.13% (Table 5.2 c). Further increase in Estercide concentrations to 0.19% and 0.27% resulted in sharp decrease in crop yield. The loss of crop yield was calculated to be 16% at the highest tested concentration of Estercide (0.27%) (Table 5.2 c). Unlike Estercide 800, 2,4-D NLCP spray formulation appeared to show a dose dependent crop phytotoxicity (Table 5.2 b). For 19 and 41 DAA the phytotoxicity ranged from 0.0 to 13.3 and 0.0 to 10.0 respectively. Though the crop phytotoxicity remained persistent up to 41 DAA, a decrease in phytotoxicity was observed along with the crop age. Plants exposed to a lower dose of 0.03% (2,4-D) could completely recover from any phytotoxicity (Table 5.2 b). Though an increase in crop phytotoxicity was observed 19 DAA at higher concentrations (0.19% and 0.27%), it gradually decreased with further crop age. It was very interesting to note the contrasting differences in the yield percentages for 2,4-D NLCP treatments when compared to untreated
controls. There was a 15.5% increase in yield for 0.03% treatments which reduced to 11.3% with the increase in 2,4-D concentration to 0.13% (Table 5.2 c). Similar to Estercide 800, further increase in concentrations of NLCP 2,4-D to 0.19% and 0.27% induced yield loss. However, the yield loss after 0.27% NLCP 2,4-D was calculated only to be 6.3% compared to 16% loss for 0.27% Estercide 800 (Table 5.2 c).

5.3.4 The control of invasive weed R. raphanistrum with 2,4-D NLCP

Control of R. raphanistrum in the T. aestivum crop following spray application of the auxin herbicide showed different patterns for Estercide 800 and 2,4-D NLCP (Table 5.3). For Estercide 800 seven DAA, the phytotoxicity of R. raphanistrum appeared to be dose-dependent for the concentrations ranging from 0.03% to 0.13%. A further increase in concentration of 2,4-D Estercide to 0.19% and 0.27% failed to exhibit a dose-dependent phytotoxicity response (Table 5.3 b). The observations recorded for phytotoxicity of R. raphanistrum progressively increased for 19 and 41 DAA, and they followed the trend earlier observed for 7 DAA (Table 5.3 b). Under the tested concentrations, it is interesting to note that the control of R. raphanistrum numbers appeared to be optimal at 0.13% Estercide, with a control percentage of 74.6% (Table 5.3 c). The control of the weed was limited only to 64.8% and 46.8% with further increase in concentration of Estercide to 0.19% and 0.27% respectively. The total number of R. raphanistrum counted per plot correlated back to the phytotoxicity observations made for 7, 19 and 41 DAA (Table 5.3 b). The number of seed podding and non-podding R. raphanistrum were consistent with the trend earlier observed for weed phytotoxicity and weed counts (Table 5.3 c).

The spray application of 2,4-D NLCP also resulted in a dose-dependent response with high toxicity observed for the applied concentration of 0.27% 2,4-D. The lower doses of 2,4-D
Chapter 5 Efficacy of nanostructured liquid crystalline particles for the delivery of 2,4-D
Figure 5.4 The effect of 2,4-D, delivered using NLCPs or surfactant on the biomass of *A. thaliana* (column on left) and *L. angustifolius* (column on right). (a) The fresh weight of *A. thaliana* shoots after 2,4-D delivery using NLCP 0.1% was found to be similar to that of empimine 2,4-D and empigen 2,4-D delivery at similar concentrations. (b) *L. angustifolius* fresh weight measurements made after 2,4-D NLCP treatments were similar to 2,4-D treatments with empigen and empimine at 0.01% and 0.1%. (c) The dry weights of *A. thaliana* after NLCP and surfactant treatments were similar. (d) *L. angustifolius* dry weights after various treatments are similar to each other when compared to respective controls. (e) In comparison to controls the amount of water in the shoots of *A. thaliana* decreased with increase in concentration of 2,4-D across the treatments. (f) Water content for all the treatments was similar except for empimine 0.1% 2,4-D treatment. Columns in the graphs indicated by the same letters are not statistically different (Duncan’s posthoc test).
Table 5.2 Assessment of crop phytotoxicity after the spray application of 2,4-D NLCP in comparison with Estercide 800. The initial crop phytotoxicity observed 7 DA-A for both Estercide 800 and 2,4-D NLCP was lost along the crop development when applied at low concentrations. At higher concentration, crop plants applied with 2,4-D NLCP showed a dose dependent increase in crop phytotoxicity, which persisted till the harvest. The crop yield however remains unaffected for the crop plants applied with 2,4-D NLCP, indicating the potential use NLCP as agrochemical delivery vehicle. Statistical analysis was separately carried out for tables (b) and (c). Means followed by same letter are not statistically significant (P=.05, LSD); UTC = Untreated control; DAA = Days after application.
### Chapter 5 Efficacy of nanostructured liquid crystalline particles for the delivery of 2,4-D

<table>
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<tr>
<th>Treatment</th>
<th>Concentration</th>
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<th>Crop height reduction (0-100)</th>
<th>Yield 82 DA-A</th>
<th>% difference in yield against UTC</th>
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</table>
Table 5.3 Effect of 2,4-D NLCP and Estercide 800 on the control of the invasive weed *R. raphanistrum*. The phytoxicity towards the weed progressively increased along with the crop age. For Estercide 800 and 2,4-D NLCP there was higher control of weed numbers when applied between 0.03% to 0.13%. However a dose dependent trend was not observed at higher concentrations. Lower concentrations of 2,4-D NLCP appeared to achieve better control of the weeds in comparison to higher concentrations. Total and podding wild radish numbers were not significantly different for both Estercide 800 and 2,4-D NLCP spray applications. Statistical analysis was separately carried out for tables (b), (c) and (d). Means followed by the same letter are not statistically significant (P=.05, LSD); UTC = Untreated control; DAA = Days after application.
### Chapter 5 Efficacy of nanostructured liquid crystalline particles for the delivery of 2,4-D

#### Table 5.1: Efficacy of Nanostructured Liquid Crystalline Particles for the Delivery of 2,4-D

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>R. raphanistrum control (0-100)</th>
<th>Total R. raphanistrum counts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Commercial 2,4-D (Esterase 800)</td>
<td>41 DA-A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#/plot</td>
</tr>
<tr>
<td>UTC</td>
<td>0.00%</td>
<td>7 DA-A 19 DA-A 41 DA-A</td>
<td>33.7a</td>
</tr>
<tr>
<td>Esterase 800</td>
<td>0.03%</td>
<td>10.0a 23.3b 30.0c</td>
<td>18.7b</td>
</tr>
<tr>
<td>Esterase 800</td>
<td>0.06%</td>
<td>13.3a 23.3d 36.7e</td>
<td>17.3be 49.5bc</td>
</tr>
<tr>
<td>Esterase 800</td>
<td>0.13%</td>
<td>20.0a 43.3b 63.3bc</td>
<td>8.3b 74.6bc</td>
</tr>
<tr>
<td>Esterase 800</td>
<td>0.19%</td>
<td>20.0a 46.7bc 63.3bc</td>
<td>11.7bd 64.8bcd</td>
</tr>
<tr>
<td>Esterase 800</td>
<td>0.27%</td>
<td>16.7a 43.3b 90.0b</td>
<td>16.3be 46.8be</td>
</tr>
<tr>
<td>2,4-D Phytoecidol</td>
<td>0.03%</td>
<td>16.7a 26.7bd 36.7e</td>
<td>12.7b 58.3bcd</td>
</tr>
<tr>
<td>NLCP</td>
<td>0.00%</td>
<td>23.3a 33.3bcd 43.3fg</td>
<td>10.3bcd 68.2abc</td>
</tr>
<tr>
<td>NLCP</td>
<td>0.03%</td>
<td>20.0a 36.7bcd 53.3bcd</td>
<td>11.3bcd 63.6bcd</td>
</tr>
<tr>
<td>NLCP</td>
<td>0.13%</td>
<td>20.0a 45.0bc 73.3cd</td>
<td>7.7bcd 76.8ab</td>
</tr>
<tr>
<td>NLCP</td>
<td>0.19%</td>
<td>25.3a 55.0b 76.2abc</td>
<td>8.0bcd 75.7ab</td>
</tr>
<tr>
<td>NLCP</td>
<td>0.27%</td>
<td>6.3a 9.5a 12.4b</td>
<td>6.5a 18.5</td>
</tr>
</tbody>
</table>

*Note: Values followed by different letters indicate statistically significant differences.*

---

*Chapter 5 Efficacy of nanostructured liquid crystalline particles for the delivery of 2,4-D*
NLCP, 0.03% and 0.06%, induced greater phytotoxicity when compared to similar concentrations of Estercide 800. The observed phytotoxicity did not, however, exhibit a dose dependent increase for the concentrations of 0.19% and 0.27% (Table 5.3 b). For seven DAA, though there was a slight increase in phytotoxicity after treatment with 0.27% 2,4-D NLCP, when juxtaposed against Estercide 800 all the treatments appeared to be statistically similar. All the observations made at 19 and 41 DAA were consistent with that of 7 DAA (Table 5.3 b). It appeared that 2,4-D NLCP had better efficiency of control of the weed numbers per plot at the tested concentrations. At low concentrations, 0.03% 2,4-D NLCP, almost 58% of the weed was controlled and is higher by 11% when compared to Estercide 800 (Table 5.3 c). Similarly, 0.06%, 0.19% and 0.27% 2,4-D NLCP showed an increase in control of weed numbers by 18.7%, 12.0% and 28.9% respectively. However, it was surprising to note that for 0.13% 2,4-D NLCP efficiency was reduced by 11.0% compared to that of Estercide tested at similar concentration (Table 5.3 c). Podding counts of *R. raphanistrum* for each plot after the various treatments were not statistically different from the observations made for Estercide 800 (Table 5.3 d). The observations made for phytotoxicity 7, 19 and 41 DAA, were reflected in the level of podding of *R. raphanistrum* for each plot.

5.3.5 Epicuticular wax micromorphology after 2,4-D NLCP treatments in the field

In order to assess the impact that treatment with NLCPs had on the leaves of the crop plants leaf surfaces were observed under the SEM after the spray application of 2,4-D NLCP. The epicuticular wax plates appeared to be fused consistently giving rise to irregular and random arrangements (Fig 5.5 a). The epicuticular wax plates were identified as crystalloids and can be assigned to the sub-group of irregular crenate platelets. For untreated controls, there was no observed epicuticular wax solubilisation. Similar to the earlier observations after 2,4-D NLCP
spray application, a dose dependent wax solubilisation was evident on the phylloplane of *H. vulgare*. After the application of 0.03% 2,4-D NLCP (27 DAA) a moderate wax solubilisation was recorded which increased with increase in the concentration of 2,4-D (Fig 5.5 b). For 0.27% 2,4-D total epicuticular wax solubilisation was evident (Fig 5.5 f). Image analysis was further performed on the SEM images obtained for *H. vulgare* to quantify the epicuticular wax solubilisation. It was observed that the area of epicuticular waxes was highest for untreated controls and significantly decreased with increase in the concentration of spray application (Fig 5.6). It was further interesting to observe disparities in the spray application of 2,4-D NLCP (Fig 5.7). From the experience of observing epicuticular wax solubilisation after surfactant sprays a uniform effect on the epicuticular waxes was expected. But, clear, isolated areas of wax solubilisation was observed for 0.06% and 0.13% 2,4-D NLCP applications. These isolated areas had epicuticular waxes totally solubilised leaving them contrastingly different from surrounding epicuticular waxes (Fig 5.7).
Chapter 5 Efficacy of nanostructured liquid crystalline particles for the delivery of 2,4-D
Figure 5.5 Wax solubilisation after the use of 2,4-D NLCP on *H. vulgare* in a field trial. For the evaluation of leaf damage on *H. vulgare*, leaf samples of the treated plants were imaged under SEM. (a) The arrows show the epicuticular wax crystals identified as irregular crenate platelets. The arrows on all the other images show the solubilisation of crenate platelets with the increase in the concentration of the 2,4-D and phytantriol (phyt) from (a) 0.03% 2,4-D (0.13% phyt) to (f) 0.27% 2,4-D (1.30% phyt). Wax micromorphology was considerably altered across the range of concentrations tested, as shown from (a) to (f). Scale bar is 1.7 μm.

Figure 5.6 Area of epicuticular waxes after NLCP 2,4-D spray application on *H. vulgare*. The area of the wax micromorphology was calculated on SEM images collected for the *H. vulgare* leaves after the spray treatment of the NLCP 2,4-D formulation. Area of epicuticular waxes reduced with increase in concentration of the spray application as proceeded from untreated control (UTC) to 0.27% 2,4-D. Letter on each column indicate significantly different homogenous subsets tested for Duncan’s posthoc tests after analysis of variance (ANOVA).
Figure 5.7 NLCP spray distribution on the surface of *H. vulgare*. The SEM images show the uneven spray distribution of the NLCP spray formulation at (a) 0.06% (0.27% phyt) and (b) 0.13% (0.58% phyt). A clear difference in the wax micromorphology is indicated by the arrows that show the wax solubilisation regions limited to the area of contact of spray formulation with the leaf surface. Scale bar is 13 μm for (a) and (b).
5.4 Discussion

Traditionally, agrochemicals have been used at various stages of crop development to control invasive weeds and pests. Most often agrochemicals are mixed with surfactants that improve the efficacy of the active ingredient and further to assist the dispersion and penetration of the spray formulation (Chitband et al., 2013; Castro et al., 2014). The use of surfactants is strongly associated with phytotoxicity, off target effects and environmental pollution (Chuah et al., 2013; Nagy and Deák, 2013; Yilmaz and Dane, 2013). To overcome the problems posed by surfactant-based agrochemical spray formulations, nanostructured liquid crystalline particles have been put forward as an alternative to deliver agrochemicals (Dong et al., 2011; Chang et al., 2012). Therefore, in this study the efficiency of NLCP to deliver the auxin herbicide 2,4-D was tested in comparison with a commercially available auxin herbicide formulation that is known to contain a surfactant. In-vitro studies on *A. thaliana* and *L. angustifolius* showed that the performance of 2,4-D NLCP at 0.1% was similar to that of surfactant based 2,4-D delivery. The field studies on *T. aestivum* and *H. vulgare* have now also indicated the potential of 2,4-D NLCP as a future herbicidal spray formulation. In the field, the 2,4-D NLCP formulation was effective in the control of the weed *R. raphanistrum*, when applied at a low concentration of 0.03% and 0.06% when compared against Estercide 800. Further, the yield was increased up to 15% at the tested lower concentrations. At higher concentrations, the control of the weed was not any more effective than the commercial surfactant-based herbicide formulation. NLCP herbicidal delivery is a step forward in the science of agrochemical delivery to crop plants by eliminating the risk of environmental pollution while efficiently delivering active ingredients to crop plants.
The *in-vitro* studies conducted on *A. thaliana* clearly showed a dose dependent response to 2,4-D spray formulations. The petiole curling and lamina folding can be explained as a consequence of the hormonal effect of 2,4-D when tested at low concentration of 0.01% 2,4-D NLCP (Qin et al., 2005; Lim et al., 2007). It should be noted that the spray application of 2,4-D was performed on the adaxial leaf surface where 2,4-D application lead to proliferation of adaxial cells of the phylloplane in comparison with cells on the abaxial surface. This difference in the rate of cell division and therefore leaf expansion ultimately leads to the development of epinastic leaves and curling of the plant (Qin et al., 2005; Song, 2014). The *A. thaliana* plants treated with 0.01% 2,4-D NLCP and 2,4-D by itself appeared much greener compared to their counterparts treated with surfactants. This difference may be explained as a consequence of a delay in leaf senescence due to the hormonal effects of auxin (Lim et al., 2007; Grossmann, 2010). The death of *A. thaliana* plants in all other treatments was a result 2,4-D herbicidal effects that involve tissue decay, growth inhibition, inhibition of CO\textsubscript{2} assimilation and transpiration and over production of hydrogen peroxide and hydrogen cyanide (Grossmann, 2010; Song, 2014). Though auxins play an important role in the development of plants it is not clear how different plants confer resistance to auxin herbicides (McSteen, 2010). This holds true for the lack of herbicidal effects on *L. angustifolius* after spray application of 2,4-D formulations. However, the observed stem curling and stunting of the shoot tips may be a consequence of disruption of phytohormone homeostasis in shoot tips (Ljung et al., 2001).

The physiological performance of *A. thaliana* and *L. angustifolius* following treatments could be described by measuring fresh weight, dry weights and total water content (Jolly et al., 2014; Yang et al., 2014). For *A. thaliana* fresh weight and total water content for 0.01% 2,4-D NLCP treatment appeared to be similar with that of controls treated with 2,4-D 0.01% and 0.1% (in
water) treatments, did not show significant differences for dry weight. These results may indicate that the total cell mass remained constant across various treatments. Further, due to rapid cell expansion the cell growth was characterised by amount of water accumulated rather than cell content, which is termed as tissue swelling (Grossmann, 2003). Though the visual symptoms and phytotoxicity rating indicate severe change in the morphology of the *A. thaliana* plants when applied with 0.01%2,4-D NLCP, they appeared to be less effective when weighed against similar surfactant based treatments. Phytotoxicity ratings and water content suggest that *A. thaliana* can resist the herbicidal activity of 2,4-D NCLP when applied at low concentrations of 0.01%. It was earlier found that *Sinapis spp* (Wild mustard) exhibited 10 to 124 fold resistance to different auxin herbicides (Mithila et al., 2011). It is widely discussed that auxin herbicide resistance may be conferred via multiple pathways (Bayley et al., 1992; Deshpande and Hall, 2000) and further, it is not completely understood (Grossmann, 2010; Song, 2014). Therefore, *A. thaliana* being a *Brassicaceae* family member like *Sinapis spp* may have elicited mild resistance via multiple pathways to very low concentrations of externally applied 2,4-D.

The amount of 2,4-D delivered systemically can be comparatively different for 2,4-D delivered via NLCP and commercial surfactants because of the way in which the NLCP formulations are made. However, the efficiency of NLCP to adsorb to hydrophobic biorelevant surfaces, earlier shown by Chang et al., (2012) and Dong et al., (2011), rules out the possibility of weak interaction between NLCP and plant surfaces. The packing of 2,4-D and formation of cubosomes after high energy dispersion was stabilised by pluronic F127 which is attached on the surface of NLCP (Dong et al., 2012). Further, 2,4-D as an ethylhexylester is hydrophobic and is insoluble in water, which may also contribute towards tight packing either within the NLCP or on the surface of NLCP which are made up of phytantriol, a synthetic lipid (Rizwan...
et al., 2007). Thus, the number of 2,4-D molecules reaching the phylloplane may actually be higher in comparison to surfactant-based formulations at similar concentration. Precise investigation for evaluating the packing efficiency of 2,4-D in NLCP, 2,4-D molecules reaching the plant surface and 2,4-D molecules delivered systemically into the plant surface may provide further insights into the efficiency of NLCP applied at low concentrations. Evaluating *A. thaliana* for an extended period of time may also provide greater insights into the mode of action of NLCPs. On the other hand, with surfactants cuticular plasticising effects coupled (Rutter et al., 1990) with modification of physicochemical properties of both active ingredient and cuticle (Stock and Holloway, 1993; Castro et al., 2014) may have increased the number of 2,4-D molecules reaching the plant systemically when applied at low concentrations of 0.01%. It should, however, be noted that damage to epicuticular waxes is irreversible (Tamura et al., 2001) and damage to the cuticle makes the plant more susceptible to both biotic and abiotic stress (Castro et al., 2014). For 0.1% 2,4-D NLCP, the herbicidal effects were in good agreement with similar treatments made with surfactants. Increased numbers of 2,4-D molecules loaded on to NLCP, reaching the surface of the phylloplane may have contributed to enhanced systemic delivery of 2,4-D resulting in herbicidal activity that is similar to that of surfactant-based 2,4-D delivery.

For Estercide 800, a commercially available auxin herbicide, it is likely that the formulation is amalgamated with surfactants and adjuvants, though due to commercial in confidence considerations, their amounts remained unknown. It is therefore plausible to attribute the observed phytotoxicity to 2,4-D and the surfactants and adjuvants (Tamura et al., 2001; Yilmaz and Dane, 2013). SEM observations revealed that *T. aestivum* and *H. vulgare* had a similar epicuticular wax micromorphology characteristic of the Poaceae family and they also belong
to the same Triticeae tribe (Barthlott et al., 1998; Jeffree, 2007). Further, the phytotoxicity observations on *T. aestivum* were in agreement with epicuticular wax solubilisation observed for *H. vulgare*, meaning a dose dependent wax damage was recorded for Poaceae members. The phytotoxic response observed for both *T. aestivum* and *H. vulgare*, after the use of 0.3% 2,4-D NLCP was unexpected as this formulation contained phytantriol of only 0.13%. Earlier investigations carried out on the phylloplane of *T. aestivum* (as discussed in sections 3.3.1.1 and 3.3.2 of this thesis) showed no signs of phytotoxicity or change in epicuticular wax micromorphology even after application of 1.0% NCLP (without 2,4-D). Therefore, the effect of 2,4-D alone on phytotoxicity and epicuticular wax micromorphology cannot be ruled out for both Esterce 800 and NLCP applications on the plant species tested. Investigating the effect of 2,4-D for damage of the epicuticular wax micromorphology of crop plants can be conclusive. Though the crop phytotoxicity appeared to be higher for 2,4-D NLCP spray applications, a better control of weed *R. raphanistrum* was achieved after 41 DAA. Weed control for Esterce 800 and 2,4-D NLCP ranged between 46.9% to 74.6% and 58.3% to 76.8% respectively. The better performance of 2,4-D NLCP can be a consequence of loading 2,4-D on NLCP resulting in delivery of a greater number of 2,4-D molecules per unit area. These results were in agreement with the yield percentage, which was again higher for the *T. aestivum* crop treated with 2,4-D NLCP. Treatment with 0.03% 2,4-D NLCP showed a yield improvement of almost 15% above that of untreated controls and yield percentages decreased with increase in crop phytotoxicity. Decrease in crop phytotoxicity and reduction in the competition of invasive weeds is a likely reason for the increase in yield observed. It was clear that an increase in phytotoxicity led to reduced yields.
In conclusion, 2,4-D NLCP were tested both in-vitro and in field trials. For in-vitro studies *A. thaliana* and *L. angustifolius* were screened for the herbicidal effects after spray application of 2,4-D NLCP. It appeared that at the higher concentration tested (0.1%) the performance of 2,4-D NLCP to kill *A. thaliana* was very similar to that of surfactant-based agrochemical delivery. However, *L. angustifolius* remained resistant to the herbicidal effects of 2,4-D. Field trials were conducted to observe the herbicidal effects of 2,4-D NLCP on *R. raphanistrum* and showed that greater control of the weed could be achieved when 2,4-D NLCP were applied at 0.03% and 0.06%. At similar concentrations, crop phytotoxicity was observed to be reduced when compared to the commercial herbicidal formulation. The combined effect of reduced crop phytotoxicity and controlled weed numbers lead to improvement of yield by up to 15%. The delivery of 2,4-D via NLCP was promising to be a potential alternative delivery strategy. I propose that employing NLCP for agrochemical delivery can eliminate the use of toxic chemicals like adjuvants and surfactants.
Chapter 6: GENERAL DISCUSSION

6.1 Summary

Research presented in this thesis has investigated nanostructured liquid crystalline particles (NLCP) as a novel alternative to classical surfactant-based delivery systems. Surfactants are one of the principle components of agrochemical spray formulations, yet they are responsible for changing the physicochemical properties of both the agrochemical active and the plant surfaces to which they are applied. In particular, in the course of their action surfactants irreversibly alter the cuticle enhancing susceptibility to abiotic and biotic stress. To overcome this problem NLCP were investigated as an alternative to surfactants. NLCP act by adsorbing onto the plant surface, interacting with the cuticle and then delivering the agrochemical active.

The epicuticular wax layer forms the outermost layer of the plant cuticle and is the first cuticular layer that comes in direct contact with the NLCP. For *T. aestivum*, *Z. mays* and *L. angustifolius* NLCP were shown to interact with this layer without altering the epicuticular wax micromorphology. Unlike surfactants, the NLCP formulations when sprayed at the relatively high concentration 0.1% (v/v), did not cause epicuticular wax solubilisation. Damage and stress to underlying leaf cells that may have been caused by NLCP application was then investigated using UV radiation as an abiotic stress factor and cellular responses monitored via an analysis of stress responsive production of the wounding- and pathogen-induced callose and hydrogen peroxide. It was shown for *L. angustifolius* that NLCP do not enhance an abiotic stress response following exposure to UV-C light. Surfactants, when tested at similar concentrations to the NLCP, however, led to greatly enhanced stress responses in leaf cells of *L. angustifolius*. Further, to test the efficacy of delivery of a herbicide (2,4-D) via NLCP the invasive weed...
Raphanus raphanistrum in a T.aestivum crop was treated with NLCP at different concentrations. 2,4-D loaded NLCP sprayed at low concentration (0.03% 2,4-D) achieved an 11% increase in kill rate in comparison to a commercial 2,4-D herbicidal spray formulation. These results, clearly demonstrate the potential of NLCP to systemically and safely deliver agrochemical actives to a crop without the toxic side-effects posed by surfactant use.

6.2 Characterisation of the epicuticular wax micromorphology

Monochromatic staining techniques performed on the cryosections obtained for T. aestivum, Z. mays and L. angustifolius resolved the cuticular layer into cuticle proper (CP), external cuticular layer (ECL) and internal cuticular layer (ICL) (Buda et al., 2009). CP was consistently observed as a bright layer external to ECL, while ECL was sandwiched between CP and ICL, and ICL was continuous with the underlying epidermal cell wall. Dichromatic staining further delineated the boundaries of cuticle and underlying cell wall. Cuticular overhangs that were also consistently observed for stomata on each species investigated. Different epifluorescence microscopy techniques were employed to show that cuticular material was localised in the anticlinal cell walls of the epidermis of T. aestivum and Z. mays, while that of L. angustifolius was limited to periclinal cell walls. The cuticular organisation on T. aestivum, Z. mays and L. angustifolius at a high resolution was, to my knowledge, shown here for the first time.

The intricacies of the epicuticular wax micromorphology were deciphered by employing high resolution scanning electron microscopy (SEM) on the phylloplane of T. aestivum, Z. mays, L. angustifolius and A. thaliana. Epicuticular waxes were observed to cover the surface of each species, A. thaliana, however, was covered with an amorphous wax sheath. The epicuticular waxes observed on the other species were identified using the classification proposed by Barthlott et., (1998), as entire platelets (EP), irregular crenate platelets (ICP) and membranous
platelets (MP). The epicuticular wax crystalloids found on *T. aestivum* were identified as ICP and that of *L. angustifolius* were MP. All the three types of wax crystals were observed on the phylloplane of *Z. mays* giving rise to a unique ‘syntopic’ arrangement of wax crystals. The epicuticular wax micromorphology strongly contributes towards the surface hydrophobicity of plant surfaces. The surface hydrophobicity in turn protects the plant from harmful anthropogenic substances and the attachment of microbial spores (Barthlott and Neinhuis, 1997).

Although plant cuticles have been studied extensively there are few, if any, methods currently available to describe the structural integrity of epicuticular waxes. To fill this gap and quantify the epicuticular waxes on the surface of leaves, a novel image analysis procedure was developed and employed on SEM images collected from the species under study. The procedures developed for analysing the area of epicuticular waxes were sensitive to subtle differences observed on leaf surfaces. For example, the area of epicuticular waxes was found to be higher for *Z. mays* when compared to *T. aestivum* and *L. angustifolius*. It is likely that the syntopic arrangement of epicuticular waxes on *Z. mays* leaves contributed to the large area of epicuticular waxes found for this species. Further, the differences in the chemical composition of the epicuticular waxes no doubt contributed to the presence of different types of epicuticular waxes on leaves of the species examined.

The composition of epicuticular waxes was analysed by gas chromatography and fatty acids, primary alcohols, alkanes and ketones were consistently found in the waxes of each of the species examined. It is very likely that the differences in the chemical composition would have contributed to the differences in wax micromorphology. Octacosanol was predominantly found in the wax extracts of *T. aestivum* and have been shown by Koch et al., (2006) to be responsible
for the formation of ICP on its phylloplane. Together, elucidating the micromorphology of the epicuticular waxes were instrumental in screening the interactions of various chemical agents with the plant surfaces. For example NLCP interactions with epicuticular waxes could be investigated. Further, knowledge on the self-assembly structures can lead to novel research in the fields of biomimetics. For example super hydrophobic surfaces can be built mimicking the plant surface (Michael and Bhushan, 2007).

6.3 Interaction of nanostructured liquid crystalline particles with leaf surfaces

Surfactants are one of the key components of agrochemical spray formulations that act on the plant surfaces by permanently solubilising the wax crystals and plasticising the plant cuticle for the effective, systemic delivery of the active ingredient (Castro et al., 2014). This irreversible damage to the cuticle leads the plant to stress and may by itself result in yield losses. To overcome this problem NLCP made of lipid phytantriol, were proposed as an alternative to surfactant based agrochemical delivery system. The idea arose from earlier work by Dong et al., (2011) and Chang et al., (2012), who demonstrated that NLCP can strongly bind to hydrophobic tristearin or silicon model surfaces through adsorption.

In the current study NLCP were tested at different concentrations against the commercial agrochemical surfactants empigen, a cationic, alkyl dimethyl betaine and empimin, an anionic, sodium di(2-ethylhexyl) sulfo succinate on the leaves of T. aestivum, Z. mays, L. angustifolius and A. thaliana. Though both empigen and empimin surfactants induced a dose-dependent phytotoxicity and wax solubilisation there were, however, some subtle differences as a consequence of differences in the physiochemical properties of the surfactants. For example, leaf necrosis after empimin treatment was much extended beyond the site of application while necrosis following empigen treatment was limited to the site of application. Empimin was
found to be more phytotoxic than empigen. The presence of ring like phytotoxicity with a central undamaged area on *T. aestivum* and *A. thaliana* was a characteristic of 0.1% empigen application and was likely a consequence of surface tension and evaporation (Deegan et al., 1997). Unlike surfactants, NLCP application at concentration of 0.1% and 1% did not show any phytotoxicity symptoms on *T. aestivum* and *Z. mays*. Increasing the NLCP concentration to an unrealistically high 5% resulted in phytotoxicity symptoms on *T. aestivum* and *Z. mays*. Strong tensile strength observed in the leaves of *Z. mays* could have contributed to the resistance of this species to NLCP (Balsamo and Orkwiszewski, 2008). More broadly, the presence of an anatomically rigid and robust leaf with a complex and dense wax layer could facilitate monocots being relatively unaffected by higher amounts of NLCP application, a feature that may be advantageous in comparison to the surfactants. *L. angustifolius* and *A. thaliana* and potentially dicots more broadly have a more fragile leaf structure with lower wax complexity that may make them more prone to damage following application of chemicals (Dominy et al., 2008).

For obtaining NLCP after high energy dispersion the block polymer F127 is critical to ensure colloidal stability of the particles. As it would not be possible to obtain NLCP dispersion without the use of F127, phytantriol alone was not tested for phytotoxicity and wax solubilisation on the plants. 0.1% F127 was, however, tested for phytotoxicity as 1% NLCP solution contains 0.1% F127. At a concentration of 0.1% F127 did not elicit phytotoxicity, but it was surprising to find that it induced severe wax solubilisation on the phylloplane of *T. aestivum* and *Z. mays*. For the dicots *L. angustifolius* and *A. thaliana*, both 1% NLCP and 0.1% F127 when tested separately induced phytotoxicity. This difference in response to F127 among the different species is likely a consequence of physiological sensitivity among dicots and

*Chapter 6 General discussion*
monocots and would be worthy of further investigation. The differential epicuticular wax solubilisation found after treatment with 1% NLCP containing an equal amount of F127 was surprising. It has been reported that in NLCP solution, F127 was strongly bound on the surface of NLCP leaving behind very limited or no F127 to interact with the wax micromorphology (Dong et al., 2011).

6.4 Physiological stress response of plants after NLCP spray application

It was shown that the epicuticular waxes are critical for protecting plants from abiotic (Pfündel et al., 2007) and biotic stress (Metraux et al., 2014). Therefore to check if NLCP or surfactant sprayed on Z. mays, L. angustifolius and A. thaliana makes these species more susceptible to UV stress, the production of the stress markers, callose and H$_2$O$_2$, was investigated. The control group treated with UV alone was found to have a larger total leaf area when compared to treatment groups that were spray applied with NLCP or surfactants followed by UV exposure. The effect of UV to developmentally trigger plant growth has been suggested (Kunz et al., 2006) and may have contributed to the increase in leaf surface area found. The A. thaliana plants treated with both NLCP or surfactants followed by UV exposure were doubly stressed leading to much reduced plant growth. It is possible for initial NLCP or surfactant spray applications to trigger a systemically propagating H$_2$O$_2$ signal that helps in development of systemic acquired acclimation (SAA). The following UV dose could have further triggered energy dependent SAA. Thus a gene level negative regulation of growth and development coupled with energy spent for abiotic stress recovery may account for the reduced growth in NLCP or surfactant and UV treated plants.

The callose extracted from leaves after low level UV treatments following NLCP or surfactant spray applications yielded less callose compared to leaves treated with the higher UV doses.
The developmental cues provided by low dose UV treatments may have enabled the plant to better recover from NLCP and surfactant stress, ultimately leading to production of less callose. Apart from the existing stress induced by NLCP and surfactant spray applications, the UV stress created by higher irradiation levels may have meant an energy diversion to production of high amounts of callose and as result severely altered (reduced) plant growth and development. It has been previously shown for *A. thaliana* that UV-C irradiation at 1000 J m\(^{-2}\) s\(^{-1}\) led to large increase in the production of callose and lignin, along with leaf curling and lesion formation (Mintoff, 2014). In the current work the results obtained using callose image analysis were also confirmed using an assay to quantify the amount.

For *L. angustifolius* it was interesting to observe that NLCP spray formulations followed by UV exposure did not stress the plants and callose production was comparable to that of control plants treated with UV alone (no NLCP or surfactant spray treatment). It was earlier shown in chapter 3 of this thesis that, epicuticular wax solubilisation after NLCP application was much reduced when compared to that of surfactant treatments. Further, the epicuticular waxes present on the phylloplane may have reflected the UV radiation, analogous to specular reflectance (Pfündel et al., 2007), ultimately preventing plant stress and consequently less callose production (Grant, 1987). Further, the absence of epicuticular waxes on the surface of *A. thaliana* may have led to reduced specular reflectance and as a consequence an enhanced stress response after NLCP, surfactant and UV treatments. A minor proportion of leaf diffuse reflection can also stimulate callose production in *A. thaliana* leaves. It is thus likely that syntopism, leaf specular reflectance and anatomical toughness of *Z. mays* leaves (Dominy et al., 2008) together contributed towards the resistance of this species to UV irradiation after NLCP or surfactant spray application. Consistent with the results for callose production that
was observed for *Z. mays*, *L. angustifolius* and *A. thaliana*, the production of H$_2$O$_2$ was also less following NLCP spray applications followed by UV exposure compared with that found after surfactant treatments. The callose and H$_2$O$_2$ production results align with the idea that NLCP, unlike surfactants, do not plasticise and alter the physiochemical properties of the cuticle but interact with the cuticle by strong adsorption while leaving it relatively free from damage.

In a similar way to the examination of the impact of an abiotic stress following NLCP or surfactant treatment an examination of the impact of a biotic stress was also carried out. For this purpose the plant-pathogen system of *A. thaliana* with *Hyaloperonospora arabidopsidis* was utilised. No differences in the pathogenicity of *H. arabidopsidis* was observed for any of the treatments. *H. arabidopsidis* is a biotrophic pathogen that thrives in the mesophyll cell layers but not on the phylloplane although it requires spores to alight on the leaf surface, germinate and then for the germ tube to find its way to a stomatal pore to access the leaf. It appears that any changes brought about to the leaf surface by NLCP or surfactant applications did not alter this interaction. This research could now be extended into the analysis of the other broad group of fungal pathogens the necrotrophs. These pathogens often first kill and colonise epidermal cells prior to entering the mesophyll, it is likely that cells killed by surface treatments will act as a ready nutrition source for such pathogens and enable their more rapid colonisation of the host.

These results together provide evidence for the potential of NLCP to interact with the cuticle without altering its biophysical properties. It was also shown that the lipophillic and lipophobic channels in NLCP facilitate the loading of either water soluble or lipid soluble active ingredients (Dong et al., 2012). A further aspect of the current study was to test the
ability of NLCP to deliver agronomic actives, more specifically the lipid soluble herbicide 2,4-dichlorophenoxyacetic acid (2,4-D).

6.5 Efficiency of NLCP to deliver the auxin herbicide, 2,4-D

2,4-D is an auxin herbicide and when applied at sufficient doses to susceptible plants disrupts the homeostasis of the phytohormones ethylene and abscisic acid leading to plant death. *In-vitro* studies on *A. thaliana* and *L. angustifolius* were aimed to screen the efficacy of NLCP based 2,4-D delivery in comparison with surfactants (empimin and empigen). For *A. thaliana*, the herbicidal action observed for 2,4-D spray applied at 0.01% and 0.1% using NLCP was similar to that of empigen. At similar concentrations tested, empimin-based 2,4-D delivery appeared to be more effective in killing *A. thaliana* than empigen and NLCP-based 2,4-D delivery. The plants treated with 0.01% 2,4-D NLCP were healthier than counterparts treated with 2,4-D/empimin and 2,4-D/empigen. NLCP mode of interaction coupled with delay in senescence and differences in susceptibility together may account for the observation of healthier plants after 0.01% 2,4-D NLCP spray application. Fresh weight, dry weight and water content measurements on *A. thaliana* (Grossmann, 2003) after 2,4 D NLCP treatments clearly showed tissue swelling rather than an increase in number of cells in the plant tissue. These results indicate the herbicidal effects of 2,4-D NLCP. *L. angustifolius* on the other hand was resistant to 2,4-D herbicidal action, however auxin overdose symptoms such as stem curling and shoot tip stubbing were evident.

Field trials were also conducted to investigate the efficiency of 2,4-D loaded NLCP in killing the invasive weed *R. raphanistrum* in fields of *T. aestivum* and *H. vulgare* against a commercially available 2,4-D formulation, Estercide 800. Phytotoxicity symptoms observed after estercide formulation application were most likely induced by the surfactants and
adjuvants mixed within the estercide formulation, however the actual concentration of the chemical mixture remains unknown. Scanning electron microscopy (SEM) and crop phytotoxicity observations made for *T. aestivum* and *H. vulgare* separately remains plausible for both the crop plants as they belong to same family *Poaceae* and tribe *Triticeae*. The observed phytotoxicity after spray application of 2,4-D/NLCP on *T. aestivum* and *H. vulgare* may have been an effect of the 2,4-D rather than the NLCP formulation. It was shown in Chapter 3 of this thesis that when the NLCP formulation was applied at 1% on *T. aestivum* and *Z. mays* it did not alter the epicuticular wax micromorphology (Nadiminti et al., 2013). The phytotoxicity observations were further confirmed by image analysis performed on SEM images collected for *H. vulgare*. Weed control ranged between 46.9% to 74.7% and 58.3% to 76.8% for estercide 800 and 2,4-D NLCP respectively. The control of weed *R. raphanistrum* was observed to be efficient for 2,4-D NLCP with a yield improvement of almost 15% for the low dose application of 2,4-D NLCP at 0.03%. This enhanced control was likely due to the number of 2,4-D molecules reaching the plant surface per unit area being greater for 2,4-D NLCP than for 2,4-D applied via Estercide, although I did not directly test this. Additionally, a decrease in crop phytotoxicity along with a reduction in weed numbers could also have contributed towards the yield increase observed for these two crops.

6.6 Final conclusion

The NLCP-plant interactions were thoroughly investigated with the aim of using NLCP as next generation agrochemical delivery vehicle and as an alternative to the traditional agrochemical surfactants. The application of NLCP on *T. aestivum* and *Z. mays* clearly show their potential to safely interact with plant surfaces even when applied at a relatively high concentration.
micromorphology of the plant surface will be required. For example, alternatives to phytantriol such as oleyl glycerate and monolinolein and to that of F127, polyethylene glycol (PEG) could be tested (Lancelot et al., 2014). The research presented in this thesis is a step forward for providing a platform on which the applicability of nanoparticles, especially lipid-based particles, to agronomic problems posed by surfactant use, can be addressed. Due to the nanostructures that one works with there is the need to develop further techniques, tools and procedures to successfully investigate their interactions with plants. For example, advanced microscopy techniques such as confocal raman microscopy may be employed to screen the localisation of various nanoparticles. Simultaneously, automated image analysis procedures for batch processing of visual data would provide deeper insights into large data sets within short time frames. Our understanding of nanoparticles and how we may benefit from their use in plant science is a new area of research and one which holds great promise for future agriculture.
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