Chapter 1: Introduction.

1.1. Problems and challenges in food security.

An average of 35% of potential pre-harvest crop loss globally is due biotic stresses which includes pests, pathogens and herbivorous feeders (Popp et al., 2013). Ten % of attainable yield is lost because of diseases which severely limits maximum yield and increases the yield gap. Plants are subject to diseases all over the world, but this varies geographically with an estimated 50% higher yield gap due to pests and pathogens in the tropics compared to temperate climates (Guest, 2008). Plants and pathogens/pests have co-evolved together for 400 million years so have both equipped various strategies to mitigate each other. Pathogens/pests co-exist with crops due to consumption, lifecycle habits or act as mediating vectors for plant pathogens (Labandeira, 2013). During domestication of crops, the gene pool has become more limited for crop defences and moving crops to new locations resulted in introduction of co-evolved pests and pathogens in new locations. The problem is exacerbated in the modern world, due to globalisation, resulting in increased trade and travel which encourages spread of pests and pathogens. Climate change is an added pressure which favours opportunistic niches for pests/pathogens, often bringing emerging problems in new locations, as previously inhabitable conditions become more optimum (Walsh et al., 2011). High resource inputs including: pesticides, fertilisers and high-yielding varieties of crops have been used during the first Green revolution, helping to improve crop yields and double agricultural food production to feed the growing population between 1960-2010 (Popp et al., 2013). However in 2010, the number of malnourished people was still 925 million worldwide, with the highest percentage (98%) in developing countries, showing the main issue is unequal access to food (FAO, 2012). Consumption will also increase as the population is expected to reach 9.7 billion by 2050 (UN, 2015) and as countries become wealthier, there is more of a social demand to eat high calorie meat-based diets, which requires more resources to produce. Global scale reliance is on pesticides as an easy to apply, cheap method to help growers achieve acceptable economic production levels (Oerke, 2006).

1.1.1. Types of agrochemicals.

Pesticides are a type of agrochemical and includes: herbicides, fungicides, insecticides, bactericides, viricides, nematicides, miticides, acaricides, rodenticides, which are named according to their target organism with latin suffix -*cide* meaning to kill. Pesticides may be contact or systemic pesticides, dependent on their mode of actions (MoAs). In spray applications to leaves, contact pesticides require direct contact with the target organism *e.g.* fungus or insect, which means there is time dependency on the leaf surface. Contact pesticides should adsorb to the leaf but ideally should not be absorbed by the leaf. Products which tend to be contact-acting include older fungicides such as: captan, chlorothalonil, mancozeb, copper and sulphur (FRAC, 2019). Uptake into the plant is required for systemic pesticides which require translocation to other plant parts via the xylem/phloem or translaminar movement to the opposite side of the leaf to achieve their MoAs (Steurbaut, 1993). Systemic insecticides, fungicides and herbicides require uptake into the cuticle,

desorption from the cuticle and then translocation to the area required in the plant or to the site of action. Each of these is a rate-limiting step, which lowers the effective dose of product reaching a target area (Zabkiewicz, 2002). For systemic pesticides, hydrophobic-lipophilic values (HLB) are used to measure the ratio of polar and lipophilic chemical groups to predict the affinity for water or oil. Log Ps are another useful parameter used for systemic herbicides as a measure of the ratio partitioned into either water or octanol solvent. Solubility in octanol solvent versus water is used as a model to predict association with oils and waxes in the cuticle and tendency for dissociation into the water-based apoplast. A negative Log P suggests a preference for water matrices and although this means there is a preference for the water transport system in plants, these types of compounds tend to be rate-limited by their diffusion across the wax-based plant cuticle. Lipophilic compounds adsorb onto and diffuse across the cuticle quicker but a higher, positive Log P may mean their desorption out of the cuticle and movement to the target area of the plant is the limiting step (Schönherr and Riederer, 1989). Plant protection products which form a uniform coverage such as a heat protection barrier product would be required to stay on a leaf as a film (Glenn et al., 1999). Products which require ingestion by an insect, such as the *Bt* toxin, are also contact pesticides but are more effective as clumps at higher dosage rather than a uniform coverage of less concentrated *Bt* toxin (Ebert et al., 1999). Different agrochemicals have different requirements to achieve their optimum effective delivery to plants.

1.1.2. Application of agrochemicals.

Agrochemicals can be applied either to the soil, sprayed onto the aerial parts of a plant (such as leaves, flowers and fruit) or more recently, supplied as a pre-seed treatment. Foliar application refers to the spraying of leaves and is a very inefficient process (Faers and Pontzen, 2008, Taylor, 2011). Spraying of a pesticide can result in losses of between 30 – 65% due to the inherent inefficiencies of the spray process (Pimentel, 1995). Flow rate, pressure rate, distance of trajectory, angle of sprayer and droplet sizes all affect deposition on the target (Cross et al., 2001, Wang et al., 2018, Musiu et al., 2019). Spraying requires a chemical solution to come into contact with a nozzle head under pressure, which forms a spray sheet. The spray sheet travels through the air and eventually breaks into different size droplets with various kinetic energies. Droplets experience impact as they meet a target leaf surface where they will either adhere and be retained and spread over the leaf or they bounce off and are wasted into the environment (Akesson and Gibbs, 1990, P.C.H. Miller, 2000, Faers and Pontzen, 2008, Forster et al., 2005, Gimenes et al., 2013, Dong et al., 2015). Small droplet sizes (<1.8 µm) have less mass and are carried longer distances in the air stream and tend to miss their targets (Vernay et al., 2016, de Oliveira et al., 2019). In spray nozzle heads that produce fine mist sprays a high percentage of droplets in the spectra are small, and the fates of these droplets tend to miss their target sites. Spray drift refers to the loss of droplets off-target, which is severe in adverse weather conditions and is a major cause of environmental pollution (Valcore, 2007). Spray drift causes issues to non-target crops, humans, aquatics and terrestrial organisms (Hilz and Vermeer, 2013). Spray drift also results in lower efficiencies of an agrochemical application as

chemical does not reach the target and may need to be re-applied, which means more input costs for the grower and more water to re-apply chemicals.

Run-off is another source of losses of agrochemicals from a target. In this case, droplets meet a target surface but cannot overcome their kinetic energies and therefore do not stick and are not retained (De Cock et al., 2017). Large droplets containing pesticide have high kinetic energy and may bounce off, or shatter on impact which produces smaller droplets which run-off (Hilz and Vermeer, 2013). Large droplets also tend to coalesce with other large droplets on the surface (P.C.H. Miller, 2000). If the leaf surface is already saturated with a spray or with water for example after a rainfall event, droplets tend to reflect off and are lost. Most chemical labels advise users to spray until point of run-off for this reason. Figure 1 shows an overview of some of the droplet losses from an agrochemical spray, the impact of environmental and plant factors on efficiency and how these losses effect the grower.

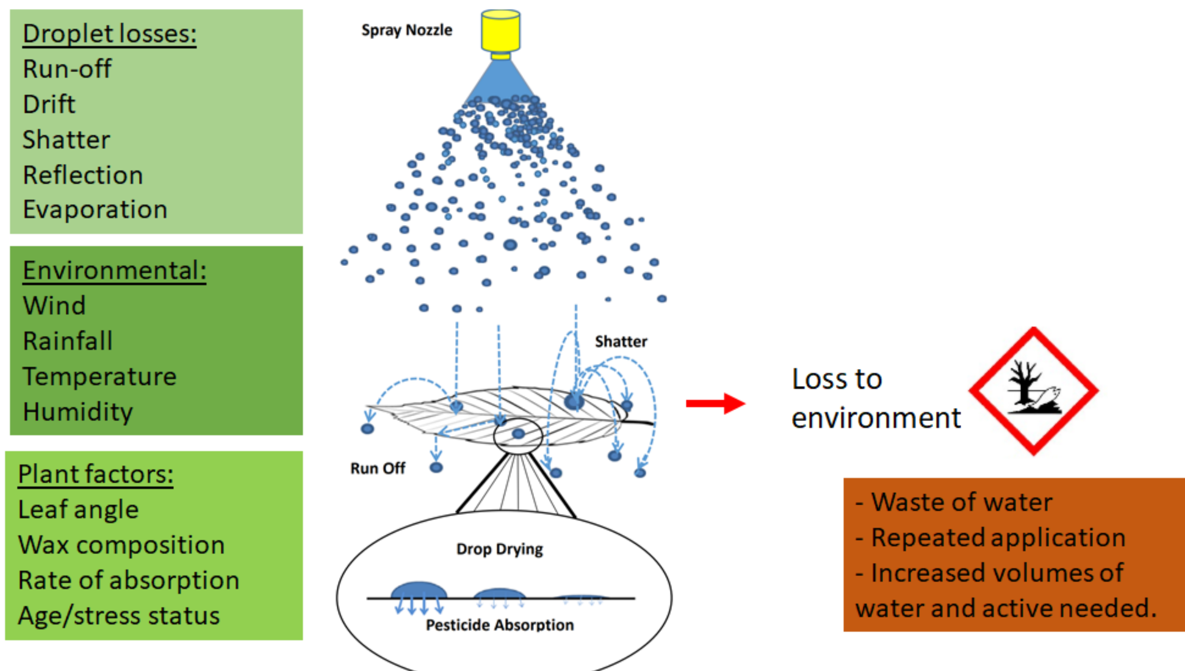


Figure 1: Process of spray application and droplet fates during spraying. *A liquid sheet is ejected from the spray nozzle and perforates to form various sizes of droplets. Droplets can be lost by different processes which are affected by environmental and plant factors. Pesticide products are only delivered into the leaf by droplets which stick and are absorbed. Pesticides losses have a direct impact to both the grower and the environment. Image modified from Attune – Adj G technical bulletin.*

After droplets have adhered to their target leaf surface, droplets containing systemic pesticides which have their MoAs within leaves still need to be absorbed and, in some cases, translocated and come into contact with another organism to have their targeted effects. One such example is the neonicotinoid family of systemic insecticides which are ingested by phloem-feeders from within the transport system of the plant.

For spray applications, water is used as a carrier system to solubilise and deliver agrochemicals to a target leaf. However, water does not wet or stick to the hydrophobic wax surface of a plant leaf because of its high surface tension. Aqueous droplets also have limited lifetimes on a leaf because water evaporates from the droplet, especially in high temperatures with low humidity. Penetration of compounds into the cuticle and epidermal cells of the leaf may also be limited if they are not highly soluble in water. It has been previously claimed that penetration stops once the water phase has evaporated and solid active ingredients (A.Is) may precipitate and crystallise to form solid deposits on the leaf surface which can no longer penetrate and may be blown off by wind (Gimenes et al., 2013). The ability of an agrochemical to adsorb and move across the plant cuticle, desorb and translocate within the plant are limited by the nature of the agrochemical. Agrochemicals consists of an A.I to give a product it's MoA, but A.Is tend not to be optimal for all parts of the spraying and translocation processes, for example if it is a highly lipophilic compound with low solubility in water.

1.2. Adjuvants.

The functionality of pesticides is dependent on the MoA of the given agrochemical product and maximum efficiency can only be achieved when an appropriate dose of the given product is sufficiently present in the area where it is required to work. Adjuvants are intended to be mixed with agrochemicals to improve their functionality. Adjuvants can be formulation adjuvants or tank-mix/spray adjuvants.

Formulation adjuvants includes stabilisers or surfactants supplied as part of a formulation of a pesticide. These may be used to improve compatibility of an A.I and water to keep them in a solubilised active form. Adjuvants may be used to buffer pH or improve water hardness to prevent hydrolysis or dissociation of A.Is. They may act as anti-foamers to improve mixing and spray applications. Adjuvants can improve homogeneity of a complex mixture stabilising solids in suspension to allow equal delivery of A.Is within droplets. (Hazen, 2000, Zollinger, 2012). Adjuvants may be used to modify spray sheet properties during spray application by lowering surface tension of liquids contacting the nozzle head or travelling in a sheet, which modifies deposit coverage on the target. Drift-control agents modify the droplet spectra during spraying to improve the ratio of medium-sized (~2.5 µm) droplets in a spray, to reduce drift and improve targeting on a given plant organ *e.g.* leaf or fruit (Foy, 2017). Examples of these types of adjuvants are oils, emulsifiers and carbohydrate-gum based products.

Spray or tank-mix adjuvants are added to an agrochemical just before crop application. Sticking adjuvants can improve adsorption of droplets after spraying so fewer droplets are lost on impaction. Adjuvants can lower surface tension thereby giving the ability of droplets to spread, giving increased contact with the leaf surface. Adjuvants can improve droplet lifetimes (lower evaporation rates) on a surface maximising time for diffusion to occur into the leaf. Adjuvants can also enhance

penetration of an agrochemical product through the cuticle, which is the primary barrier for entry of systemic hydrophobic pesticides into the leaf (Knoche and Bukovac, 1992, Petracek, 1998, Orbovic et al., 2007).

1.2.1. Types of spray adjuvants.

1.2.1.1. Spreaders/wetters.

A type of adjuvant which increases spreading over a leaf surface is called a surfactant. Droplet spreading is a result of surface free energy at the interface between leaf surface, droplet and air and the product of this interaction gives a contact angle between droplet and leaf (He et al., 2019). Figure 2 shows the equation for determining the contact angle of a droplet on a flat surface. Surfactants work by reducing the equilibrium surface tension (EST) of a droplet and lower the contact angle (increases contact) between a droplet and a leaf and increases spreading. Surface-actants or surfactants include amphiphilic molecules which have lipophilic tails and hydrophilic head groups. The polar heads can be cationic, anionic or non-ionic depending on their charge (Hazen, 2000). A typical class of non-ionic surface-actants (surfactants) include synthetic organosilicone surfactants, which spread and reduce surface tension of water to <20mN m^{-1} thereby achieving maximum wetting on a leaf. Wetting adjuvants can lower the EST of a droplet because of their amphoteric nature, the surfactant monomers can arrange as micelles on the surface of a droplet interface with air and can help destabilise interactions between water molecules, maximising contact with leaf surface and increasing droplet diameter. Adjuvants which form micelles have a critical micelle concentration (CMC) which is the concentration of adjuvant where the surface micelle is saturated, and no more lowering of surface tension can be achieved by further increases in concentration (Zhou et al., 2017, Januszkiewicz et al., 2018). Figure 2 shows the arrangement of surfactant micelles within a droplet. The CMC value is specific to each surfactant and suggests there is an optimum concentration of adjuvant to best achieve wettability (Janků et al, 2012).

Liquids	Surface tension N/mm
Water	72
Formamide	58
n-hexane	18
Solids	Surface free energy N/mm
Nylon	38-46
Polystyrene	38
Polypropylene	29-31

γ_{sl} solid–liquid
γ_{sv} solid–vapour
γ liquid–vapour

$$\cos\theta = \frac{\gamma_{sv} - \gamma_{sl}}{\gamma}$$

Figure 2: Equation for contact angle for sessile droplets on a surface. *Equation to work out contact angle θ tangent point of contact between surface and edge of droplet. The solid surface is assumed to be flat. The ball and sticks along the top edge of the droplet represents the arrangement of surfactant micelles in an oil droplet. The balls represent hydrophilic heads and sticks show lipophilic tails facing inwards. The inverse arrangement would be true in a water-based droplet. The table gives an indication of how surface free energy varies with different solid substrates and surface tension varies between different liquids, two of the parameters which give rise to different contact angles. N/mm = Newton millimetre. Figure taken from (Brutin and Starov, 2018) with additional information for the table from (Bishop, 2015).*

1.2.1.2. Stickers/adhesives/deposition agents.

These include Latex based adjuvants and resins and are characterised by having good adhesion on the leaf surface. One characteristic of these types of adjuvants may be high viscosity, which improves sticking (Hazen, 2000). Droplets travel across the air with velocity and they must be able to overcome the loss of kinetic energy during impact to stick to a surface (Akesson et al., 1994). Dynamic surface tension (DST) is the droplet behaviour in the first 100 ms during spraying and during impact with the surface. After adhesion, droplets reach their EST which closely matches the EST for an adjuvant, when combined in droplets with pesticides (Forster et al., 2005, Faers and Pontzen, 2008, Asmus, 2016). Sticking adjuvants are able to arrange to form micelles and lower surface tension sufficiently, to stick droplets to leaves and stop droplets bouncing off (Taylor, 2011). Sticker adjuvants can also have 'rain-fastness' properties which means they can enhance retention of pesticides after rainfall events (Mulrooney, 2000, Melo et al., 2015). The insecticide Act C has good rainfastness with oil-based adjuvant rape oil methyl ester (Nauen, 2007).

1.2.1.3. Humectants and penetration agents.

Humectant adjuvants have hygroscopic properties which means they can attract water molecules. Humectant adjuvants can maintain solubility of an A.I within droplets, especially at high humidity in which case they have deliquescence properties and can absorb water from the atmosphere. Penetration agents are used to achieve even spread of A.I in droplets and the formation of micelles can act as a carrier or delivery mechanism to solubilise A.Is which may not have high solubility in water. Pesticides should be dissolved in the liquid phase to be able to penetrate the leaf and there must be a water interface between the droplet and leaf surface for diffusion to occur (Ramsey et al., 2005). Diffusion of an A.I occurs from a droplet, across the cuticle and is translocated around the plant, occurring down a concentration gradient (Schönherr and Riederer, 1989). After passing through the cuticle, compounds may move through either the apoplastic pathway (extracellular) compartment including cell walls, or through the symplastic pathway, within cells and organelles. To reach the plant transport system, compounds need to be loaded from the apoplast into xylem and phloem to be carried by mass flow to sink tissues. Adjuvants which slow evaporation time of droplets are retained longer on the leaf and can improve A.I uptake. Plasticisation is thought to be involved to alter cuticle arrangement to better facilitate diffusion of a pesticide (Mojsiewicz-Pieńkowska et al., 2016). Typical spray adjuvants include surfactants, vegetable oils, petroleum oils, mineral oils, modified oils with emulsifiers and oil-surfactant blends and are used to facilitate generation across the cuticle. Adjuvants typically contain hydrophilic water-soluble heads and hydrophobic oil-loving tail moieties. Hydrocarbons, such as lipids bonded to ethoxylated (EO) groups, is a typical example. Adjuvants can associate to form micelles of oil-in-water or water-in-oil emulsions and act as a carrier system for pesticides because of their duel properties (Aveyard et al., 2003). Non-ionic alcohol ethoxylates such as organosilicone surfactants are thought to be able to dissolve the cuticle and plasticize the membrane lipid bilayer which is suggested to aid absorption into the symplast. Organosilicone surfactants also lower surface tension enough to allow uptake to occur through stomata. Humectants/penetrants may be ethoxylated and contain various numbers of ethylene oxide (EO) groups (Ramsey et al., 2005). Ethylene oxide groups can hydrogen bond with water and are suggested to hydrate polar groups within the cuticle to facilitate cuticular absorption of polar molecules (Liu, 2004). When penetration of a pesticide is undesirable and applied with an adjuvant *e.g.* organosilicone with copper, this causes phytotoxicity as copper is a contact fungicide and should adsorb on the leaf surface but not be absorbed through it as copper is both toxic to fungi and to plants (Orbovic et al., 2007). Conversely, adjuvants that do not penetrate may also slow down penetration of an A.I as they may diffuse in at different rates (Steurbaut, 1993). The correct adjuvant should therefore be tested with an active for whether retention or penetration is desired.

Adjuvants included in formulation with a pesticide are not normally known, as manufacturers patent and protect the secrecy of these formulations. All tank-mix or

spray adjuvants should be tested in combinations with pesticides containing various active compounds to check for compatibility before recommendations can be made to growers regarding specific required functions. This creates a need to screen adjuvants in terms of their properties: whether they are to retain moisture, help stick, improve coverage or aid penetration of a systemic pesticide. It also requires the use of a range of active compounds: contacts, systemics, insecticides, fungicides, biofungicides to assess a range of compatibility for adjuvants and see whether they are robust enough to be applied with multiple products.

1.3. Factors important for surface behaviour of droplets of agrochemicals.

1.3.1. Evaporation.

When a spray droplet loses velocity, lands on a target surface and is retained, it must adhere to the surface. Adsorption between surfaces and droplets results in different degrees of wetting and polarity of droplets.(Knoche and Bukovac, 1992, Brutin and Starov, 2018). Some liquid solutions may give rise to droplet polarity on a surface, which is caused by unequal adsorption, resulting in droplets which have leading and shrinking edges. The lower the surface tension, the more of the droplet is in contact with the surface, creating a larger wetted area. This surface contact creates a pinned line or a three-phase line which describes the meeting point between the solid leaf surface, the liquid droplet and the air. The three-phase line is also where most water evaporation occurs (Deegan et al., 1997). A liquid droplet sat on a surface is described as a sessile droplet. Sessile droplets experience various kinetics during evaporation of water from the droplet, the rate of which, depends on temperature and relative humidity as well as the composition of the droplet, its shape, interaction with the leaf and other chemical characteristics such as vapour pressure and thermal conductivity (Brutin and Starov, 2018). The wax composition and heterogeneity of the leaf surface effects how much of the droplet is in contact. Hard to wet species tend to have thick epicuticular wax layers which can have macroscopic pillar like structures with air in between. There are two types of wetting phases, Cassie wetting and Wensel wetting. When a droplet is suspended over wax pillars with air inbetween it is in the Cassie phase of wetting which is an incomplete wetting phase (Xu et al., 2013). This wetting phase describes droplets on hydrophobic leaves and heterogeneous surfaces such as Brassica cv. brussel sprout leaves which are difficult to wet and are not fix adsorbed which results in droplets that easily roll off. The Wensel phase describes droplets on flat, homogeneous surfaces where full pinning is more likely to occur. Leaves which give contact angles <90˚C with water droplets (see Figure 3) tend to be easy to wet, while those >90˚C are difficult to wet and those beyond measurable parameters >150˚C are even more difficult to wet and include super hydrophobic leaves such as lotus leaves (Lin et al., 2016). Adjuvants lower surface tension of water (and pesticides) which decreases contact angles between droplet and surface and results in increased spreading of droplets which forms flatter droplets with larger surface areas.

During droplet evaporation, sessile droplets experience two types of flow within the droplet. The first is Marangoni flows or capillary flow which describe the convective motion of temperature gradients within a droplet (Xu and Luo, 2007). As droplets sit on a warm interface such as the solid leaf surface, the liquid in contact with the solid surface is the warmest part of the droplet and the apex in contact with the air is the coolest (Starov and Sefiane, 2009). Water loss to vapour tends to be from the edges of the three-phase contact line due to adsorption which drives an outward flow of replacement water from the centre of the droplet to the edges of the droplet (Deegan et al., 1997, Xu and Luo, 2007). This also results in droplet shrinkage (loss of droplet height) as droplets flatten over time as the aqueous phase is removed from the droplet (Brutin and Starov, 2018). Droplets which do not completely wet the surface or have closer surface tension to water (72 mN m^{-1}) may have ridges between the droplet and surface (higher contact angles), where the line is not properly adsorbed. This is due to high surface tension and differences in lipophilicity between the composition of the droplet and surface. Ridges at the edges of droplets can concentrate diffusing vapour between the solid/liquid/gas interface and reduce further evaporation as the vapour pressure deficit decreases. (Zhou et al., 2017) (see Figure 3 schematic). The other type of flow is caused by latent heat of vaporisation, which occurs at the perimeter of the droplet in contact with the vapour phase of air and causes outward vapour diffusion from the leaf boundary layer. The maximum rate of evaporation is affected by the temperature with the relative humidity (RH) and air speed Act Eing the droplet contributing to the gradient of vapour pressure deficit from leaf to air. The volume and density of the droplet also affect its rate of water evaporation (Deegan et al., 1997, Liu, 2004, Precipito et al., 2018). When volume is increased, droplets containing water take longer to evaporate and when density of water is decreased by addition of other compounds, evaporation is slowed at the droplet perimeter as less water molecules are present on the surface. There is an apparent increase in the evaporation flux of larger, high spreading droplets as there is more perimeter in contact with air. A droplet containing complex mixtures, such as pesticides in formulation with tank-mixed adjuvants, experience conjoining and disjoining pressures during evaporation if there are several bulk phases and instability in a droplet, which due to entropy, causes changes in partitioning in the droplet mixture during drying (Zhou et al., 2017). Adjuvants may alter partitioning of both water and A.Is which affects evaporation rates, deposition structures and potentially also penetration. As droplets dry down, the temperature, pH, concentration of solutes and viscosity also vary within a droplet which will affect penetration rates over the course of drying (Schönherr and Riederer, 1989, Knoche and Bukovac, 1992, Hunsche and Noga, 2012).

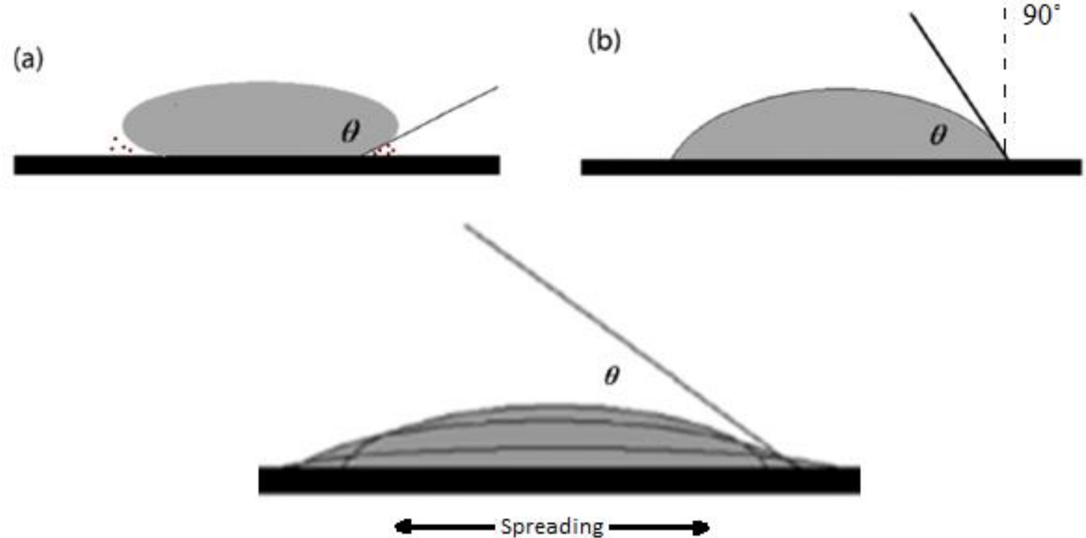


Figure 3: Contact angles for different wetting states on a surface. *a. Shows a partial wetting state where the contact angle is higher than 90˚ and the presence of ridges at the edge of the interface traps vapour (red dots). b. Partial wetting gives a contact angle <90˚ increasing contact between droplet and surface. Dotted line represents 90˚. c. Complete wetting, the contact angle is low and much closer to zero such as in the case of an addition of a wetting surfactant, the droplet is flatter and more spread over the surface. Figure adapted from (Brutin and Starov, 2018)*

Loss of water to the vapour in air is not the only loss from a droplet. Water may also diffuse into the leaf, typically through the cuticle but potentially through stomata, trichomes and other external leaf structures which form potential pathways for polar molecules. The rate of diffusion into the leaf depends on the concentration of a solute and its partitioning in the droplet. Hydrophilic/lipophilic balance (HLB) values for pesticides correlate with the diffusive potential across the cuticle, the rate of which, depends on whether the pesticide is more hydrophilic or lipophilic.

1.3.2. Spreading and deposition.

The degree of adhesion and dissipation over a leaf surface is a result of surface tension, which can be lowered by including adjuvants in the tank-mix. The contact angle between the droplet and leaf surface is a measure of surface wettability and lower contact angles are a result of more of the droplet being adsorbed on the surface. The equilibrium surface tension of pure water is 72 mN m^{-1} which gives a varied contact angle depending on plant surface. However, adjuvants typically lower surface tension to between 30 – 55 mN m^{-1} and organosilicone surfactants can lower this further to <20 mN m^{-1}. Spreading of droplets influences the evaporation rate as more droplet is in contact with the air for latent heat of vaporisation (Yu et al., 2009, de Oliveira et al., 2019). Spreading or increasing droplet wetted areas is thought to also affect penetration, as more of the droplet containing A.I is in contact with the leaf giving a higher surface area for penetration to occur. (Liu, 2004).

The association between the components within the droplet also affect the area of the droplet in contact with the surface (Webster et al., 2016). If the droplet contains similar molecules *e.g.* organic solutes in an aqueous droplet with humectants, they have a tendency for a tighter interaction, although this will change as evaporation occurs. If there are dissimilar components in the system, which is highly likely in complex mixtures, there may be a tendency to dis-associate and form more than one bulk phase (Brutin and Starov, 2018). This tendency for separation can increase on unlike surfaces *e.g.* polar molecules may align in the droplet away from the surface in contact with the hydrophobic cuticle.

After droplet adhesion and spreading and evaporation which reduces droplet height, the final step is the recession of the flat droplet during drying (Starov and Sefiane, 2009, Brutin and Starov, 2018). At this point the aqueous component is reduced, so adjuvants/active can become more concentrated in the droplet and the recession and deposition structure left behind depends on the characteristics of the mixture such as active solubility and the arrangement of the adjuvant and any active which hasn't diffused at the end of evaporation (Hunsche and Noga, 2012). Humidity, droplet volume and adjuvant type all effect deposition structures after droplet dry down. Emulsion droplets have both oil and water phases with characteristic domes or air inclusions (bubbles) which can concentrate A.Is in the centre at the top edge of a droplet (Aveyard et al., 2003, Wang et al., 2018). During droplet flattening, solids drop down under gravity and form a central deposition consisting of pockets of A.I, which have been termed 'ring islands' (Hunsche and Noga, 2012). Dissolved solids may also precipitate on the perimeter of the droplet if it is properly pinned to the surface (Wang et al., 2018). This process is driven by Marangoni flows, driving solutes to the edge and leaving a 'coffee ring' or 'o-ring' after droplet drying (Deegan et al., 1997, Truskett, 2003, Callegari et al., 2018). The links between deposit types and penetration is not well defined and more studies are needed to link surface behaviour, penetration and effects on biological activity of a pesticide (Kraemer et al., 2009).

1.4. The nature of the plant cuticle.

1.4.1. The cuticle as a barrier.

The primary barrier of leaf penetration is the cuticular membrane which is secreted by epidermal cells to form an outer layer on aerial parts of plants, including leaves, fruits, flowers and stems (Kerstiens, 1996) (Koch et al., 2008). The cuticle is the barrier in contact with the phyllosphere and has functions in biotic and abiotic protection. The waterproof nature of the cuticle slows losses of water and leachates through cuticular transpiration, to maintain control of water loss through stomatal regulation (Jetter et al., 2000, Schuster et al., 2017). The cuticle also acts as a physical barrier for pests such as insects and fungi (Gorb and Gorb, 2017). The cuticle acts as a penetration barrier for agrochemicals, fertilisers, pollutants and dust particles and highlights why the physical and chemical nature of the cuticle needs to be well understood in the context of pesticides in foliar spray application (Beattie and Marcell, 2002).

Environmental factors such as air pollution and UV-B radiation have been shown to have an impact on wax biosynthesis and structure of the cuticle. (Barnes et al, 1994, Percy et al, 1994)

A simplified structure of the cuticle is shown in Figure 4. The outermost layer consists of an epicuticular wax film which is amorphous in smooth leaf species *e.g.* vine or *C. communis* leaves. In more difficult to wet species, such as hydrophobic *Nelumbo nucifera* (lotus) leaves, the epicuticular wax is crystalline and forms 3D nanostructures of tubules, rods, rodlets, filaments, platelets and others (Beattie and Marcell, 2002, Taylor, 2011, Gorb and Gorb, 2017). The innermost layer, the intracuticular wax, is composed of cutin which is made from the polymerisation of C16 and C18 alkanoic monomers, embedded in soluble cuticular lipids (Kolattukudy, 1980) (Jetter et al., 2000, Schuster et al., 2017). The cutin layer is cross-linked to the cell wall on the internal side by the pectin lamellae (Kerstiens, 1996). The waxes in the cuticle typically consist of C20-C34 very long chain fatty acids (VLCFA) and includes: alkanes, primary and secondary alcohols, aldehydes, ketones, esters. Some species also include alicyclic compounds (triterpenoids, tocopherols, steroids) confined to the intracuticular layer (Juniper and Jeffree, 1983) (Jetter, 2006, Buschhaus et al., 2007, Jetter and Riederer, 2015). Polysaccharides (*e.g.* cellulose, hemicellulose and pectin) and phenolics are also limited to the intracuticular layer and polysaccharides have been shown to form channels between the anticlinal walls of epidermal cells up to the epicuticular layer (Kerstiens, 1996, Yeats, 2013). The intracuticular layer thickness is much more variable between species than the epicuticular layer. In the third leaf of *C. communis*, the reported wax content for the epicuticular layer is 37.2 µg cm^{-2} compared with a total cuticle wax content of 5527.9 µg cm^{-2}. (Park, 2004)

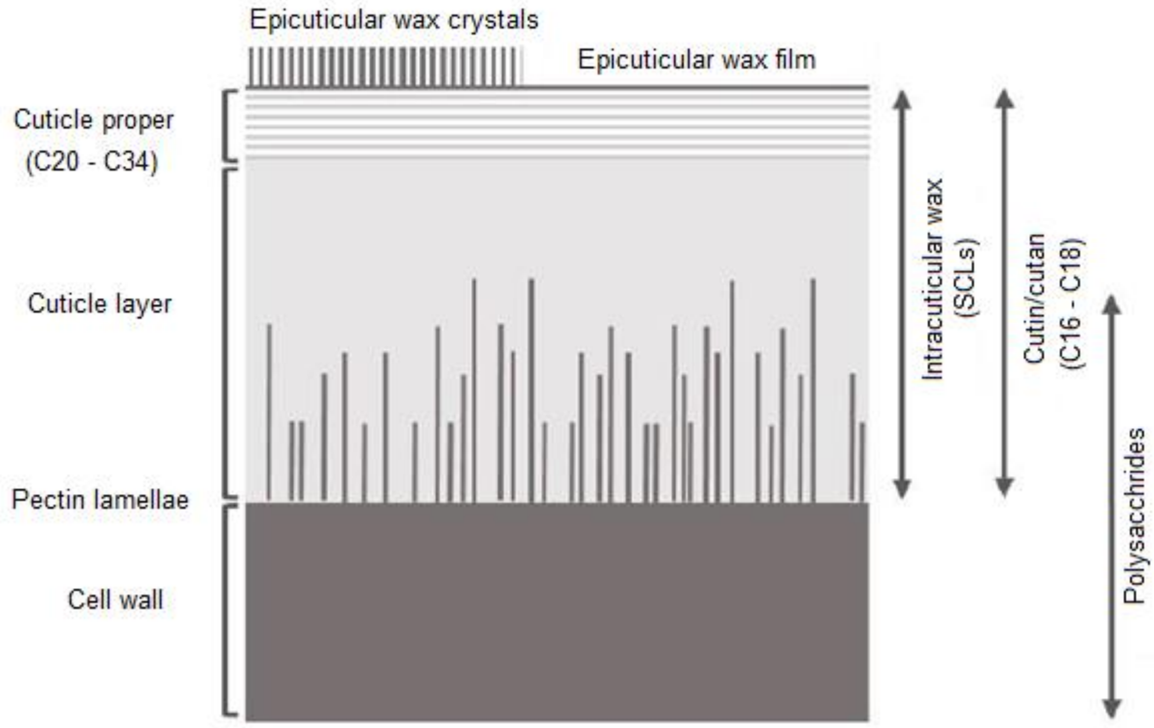


Figure 4: Simplified schematic of cuticular membrane of a leaf: *Simplified diagram of layers in the leaf cuticle. Arrows on the right indicate compositional chemical gradients across the cuticle. The cuticle proper consists of C20-C34 long chain lipids. Intracuticular wax consists of embedded soluble cuticular lipids (SCLs) and cutin/cutan C16-C18 lipids. Figure from (Schuster et al., 2017).*

1.4.2. Penetration into the leaf.

Penetration requires diffusion from the soluble phase from the droplet (donor) through the solid cuticle and then desorption at the apoplast side and translocation to the rest of the plant (receiver). Total amounts of A.I penetrating through leaf epidermises varies by 4 orders of magnitude depending on species and compound type (Schönherr and Riederer, 1989). The uptake pathway and rate will depend on type of molecule, its diffusion and partition co-efficients and the permeance across the cuticular membrane and to a lesser extent the resistance from the cell wall and plasma membranes. The chemical properties to help predict permeability include Log P as a measure of octanol-water partitioning, HLB values as a ratio of hydrophobic and lipophilic moieties in the molecule and its pKa value, the pH requirements for dissociation, which is particularly important for ionisable pesticides.

1.4.3. Nature of the lipophilic pathway.

Pesticides that are lipophilic (Log P value 2-7) can more easily adsorb into the epicuticular waxes on the surface of the cuticle and absorbance occurs through the intracuticular layer dissolved in lipid fractions including with cutin and soluble lipids (Schönherr and Riederer, 1989). Lipophilics can be limited by the thickness of the intracuticular wax layer, which is the more polar side of the membrane and as such, movement through this layer occurs 6 times slower than for hydrophilic molecules (Schönherr and Riederer, 1989). High temperature increases penetration of lipophilics through the wax layer but low temperatures can limit fluidity. Desorption on the cell wall side is the rate limiting step for lipophilics, however diffusion continues aslong as pesticide metabolism or translocation continues. (Schönherr and Riederer, 1989).

1.4.4. Nature of the hydrophilic pathway.

The proposed method of entrance into the leaf for polar and ionic molecules such as glyphosate and metal ions are through the hydrophilic route. Water-soluble and ionic pesticide movement through the wax cuticle is not energetically favourable and is slower than for lipophilic pesticides. This route is dynamic and characterised by hydrogen bonding with available non-ionised side groups of carboxyl, hydroxyl, carbonyl, esters and amines on various compounds in the cuticle, especially polysaccharides (Schönherr and Riederer, 1988). The crystalline fractions of the epicuticular wax layer which are composed of non-polar alkanes and alkyl esters (C30-C70) restrict polar transport as they are difficult to wet (Jetter, 2006, Buschhaus

et al., 2007). Water-soluble molecules are forced to move in amorphous more polar and aromatic waxes which may be limited, depending on the nature of the cuticle. Pentacyclic triterpenoids are more able to bind water and form a significant fraction of the intracuticular layer in some species (Xu et al., 2010b, Zeisler-Diehl, 2018). The epicuticular waxes form protrusion structures on the surface, which when tightly packed, limit wettability and water transport. An increase in air humidity increases from 2% to 100% cuticular permeability to water increases by a factor of 2-3 (Schreiber et al., 2001). Cuticular permeability to water loss through vapour is between $10^{-4} – 10^{-6}$ m s^{-1} however abiotic stresses such as high UV-B radiation modify the cuticle and may decrease cuticular water loss. (Kerstiens, 1996). Desorption of 2,4-Dichlorophenoxyacetic acid (2,4-D) systemic herbicide in fruit cuticles was found to be 50-80 times faster from the intracuticular layer than the outer, due to apolar and crystalline waxes in the outer layer (Schönherr and Riederer, 1988). Other research has confirmed the gradient nature of cuticles by characterising the compositions across species, in mutants and by assessing the contributions of the two layers to water permeability (Beattie and Marcell, 2002, Vogg et al., 2004, Jetter and Riederer, 2015). Physical packing of waxes, length of hydrocarbon chain and nature of waxes and the availability of un-ionised groups limits penetration of water and ionic compounds.

1.4.5. Surface roughness.

Plant lamina are not homogeneous: they may be striated and consist of cuticle overlaying epidermal cells with regular patterning of stomata and various extensional leaf structures in some species *e.g.* trichomes, spines. The homogeneity of the surface effects wetting, adhesion and evaporation of droplets during foliar spray application (Lin et al., 2016). The nature of the surface effects surface free energy at the droplet/surface interface, which results in varying contact angles of droplets. Wheat and tomato are difficult to wet and have low surface free energy because of the presence of trichomes which traps droplets with air underneath, this leads to faster evaporation of droplets on hairy surfaces (Yu et al., 2009). Cuticle thickness also varies between plants over guard cells, stomatal ledges and stomatal pores. For instance, poplar has limited cuticle thickness over stomatal pores but a thicker cuticle over guard cells (Fernandez et al., 2016). Variations in cuticle thickness and frequencies of surface structures alter surface contact, wettability and absorption but this is not well understood (Fernandez et al., 2016). Stomata have different morphologies and densities between species and between adaxial and abaxial surfaces. There are also differences between stomatal numbers and cuticle thickness at different ages of leaves of the same species in some plants (Beattie and Marcell, 2002, Park, 2004, Domínguez et al., 2017).

Entry through the stomata into the substomatal cavity has been previously thought to offer a transportation route for hydrophilic molecules, especially when stomata are open (Schönherr and Bukovac, 1972, Falk, 1994). Research has shown that penetration through stomatal margins is limited by the morphology of the stomata and solutions should have low surface tension (less than 30mN m^{-1}) and a contact angle around zero to be able to gain access and facilitate entry in through the stomata (Fernández and Eichert, 2009, Eichert and Burkhardt, 2001). Organosilicone is a synthetic ionic surfactant adjuvant composed of a polar head with Silicon oxide

groups and a lipophilic hydrocarbon tail which is ethoxylated. Organosilicone surfactants increase wetting and faster drying over the surface and presumably also faster, more efficient penetration as solutes pass through the stomatal pathway as well as the cuticular pathway (Fernández and Eichert, 2009). Although research has shown organosilicone surfactants can increase penetration of copper ions and uptake of bacteria such as *Agrobacterium tumefaciens* in plants, it has not been shown that they result in increased bioactivity of pesticides, in particular systemic herbicides (Zidack et al., 1992, Orbovic et al., 2007, Zollinger, 2012, Lizamore and Winefield, 2014).

1.5. Biocontrol methods and their limitations.

Pesticides have been relied upon by growers for at least the last 70 years (Popp et al., 2013). Long term pesticide use results in the selection of resistance in target organisms, they are linked to cancer and hormone disruption in farm workers (Wiklund, 1994, Toppari et al., 1996, Sparks and Nauen, 2015). Broad spectrum pesticide use reduces biodiversity, with harmful effects on non-target organisms such as birds, bees and aquatics (Arias-Estévez et al., 2008, Desneux et al., 2007, Wood et al., 2018). Pesticides also affect natural enemies and parasitoids. Many pesticides are being withdrawn, especially in EU countries. DDT was banned in the 20th century, Between 2012- 2014, 3 neonicotinoids were banned in the EU, chloropyrifos was banned in 2016 and glyphosate is expected to be banned in the future (Kathage, 2018, Mole, 2019). With fewer chemicals available, the emphasis has been placed on integrated pest management (IPM) strategies, which explore the use of physical, mechanical and biological/genetic alternatives to manage pests (van Bruggen et al., 2015). In the USA, organic farming limits the use of chemicals defined by the Environmental Protection Agency as “pesticides” which are subject to the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), and the Federal Environmental Protection Control Act (FEPCA) (Foy, 2017). Demand for organic food is increasing as people become more aware of harm from chemicals and seek healthier diets (Wier and Calverley, 2002). However, alternative methods of pest control to replace pesticides, such as biopesticides, still have their limitations.

The use of live organisms such as: nematodes, bacteria, fungi or viruses that parasitize plant pests, are all examples of biopesticides. The classification system also includes natural products such as plant-based (botanicals) or microbe derived extracts (macrobials) and semiochemicals *e.g.* insect pheromones (Lacey, 2003, Chandler, 2011). Biopesticides are often highly specific for a target organism *e.g.* Baculovirus for apple coddling moth or entomopathogenic nematode (EPN) species that target root weevil (Schroeder and Sieburth, 1997, Shapiro-Ilan, 2006, Shapiro-Ilan et al., 2006a). Biocontrols have experienced growth in the market, microbials have grown by 20.2% and all other types of biopesticides such as botanicals, macrobials have had between 15-20% growth (FAO, 2012). Biopesticides can give variable results in the field. Optimum biocontrol may only be achieved when pest populations are low and biopesticides are in the same target location as the pest (Chandler, 2011). Therefore, some knowledge is required about the target pest, such as location on the crop, stages of the lifecycle where they are most damaging and number of pests (Lacey, 2012). Biopesticides are also limited by high pressure during spraying, chemical residues in

spray and tank equipment and storage conditions, which can severely affect survival rates and biocontrol efficacy (Shapiro-Ilan et al., 2006a, Gasic, 2013). For foliar application in particular, biopesticides are sensitive to the environment and have limited mobility. During application, coverage over a leaf, UV protection and moisture enhancing are important factors to aid survival rates and biological control efficacy in a desiccating leaf environment.(Bailey et al., 2007, van Bruggen et al., 2015). Moisture has been shown to be limiting for soil applications of EPNs on fruit and nut weevils and research has found the optimum biocontrol occurred within a narrow boundary (Shapiro-Ilan, 2006, Shapiro-Ilan et al., 2006a). Moisture levels during storage are also limiting for fungi as they may germinate too early and be ineffective when applied to crops. Formulations such as wettable powders and wettable granules are being used to store them in the absence of water but allow high germination when water is added to activate (Lyn, 2010). Separate studies which analysed biocontrol efficacy after application of the biofungicides *Microsphaeropsis orachea*, *Penicillium frequentans* and the myco-herbicide *Colletotrichum truncatum* found efficacy was limited by the type of formulation and the number of small droplets in a spray, causing high drift losses and fast evaporation of droplets from the target leaf. High retention was necessary to promote germination and the adhesion of conidia to the leaf. High coverage over the leaf increased competition with parasites and was shown to give the best levels of pest control (Egley et al., 1993, Kessel et al., 2002, Bailey et al., 2007, Guijarro, 2018). Biopesticides have had limited use by growers previously because they are highly specific to a given plant-pest problem and knowledge of application is needed for specific plant pests (Chandler, 2011). Using formulations and the application of adjuvants may be one solution to help broader adoption of biopesticides as reliable biocontrol methods.

Gliocladium catenulatum is a biofungicide that parasitizes multiple pathogens and gives 65-88% control against damping off in bedding plants caused by *Fusarium spp*. and did well compared to other biocontrols against *Botrytis aclada* on dead onions and lilly leaves (Simay, 1988, Köhl et al., 1997, McQuilken, 2008). *G. catenulatum* has also been shown to colonise roots and have lasting biocontrol for 5 weeks against a range of soil pathogens (McQuilken, 2008, Chatterton et al., 2008, Chatterton and Punja, 2009). *G. catenulatum* can also colonise stems and leaves if provided with 12 hours of leaf moisture, and wound sites were preferred by the fungus (Chatterton and Punja, 2011). *G. catenulatum* has also shown good efficiency in field trials against anthracnose in berries (Verma et al., 2006). Adjuvants may be useful to improve moisture retention, UV protection or even penetration, to maximise biocontrol for foliar application (Schroeder and Sieburth, 1997, Shishkoff, 2002). Organic farming practices such as using biological control cannot adopt surfactant adjuvants and be considered organic. However, adjuvants that are biologically derived can be applied in organic farming. Adjuvants already improve coverage, moisture retention and UV protection for conventional pesticides so offer promise to improve biocontrol applications (Nauen, 2007).

1.5.1. Organosilicone surfactants and their limitations.

Organosilicone chemical adjuvants can be criticized as they produce fine mists and are lost through spray drift. Organosilicone adjuvants are toxic to non-target organisms

and can enter the soil and water systems through losses from application. Non-ionic organosilicone surfactants are able to penetrate the cell membrane by increasing fluidity in the membrane and causing leakage, especially in aquatic organisms (Ciarlo et al., 2012, Mullin et al., 2016, Mojsiewicz-Pieńkowska et al., 2016). These types of surfactants tend to be used with biopesticides with some known detrimental effects on colony forming units (CFUs). As organosilicone surfactants are synthetic chemicals they cannot be applied in organic farming practices. As an alternative, bio-adjuvants are a range of adjuvants which are organic and derived from plant and microbial sources, which if proven to be effective compared to organosilicone-type surfactants, can be an organic alternative for conventional spraying practices and also be applicable for biopesticides. To increase widespread use of bio-adjuvants, they first need to be compared to the silicone alternative. Biosurfactants such as rhamnolipids have shown promise in foliar penetration experiments and crop oils have been found to have fewer toxic effects to non-target organisms (Ciarlo et al., 2012, Liu et al., 2016). Ethoxylated seed oils have proven to be a more environmentally friendly alternative adjuvant for glyphosate than tallow amines (Hunsche and Noga, 2012).

1.5.2. Market trends in biocontrol applications.

Global biocontrol sales have increased from around US$ 0.6 billion in 2003 to around US$ 3 billion in 2017 and are projected to reach US$ 11 billion by 2025 (Bullion, 2017). There will be more biopesticides on the European market than chemical ones within five to ten years (Cary, 2019) and organic farmland is increasing globally at a rate of 15% per year. The most recently reported figure is 57.8 million hectares of organic farmland (Willer, 2019). Therefore, global agricultural growth patterns create a market for bio-adjuvants.

1.6. Aim of this study.

The aim of this study is to screen a range of biological adjuvants against an organosilicone standard adjuvant. Bio-adjuvants have the potential to be applied in organic farming as well as with conventional pesticides, if proven to be effective for a range of adjuvant properties, with a range of methods. Adjuvants will be analysed for their surface characteristic behaviours, deposition on the target leaf during spraying, ability to penetrate the leaf and adjuvant compatibility with a biofungicide. A range of conventional and organic pesticides will be used to assess robustness of adjuvants. The hypothesis is that there will be no significant difference between bio-adjuvant performance compared to an organosilicone standard adjuvant performance with actives. Bio-adjuvants should be able to promote retention of actives, give high coverage of an active during spraying and aid penetration of actives.

The project is centred on the following 5 objectives to achieve this aim:

1. To develop methods to analyse droplet retention times (DRTs) of adjuvants mixed with water or different actives on the leaf surface.
2. To compare adjuvant spreading (droplet wetted areass) on a leaf with different actives.

3. To compare adjuvant coverage performance on a leaf after spray application with a pesticide.
4. To measure adjuvant carrier potential using fluorescent dyes and tracking them visually within the leaf or recovering quantitative fluorescence from leaf tissue.
5. To grow a commercial biofungicide in the presence of different adjuvants and assess the effect on biomass relative to a water control over a given time.

Chapter 2: Materials and Methods.

2.1. Materials.

The adjuvants and actives used in this study, were kindly given by Adgro Consulting Ltd the project industry partner.

2.1.1. Trade names of Adjuvants.

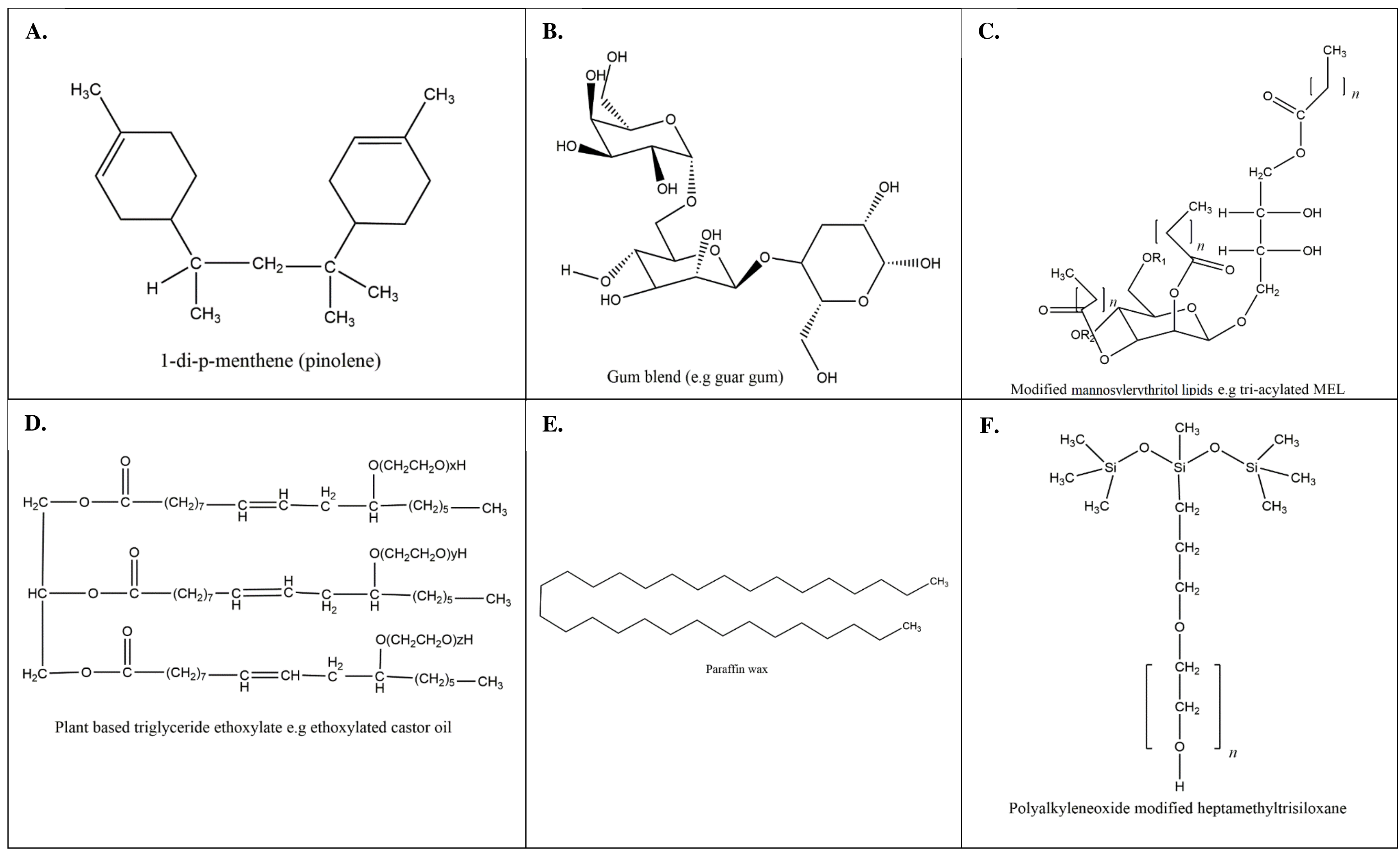
Code name	Adjuvant type	Chemistry
Adj J	Bio-adjuvant	Mannosylerythritol lipids (MELs) based biosurfactant.
Adj H	Bio-adjuvant	Mannosylerythritol lipids (MELs) based biosurfactant.
Adj G	Bio-adjuvant	Activated gum hydrocolloid blend.
Adj A	Bio-adjuvant	Plant monoterpene emulsion.
Adj 1	Bio-adjuvant	Paraffinic wax based.
Adj 3	Bio-adjuvant	Plant based ethoxylated triglyceride.
Adj 2	Synthetic	Trisiloxane superspreader surfactant.

Table 1: Description of adjuvants used in this study: *including the type and chemistry.*

2.1.2. Trade names of Actives.

Trade name	Active type	Chemistry
Act B Fungicide	Conventional	Pyriofenone (active ingredient)
Act C Insecticide	Conventional	Spirotetramat (active ingredient)
ACT E	Conventional	Kaolin, aluminosilicate mineral
Bioactive 1	Biological	*Gliocladium catenulatum* strain in milk powder
Bioactive 2	Biological	*Gliocladium catenulatum*

Table 2: Description of actives used in this study: *including the type, active ingredient and suppliers.*



2.1.3. Chemical structures and characteristics of adjuvants in this study.

Table 3: Structures of adjuvants used in the study**.** ***A.*** *Main component of Adj A. Organic monoterpene derived from wounding the bark of* Vateria indica *trees. Adj A contains the pinolene resin and a non-ionic surfactant to stabilise the emulsion.* ***B.*** *Adj G contains a mixture of carbohydrate-based polymers also described as hydrocolloid gums, e.g. guar gum, acacia gum, locust bean gum which are derived from wounding plants or from seeds/microorganisms. It also contains stabilisers and humectants in formulation which require the addition of water for activation.* ***C.*** *Developmental adjuvants Adj J and Adj H are composed of Mannosylerythritol lipids (MELs). MELs are glycolipid-based bio-surfactants composed of a mixture of 4 MELs (-A,-B,-C,-D) fermented by fungi and yeast such as from the genus* Pseudozyma spp. *and* Ustilago spp. *using a primary carbon source such as seed oil (Morita et al., 2009, Morita et al., 2015). C. Shows the structure of -D. Adj H has methylated rapeseed oil (MSO)) in the mixture but Adj J does not.* ***D.*** *Adj 3 contains a traditional plant oil forming hydrophilic tails e.g. castor oil, with the addition of variable numbers of ethoxylated groups as polar components, which give the oil hygroscopic properties. The structure above shows 3 EO groups (x, y, z) but the degree of ethoxylation in each component is unknown.* ***E.*** *Paraffin wax is a saturated hydrocarbon from the distillation of petroleum and is available as an oil-in-water emulsion (EW) formulation with the name Adj 1. F. Adj 2 – Polyalkeneoxide modified heptamethyltrisiloxane. Non-ionic silicone surfactant synthesised by the chemical industry using one of three methods (Mojsiewicz-Pieńkowska et al., 2016). This organosilicone surfactant is typically used as a wetting agent and contains the silicone polar head and various degrees of ethoxylation at the termini of the hydrocarbon tail. The degree of ethoxylation is unknown and is represented by n. All structures drawn using ChemDraw 17 (PerkinElmer).*

2.1.4. Chemical/physical structures and characteristics of actives in this study.

A. Spirotetramat	**B.** Pyriofenone
C. Kaolin	**D.** *Gliocladium catenulatum J1446*

Table 4: Structures of actives used in this study. *A, B and C. Conventional actives. D. Biological active.* **A.** *Act C Insecticide (A.I Spirotetramat). Tetramic acid derivative, chemical name : (5s,8s)-3-(2,5-Dimethylphenyl)-8-methoxy-2-oxo-1-azaspiro[4.5]dec-3-en-4-yl ethyl carbonate. Formulated as: Suspended concentrate, classified as Group 23 insecticide, systemic. Treatment of aphids, whitefly, moths and juvenile thrips on brassica, lettuce, corn, vegetables, beans, peas, cotton and onion. Treatment of scales, mealy bugs, thrips on citrus and pome fruits. M.O.A : Inhibition of acetyl-COA carboxylase and affects lipid synthesis and growth.* ***B.*** *Act B Fungicide (A.I Pyriofenone). Aromatic aryl phenyl ketone, chemical name : 5-chloro-2-methoxy-4-methyl-3-pyridinyl)2,3,4- trimethoxy-6-methylphenyl)methanone. Formulated as: Suspended concentrate, classified as B6 classified Fungicide, locally systemic. Treatment of pathogenic fungi in* Erysiphales *family including powdery mildew on wheat, barley, grapes and curcubits (Bernard et al., 2012). M.O.A : Disrupts actin/myosin or fimbrin function. Inhibits formation of appressorium and penetration of fungal hyphae pre-disease. During disease, inhibits secondary hyphae and spore production.* ***C.*** *ACT E Crop protectant and Insecticide (Kaolin mineral). Chemical name: Calcined kaolin. Formulated as : Wettable powder. Organic clay mineral which forms hydrophobic white particle film. M.O.A. Protects crops from heat stress, forms a physical and mechanical barrier to pests, affects landing, movement, ovipositioning and egg-laying of psyllids, two-spotted spider-mite, whitefly, aphids and leaf hoppers in apple, peach and pear trees and melon (Glenn et al., 1999, Liang, 2002, Peng, 2011). Used for fruit crops, pome and stone fruits, grapes, citrus, tomatoes, curcubits and pineapples.* ***D.*** *Biofungicide – A.I* Gliocladium catenulatum (syn. Clonostachys rosea f. catenulata strain J1446*). Formulated as: Wettable granules, Bioactive 1 has addition of milk powder. Hyperparasite against* Sclerotina sclerotorium, Fusarium. spp, Pythium ultimum and Rhizoctonia solani, Colletotrichum acutatum *(Huang, 1978, Verma et al., 2006, McQuilken, 2008, Chatterton et al., 2008, Chatterton and Punja, 2009, Chatterton and Punja, 2011) M.O.A. Positive chemotropism towards parasite, forms pseudo-appressorium, holds, coils around parasite and degrades cell walls to damage cells (Huang, 1978). Beta 1-3 glucanase and chitinase are released in the presence of some pathogens (Chatterton and Punja, 2012) (Chatterton and Punja, 2011). All structures drawn using ChemDraw 17 (PerkinElmer).*

2.1.5. *Commelina communis* as a model species.

C.communis was selected as a model plant for this work. It was chosen particularly for the penetration analysis of adjuvants because of its ease to visualise within tissues, relevant for systemic pesticides. *C.communis* has been used historically at Lancaster University in pioneering research in stomatal physiology and seed stocks are readily available. Bio-assays have previously been developed using epidermal peels to study stomatal responses to light, CO_2, pH, temperature and external chemicals in the incubation media. External Abscisic acid was shown to increase stomatal closure, redefining the understanding of plant signalling response to drought. (Mansfield and Jones, 1971). Stomatal aperture was found to be under the control of calcium ion flux which was downstream of Abscisic acid in the signalling pathway. (Atkinson et al, 1990). The fungal toxin fusicoccin was shown to promote stomatal opening by increasing K^+ flux into guard cells. (Squires, 1974).

The first techniques for peeling *C.communis* was described by Willmer & Mansfield, 1969 who used first fully expanded leaf tissue and dark treatments to close stomata. The inclusion of MES buffer with KOH as an optimum buffer for epidermal peels was described by Blackman and Davies, 1983. In a comparison of peels derived from different plant species, Commelina epidermal peels were shown to have high functionality of guard cells after peeling and had low contamination from the mesophyll layer. In addition to this, it is easy to peel, easy to handle and peels can be manipulated on buffer easily. (Weyers and Travis 1981). Commelina is also a fast-growing species, which germinates readily and has well characterised stomatal characteristics. Accumulation of calcium oxalate into raphides in a monocot such as Commelina means that older leaves have different leaf characteristics. (Prychid, 1999). This literature guided the design of the methods for Commelina used in this present study.

Figure 5. Four week old *C. communis* plants: *Grown in controlled growth conditions.*

2.2. Methods.

2.2.1. Methods for analysis of droplet behaviour.

2.2.1.1. Growing *Commelina communis*.

Commelina communis seeds were potted into Levington advanced M3 compost mix in individual 4" square, plastic pots. The trays beneath the pots were watered three times weekly with 500 ml water per tray of 9 plants, to maintain high soil water levels. Plants were grown in a controlled environment between 17 and 22.5˚C (Avg 19.8˚C + SE $\pm$ 5.27) at a relative humidity (RH %) between 46 and 86.5% (average 67.7% $\pm$ SE 9.02). Plants were grown under LED (Valoya NS1) lights and intensity ranged between 130 and 148 µmol m^{-2} s^{-1}. At a short day photoperiod of 10 hours day, 14 hours night. No fertiliser or pesticide products were applied to plants during growth. Fully expanded 3rd and 4th leaves from 4 week old plants were taken for droplet retention analysis, thermal imaging, droplet area measurements and penetration studies using Lucifer yellow dye and a systemic fungicide with a fluorescent active ingredient (Act B ® 300 SC Fungicide).

2.2.1.2. Droplet retention time (DRT) analysis.

Four week old fully expanded *C. communis* leaves were excised at the base. The cut ends of the leaves were immediately wrapped in tissue paper wetted with 1 ml water to maintain moisture content. Adjuvants and actives were diluted using reverse osmosis (R.O) water to working concentrations as per company recommendations and using the dilutions as shown in Table 5 and 6.Working concentrations of adjuvants and pesticides were mixed at a ratio of 1:1 *e.g.* 35 µl active + 35 µl adjuvant and then vortexed for 10 seconds prior to treatments. Excised leaves were placed adaxial side up, flat in a Petri dish and different volumes of adjuvants/active solutions added droplet-wise to one side of the midrib per leaf. For each combination of adjuvants + water or actives, droplet volumes 1, 2, 3, 4 and 5 µl were used. Droplet behaviour was observed throughout the experiment using a Leica MZ6 (Leica AG, Germany) dissecting microscope at a resolution of 6.3x, affixed with a camera (Scopetek MDCC560 5.6M px) to photograph droplets at 10 minute intervals and a stopwatch used to quantify droplet lifetimes. Lifetimes were quantified when droplets were no longer visible or in the case of certain adjuvants, after flattening, when no further shrinkage or retraction occurred. Experiments were conducted at ambient temperature of 24˚C and with a RH of 40%. n= 20-60 repeats for each adjuvant when used alone (with water), variation in numbers of repeats were due to variations in consistency between adjuvants, in particular Adj G which gave variable lifetimes so was repeated 12 times in total. n = 20 per adjuvant when used with Act C or Act B SC 300 and n = 30 for adjuvants with Bioactive 1 and Bioactive 2. Raw data were plotted separately for each active as box and whisker plots using R. R studio is available at: https://rstudio.com/products/rstudio/download/#download to show spread of data. Data were then plotted as bar graphs to combine active datasets. Two-way ANOVA and post-hoc Tukey analyses were used to statistically analyse DRTs. For the dataset with no actives added to adjuvants n = 53, for the two conventional pesticides datasets

n=32 and for bio-pesticides n = 48. All analysis tests were performed with SPSS Statistics 24 available at: https://www.ibm.com/uk-en/products/spss-statistics. Statistical significance was considered at P < 0.05.

Table 5: Recommended working dilutions for adjuvants.

Active	**Testing rate**	**g/ or µl /1 ml**
Act B	50 ml /100L	0.5 µl
Act C	40 ml /100L	0.4 µl
ACT E	5kg / 100L	0.05g
Bioactive 1	500g / 100L	0.05g
Bioactive 2	500g / 100L	0.05g

Table 6: Working dilutions of actives.

Adjuvant	**Testing rate (ml/L)**	**µl/1 ml**
Adj A	125 ml / 100L	1.25
Adj G	250 ml / 100L	2.5
Adj H	250 ml / 100L	2.5
Adj J	500 ml / 100L	5
Adj 1	100 ml / 100L	1
Adj 2	50 ml / 100L	0.5
Adj 3	150 ml / 100L	1.5

2.2.1.3. Droplet area measurements.

Droplets were applied to leaves and photographed at 6.3x using a Leica MZ6 (Leica AG, Germany) dissecting microscope affixed with a camera (Scopetek MDCC560 5.6M px). Ten different leaves were used for each adjuvant/active combination to give 10 replicates for each volume of each adjuvant/active mixture. Adjuvants alone (mixed with water) and adjuvants with Act B had between 39-52 repeats per adjuvant, Act C, ACT E, Bioactive 1 and Bioactive 2 had 50 repeats for each adjuvant. The photographs were loaded into ImageJ available at: https://imagej.nih.gov/ij/ and the droplet areas worked out in pixels using the 'oval' tool before being converted into μm^2. Raw data were plotted as box and whisker plots for each active. Active datasets were combined using a bar graph, using R studio. Data were analysed using Two- way ANOVA and Tukey post-hoc analyses with SPSS Statistics 24. Statistical significance was considered at P < 0.05.

2.2.1.4. Thermal imaging for measuring droplet lifetimes.

Four week old excised leaves were cut from *C. communis* plants and placed adaxial side up, flat in a Petri dish on a dark stage. A FLIR A325Sc infra-red camera was suspended above the stage. Leaves were not wrapped in wet tissue paper as this interfered with the cooling images on the thermal camera. Adjuvant/pesticide mixes were pre-mixed and vortexed in a ratio of 1:1 and added drop-wise to the leaf under observation. Individual droplet volumes of adjuvants with the Act B fungicide and Act C insecticide actives were added to the same leaf. Videos were set to record at 10 frames every 10 seconds until droplets were no longer visible as cooling spots. FLIR tools (https://www.flir.co.uk/products/flir-tools/) was used to record temperature measurements and import videos into WMV format.

2.2.1.5. Droplet deposition of adjuvants.

Adjuvants and actives were made up as per recommended rates in Table 5 and 6 and vortex mixed 1:1 and added to a leaf. Deposits from 1,2, 3, 4 and 5 µl deposits were photographed after droplet drying using a Leica MZ6 (Leica AG, Germany) dissecting microscope affixed with a camera (Scopetek MDCC560 5.6M px). Single 30 µl droplets of Act C active + adjuvants or water were added to strips of Parafilm and photographed the same way. Lucifer yellow dye and adjuvants or water (5mM Lucifer yellow final conc.) were also vortex mixed 1:1 and used as 30 µl droplets on the leaf for deposition visual analysis.

2.2.1.6. Percentage coverage of pesticide with adjuvants.

For the ACT E pesticide, a small-scale sprayer was used to spray the product with adjuvants or water and measure percentage leaf coverage. Four week old *C. communis* plants between 13 cm and 15 cm tall were placed on a stage with a height of 21 cm, with the 3rd fully expanded leaf 90 degrees to the sprayer which was held at a constant angle and height. The adjuvant/pesticide mix was used at the recommended rates as in Table 5 and 6 and made to a 15 ml volume of stock. 10 successive sprays were made at each plant at 2 Psi with a flow rate of 159 ml/min using an air gun with fine nozzle spray head. The leaves were left to air dry on the plants for 1 hour, before being excised and photographed on a dark background. Percentage coverage values were obtained for each leaf using Image J. The images were made binary, thresholding the white particles against the green leaf background and quantifying percentage area of white on the total leaf surface. 4 leaves were sprayed per adjuvant/pesticide mix and a mean percentage coverage obtained from the 4 measurements' average.

2.2.2. Methods for analysis of adjuvant penetration.

2.2.2.1. Lucifer yellow (LYCH) tracer dye assay.

Four week old plants with first, fully formed expanded leaves were put in the dark prior to beginning of experiments for 6 hours. Then one leaf per plant was excised and individual leaves were placed in closed containers with wet tissue paper wrapped around the petioles. Adjuvants were made fresh according to manufacturer's recommendations (Table 5). Lucifer yellow CH (dipotassium salt; Sigma Aldrich, U.K) was made into a 10 mM stock and was pre-mixed with adjuvants just before the experiment in a ratio of 1:1 to give a final concentration of 5 mM LYCH. Two selected areas on the adaxial surface, one on each side of the midrib on each leaf, was marked with 5 mm pieces of circular plastic drinking straws affixed with Vaseline, to create a well in which to add the solutions. Adjuvant/LYCH solutions were vortexed for 15 secs just before addition to leaves. 30 µL droplets of adjuvant/LYCH mixtures were pipetted into the centre of the wells and left for 1, 3, 6or 22 hours. Leaves were kept in the dark through the duration of the experimental treatment times and maintained at a high humidity and with an average temperature of 22.4˚C throughout the experiment.

2.2.2.2. Spectrofluorometric analysis to quantify Lucifer yellow (LYCH) recovery from leaves.

One leaf disc per leaf was cut from the treated area using a scalpel, into 10 mM MES buffered to pH 6.2 using KOH. Leaf discs were briefly washed in water baths containing fresh water, dried with tissue paper and added to 1.5 ml microcentrifuge tubes containing 100 µL 10 mM MES buffer and stored frozen at -20˚C. The leaf discs were ground into the MES solution using microhomogenisers and the solid left to settle to the bottom of the tube, before the supernatant was pipetted into black NUNC 96 well plates and read by an FLUOStar Omega Microplate reader (BMG Labtech, Germany). Plates were excited with 485 nm light and emission read at 520 nm for detection of LYCH. For the 22 hour treatment set of leaves, any remaining solution on the surface, was recovered and diluted in 100 µL water and read in the same way. The 5 mM stock solutions of LYCH + adjuvants or water only were diluted by 10 and read in the same way.

2.2.2.3. Fluorescence microscopy of Lucifer yellow (LYCH) in leaf tissues.

Each leaf was washed in fresh water and patted dry with tissue paper. A piece of leaf blade from one side of the midrib was cut into a rectangle using a fresh razor blade into either mineral oil (1, 3, 6 hours) or 10 mM MES buffer (22 hour samples). Time points were chosen to measure fluorescence in tissue samples before droplets had dried down, around droplet dry down and hours after droplets had dried. The blade was cut so to have the treated area in the centre of the rectangle. The edge of the adaxial surface edge was scored with a blade and the lower epidermis peeled off. Both the tissue with the mesophyll surface facing up and the lower epidermal peel were mounted on a clear microscope slide in either mineral oil or 10 mM MES buffer and sealed under a coverslip using non-UV varnish. Microscope slides were stored in the

dark at 4˚C. Slides were viewed on a Nikon Diaphot 300 epi-fluorescence microscope powered with a Cairn LED system using 430 nm excitation and emission was limited to 505 nm using 505 long pass filter. Confocal Z-stack images were taken of the vasculature/mesophyll and epidermis of each slide of treated tissue using a Zeiss LSM800 laser detection confocal microscope. Excitation was set at 405 nm and emission ranged from 509 nm to 581 nm for LYCH and a separate channel added also at 405 nm, collecting emission at 700-760 nm for Chlorophyll A. Image stacks were later compiled into 3D projections using Image J default settings.

2.2.2.4. Detection of Act B: Pyriofenone fluorescence analysis.

4 week old plants were treated using a similar assay to the Lucifer yellow described above but instead used adjuvant:Act B mixes also in ratio of 1:1, made according to manufacturer's recommended concentrations and vortexed just before use. Treated leaf discs (time course every hour from 2 hours to 27 hours) were harvested and read using the FLUOStar Omega Microplate reader (BMG LABTECH, Germany) at either an excitation of 355nm and emission of 460nm or with an excitation of 485nm and emission of 520nm to attempt to recover and detect Pyriofenone from leaves. Leaf areas were treated for 22 hours, then each leaf was cut, peeled and embedded (as above). The Zeiss LSM800 confocal microscope was used to attempt to detect pyriofenone in leaf tissues using excitation at 405 nm and emission within 430-460 nm or 440-550 nm. Fluorescence lifetime image mapping (FLIM) were recorded on a MicroTime 200 (PicoQuant, Germany) time resolved confocal fluorescence microscopy system consisting on an Olympus IX73 confocal microscope fitted with a x100 oil objective excited with a picosecond pulsed laser 405 nm PicoQuant laser to attempt, to resolve pyriofenone based on time resolution of its fluorescence in prepared leaf tissue samples. Preliminary fluorescence analysis of pyriofenone (excitation and emission spectra) of Act B was performed on a Cary Eclipse in DCM solution. 1H Nuclear magnetic resonance (NMR) of Act B was recorded on a Bruker Advance 400 MHz spectrometer in a 5 mm NMR tube in solution in $CDCl_3$.

2.2.3. Methods to assess compatibility of adjuvants with biofungicide.

2.2.3.1. Growth of Bioactive 2 on adjuvant-adjusted agar.

To see whether Bioactive 2 (*Gliocladium catenulatum* as wettable granules) could grow on potato dextrose agar (PDA) plates in the presence of adjuvants or two actives, PDA plates were made containing 24 ml PDA and 1 ml adjuvant or active concentrations as recommended for a total of 25 ml solution. When the PDA was semi-cooled, the adjuvant mixes were added and swirled to mix. Bioactive 2 mycelium inoculum was taken from a fresh PDA plate and 0.02 g added to the centre of each PDA plate containing adjuvants. The plates were grown at 22˚C in the dark for up to 5 days (120 hours) to monitor the expansion of the initial inoculum across the plate in the presence of adjuvants/actives. The plates were photographed at 0, 2 and 5 days and loaded into Image J. Image J was used to measure the width of the inoculum relative to the plate. One plate per adjuvant/active was made to test initially whether adjuvants or actives had any obvious inhibition of plate spreading capability of Bioactive 2.

2.2.3.2. Growth of Bioactive 2 in liquid media after adjuvant incubation.

Bioactive 2 commercial powder was weighed as 0.5 g and diluted into 5 ml stock solutions of different adjuvants or in triple strength Act B or 70% ethanol. Adjuvants were made up as detailed in Table 5 and initial stocks of Bioactive 2 were made up at 2 x 10^8 cfu/ml. These solutions were incubated for either 10 mins (n= 3) or 60 mins (n= 6) at 29˚C at 250 rpm in the dark. Stocks were then vortexed and diluted to 1 x 10^7 cfu/ml in sterile water. Empty tubes were pre-weighed, before 2 ml nutrient broth (NB) was added to each tube using sterile technique. Each Bioactive 2/adjuvant or active mix was vortexed for 30 seconds and 20 µL of each mix added to tubes of NB. Triplicate tubes of Bioactive 2/water mix were initially centrifuged at 4000 rpm for 10 mins to pellet the solid at the start of the experiment. The supernatants were discarded and tubes were left to air dry for 24 hours before being re-weighed to account for the initial weight of 20 µL spores added to the tube, prior to any growth. The remaining tubes containing Bioactive 2 were grown in the dark at 29˚C at 250 rpm for 3 days (72 hours) before they were also pelleted at 4000 rpm for 10 mins, supernatant discarded and the pellet air dried for 24 hours. The weight change between day 0 and day 3 was calculated for each tube to measure growth.

Chapter 3: Results.

3.1. Surface behaviour of adjuvants.

Adjuvant DRTs on *C. communis* leaf surface.

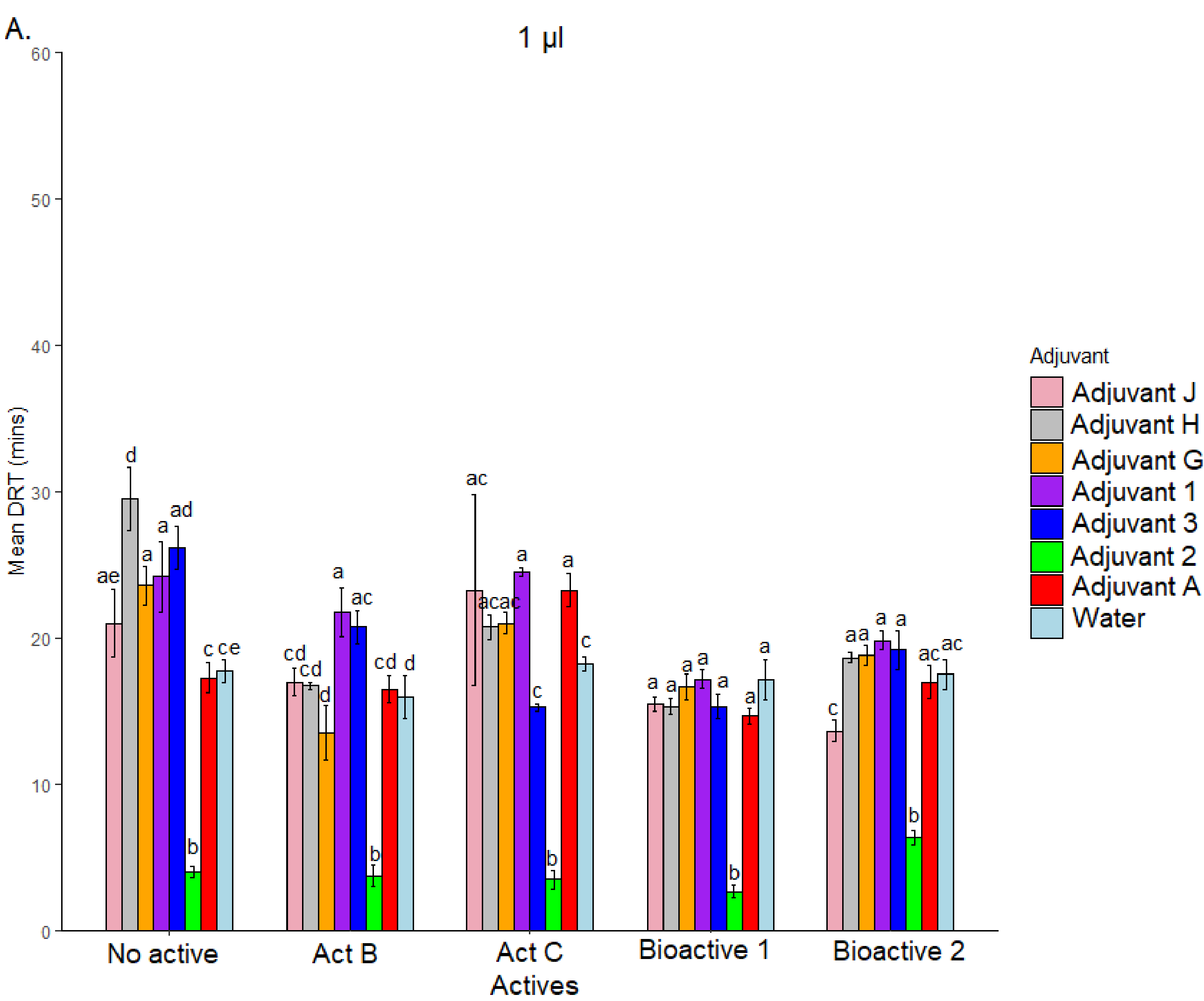
How long droplets containing adjuvants spend in the liquid phase on a surface of a leaf is an important factor when considering how much of an A.I dose may be delivered into the leaf. Different volumes of droplets were chosen to represent volumes applicable to agriculture. Different actives were mixed with different adjuvants and mean DRTs of these droplets measured, to be able to better understand adjuvant/active compatibility and identify combinations with maximum retentions.

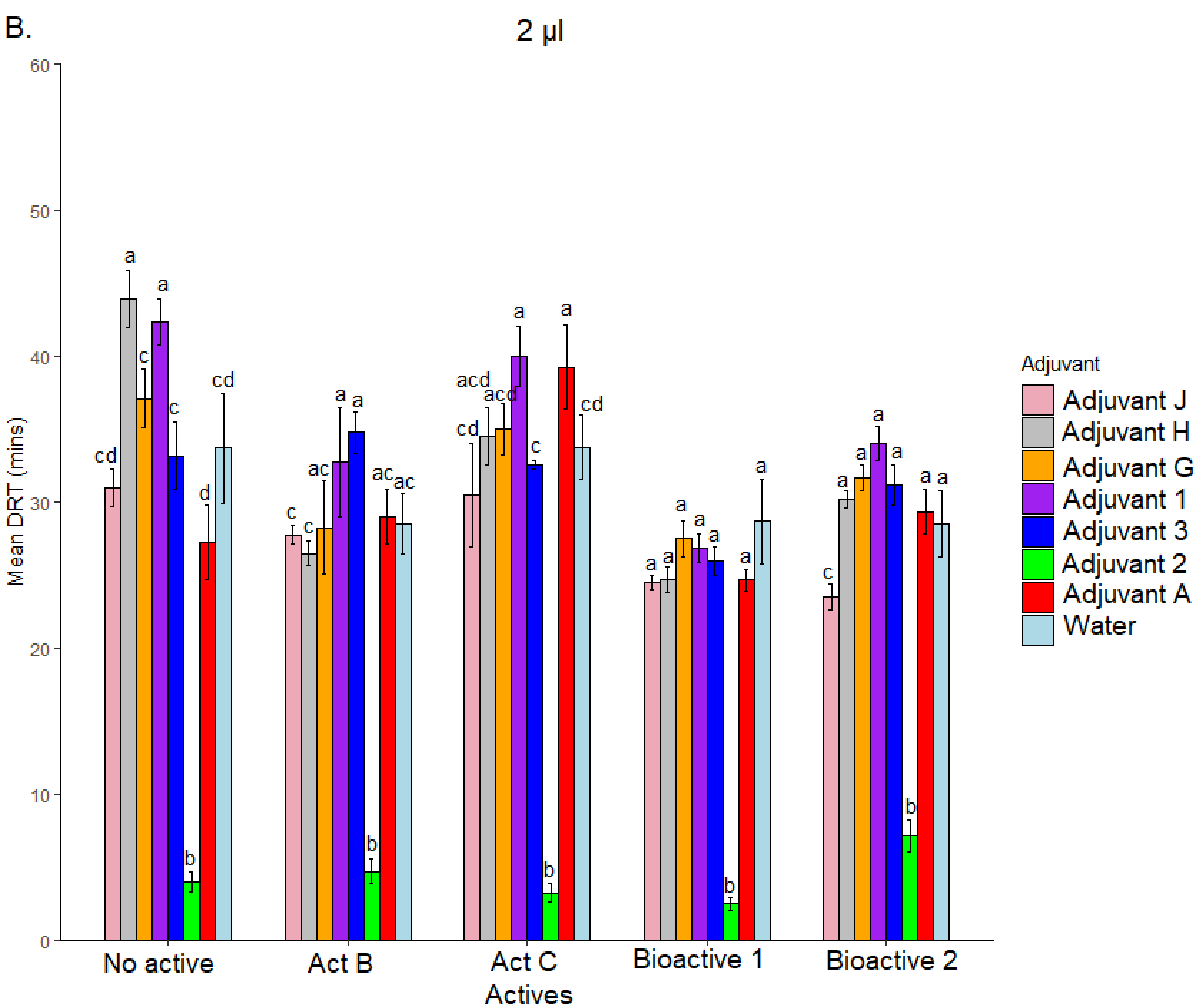
Variation in DRTs between adjuvants.

Adjuvant droplets were first compared without actives to see adjuvant effect on DRTs. As expected, retention times of droplets containing adjuvants increased linearly with volume (Appendix 1). The only exception was Adj 2, which dried significantly quicker than all other adjuvants, with a drying time ~ 10 mins or less, irrespective of volume applied. Relative to water, DRTs were increased by the addition of 5 of the adjuvants at 1 µl, 3 adjuvants at 2 µl and 4 adjuvants at 3 µl (Figure 6). Two adjuvants which had consistently higher droplet lifetimes than water at all volumes were Adj H and Adj 1. Adj 1 and Adj H also had higher DRTs than the majority of other adjuvants in 2 and 3 µl droplets. Adj A had significantly lower DRTs than water and all other adjuvants apart from Adj 2.

The effect of adjuvants on the DRTs of actives.

Adjuvants and actives both had a significant effect on DRTs of droplets (Table 7). However, adjuvants also varied in their effects on DRT, which is shown by the consistent significant interaction between adjuvants and actives (Table 7). Individual box and whisker plots can be found for DRTs for each active separately with adjuvants in Appendices 2, 3, 4 and 5. Figure 6 shows that droplets containing actives dried at a similar rate in water (except for Act C in 3 µl droplets). Actives also dried at a similar rate in Adj 2 but had between 3-5 times lower DRTs than when in water. Adjuvants had variable effects on active drying time. Adj 1 was the adjuvant which had some of the highest DRTs compared to water and different adjuvants with a range of actives. When combined to give 1 µl droplets with Act B, lifetimes were higher than 5 other adjuvants and water, higher than 4 adjuvants and water at 3 µl but was only significantly higher than 2 adjuvants and was no longer significantly higher than water when using 2 µl drops. In 1 µl droplets of Adj 1 with Act C, mean DRTs were significantly higher than Act C with 2 adjuvants and water, increasing to 3 adjuvants and water in 2 µl droplets and to 4 adjuvants and water at 3 µl. With Bioactive 2, Adj 1 DRTs at 1 µl and 2 µl were significantly higher than Bioactive 2 with 2 other adjuvants and water and this increased to 4 adjuvants and water at 3 µl.


A.
1 µl
Mean DRT (mins)
Actives
No active
Act B
Act C
Bioactive 1
Bioactive 2
Adjuvant
Adjuvant J
Adjuvant H
Adjuvant G
Adjuvant 1
Adjuvant 3
Adjuvant 2
Adjuvant A
Water


B.
2 µl
Mean DRT (mins)
60
50
40
30
20
10
0
No active
Act B
Act C
Bioactive 1
Bioactive 2
Actives
Adjuvant
Adjuvant J
Adjuvant H
Adjuvant G
Adjuvant 1
Adjuvant 3
Adjuvant 2
Adjuvant A
Water

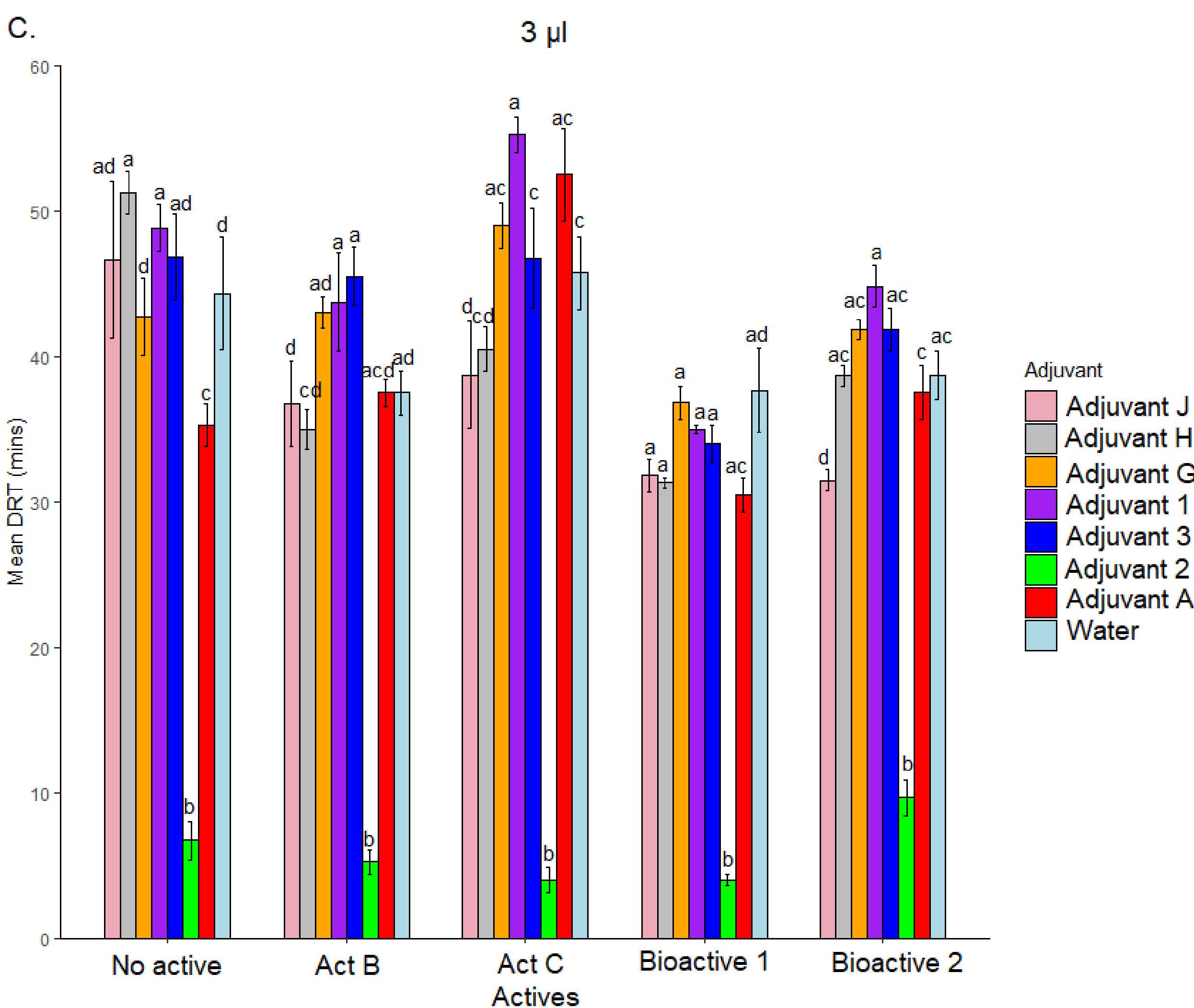

C.
3 µl
Mean DRT (mins)
0
10
20
30
40
50
60
No active
Act B
Act C
Bioactive 1
Bioactive 2
Actives
Adjuvant
Adjuvant J
Adjuvant H
Adjuvant G
Adjuvant 1
Adjuvant 3
Adjuvant 2
Adjuvant A
Water

Figure 6: Average DRTs of adjuvants with actives on *C. communis* leaf: *A. 1 µl. B. 2 µl. C. 3 µl. Adjuvants include two developmental MEL adjuvants Adj J, Adj H, humectants Adj G and Adj 3, Adj 1 emulsion, Adj A resin and Adj 2 organosilicone surfactant and water. Actives from left to right, No active (adjuvant used with water), Act B fungicide, Act C insecticide and two biofungicide preparations Bioactive 1 (*Gliocladium catenulatum *+ milk powder) and Bioactive 2 (*Gliocladium catenulatum*). Error bars show standard error SE. Letters above bars represent statistically significant groupings of adjuvants within each active grouping.*

	1 µl		**2 µl**		**3 µl**	
	F- value	***P- value***	***F- value***	***P- value***	***F- value***	***P- value***
Adjuvant	61.64	9.63×10^{-44}	124.18	4.83×10^{-64}	154.74	8.80×10^{-71}
Active	23.53	1.51×10^{-15}	28.83	2.30×10^{-18}	35.06	2.12×10^{-21}
Adjuvant*Active	3.74	4.52×10^{-8}	3.64	8.71×10^{-8}	3.94	1.21×10^{-8}

Table 7: Two-way ANOVA reported significance of mean drying times of adjuvants with actives: *Two-way ANOVA significance considered at 0.05%.*

The effect of actives on the DRTs of adjuvants.

As indicated by the two-way ANOVA results in Table 7, actives also had a significant effect on DRTs. Droplets containing Bioactive 1 caused the largest reduction in DRT. When Bioactive 1 was mixed with different adjuvants, droplets were retained for a limited time. With the exception of Adj 2, none of the adjuvants or water in 1 µl and 2 µl droplets, had mean DRTs that were significantly different from each other. In 3 µl droplets, as shown in Figure 6C, there was an increase in variation in DRTs, whereby the retention average of water with Bioactive 1 was significantly higher than Bioactive 1 with 4 adjuvants. This is the only active in which droplets containing water had higher DRTs than the active with adjuvants instead. The effect of Bioactive 1 can also be seen when comparing all combinations of Adj 1 with actives, mean lifetimes were significantly shorter for Adj 1 + Bioactive 1 than all other actives at all 3 volumes.

The addition of actives to the two developmental adjuvants Adj H and Adj J had a significant effect on DRTs. Adj H droplets had longer droplet lifetimes than all other adjuvants when no actives were present. However, both Adj H and Adj J adjuvants typically had shorter droplet lifetimes than water and other adjuvants, with a range of different actives (exception at 1 µl with Act C + Adj J). The addition of Bioactive 2 to adjuvant Adj J had significantly lower DRTs than all other adjuvants (apart from Adj 2) at 2 µl and 3 µl.

The addition of the Act C active to different adjuvants had a positive effect on mean drying times. At all volumes, Adj A had significantly higher mean DRTs with Act C SC than when used with any other active or alone. The effect of the addition of Act C to Adj A was more significant as volume increased. Figure 6C shows that 3 µl droplets of Adj A + Act C droplets lasted between 15-22 mins longer on the surface of the leaf than any other active with Adj A or using Adj A alone. The addition of Act C to Adj 1 gave the highest mean DRT of all other active combinations, at 3 µl. Similar to the behaviour with the Adj A adjuvant, the DRTs of Adj 1 and Adj G adjuvants were extended when Act C was present in the droplet.

Actives showed little effect on the drying time of Adj 2. At all 3 volumes, none of the mean drying times significantly differ between Adj 2 alone and between actives (Act B, Act C and Bioactive 1). In 3 µl droplets, the mean DRT for Adj 2 with Bioactive 2 was significantly higher than that of Bioactive 1 ($p=0.048$) but not significantly different from any of the other active combinations. Water droplets also had little variation with addition of actives, with only a slight increase in DRT at 3 µl when Act C is present in the droplet.

Thermal imaging as a method to compare DRTs.

Thermal imaging was used to measure adjuvant/active changes in droplet temperatures relative to the leaf surface during drying. It was theorised that the temperature difference due to evaporative cooling may be a proxy for DRT. Temperature differences were measured for both Act B fungicide (3 µl droplets shown in Figure 7) and for Act C iinsecticide (data not shown) with different adjuvants at different volumes.

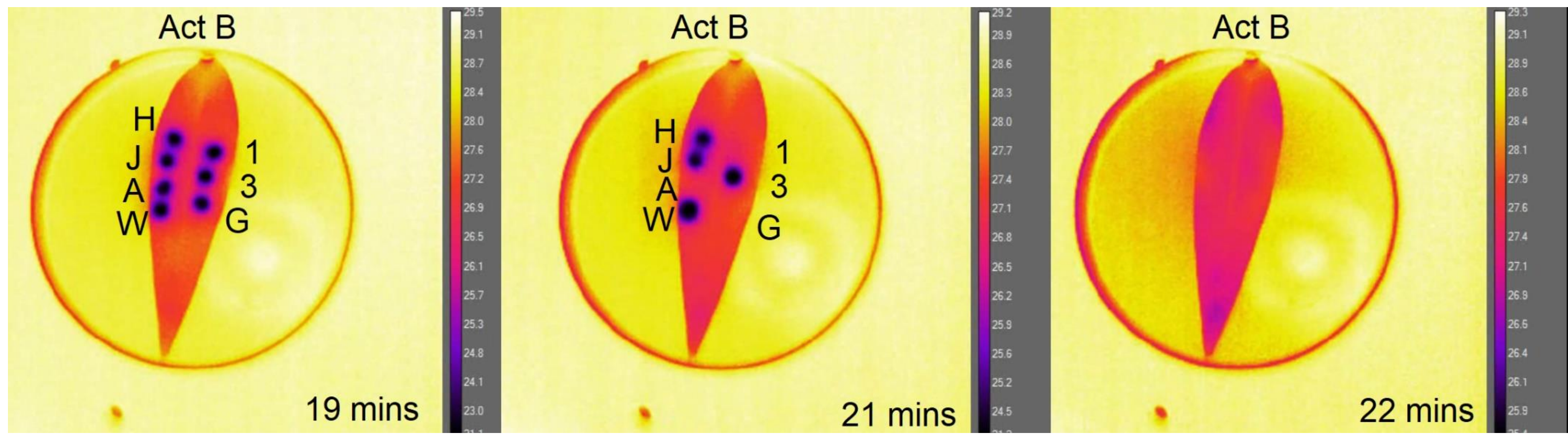


Figure 7: Time sequence of drying 3 µl droplets of Act B + adjuvants: *False colour scheme pictures represent changes in temperature and are coloured according to the bars on the right, typically temperature variation was between 20°C and 30°C. 3 µl droplets of adjuvants + Act B fungicide on* C. communis *leaf after 19 mins, when all droplets are cooler than leaf temperature, 21 mins when droplets and leaf surface start to lose their temperature differences and 22 mins when droplets and leaf surface are the same temperature. Adjuvants include: Adj H (H), Adj J (J), Adj A (A), Water (W), Adj 1 (1), Adj 3 (3) and Adj G(G). Adj 2 was not included in this work as rapid droplet spreading meant it could not be used as a single droplet on a leaf with other adjuvants.*

At the start of the thermal imaging experiment, all droplets were at a lower temperature than the leaf surface, confirming the effect of evaporative cooling (see Figure 7). However, at 19 mins, the difference between droplets and leaf temperature started to diminish and droplets were similar to leaf temperature and no longer visible by 22 mins, showing that droplets reached leaf temperature within a few minutes of each other. This data was in contrast to the visual inspection on the Leica MZ6 which detected drying time differences of up to 20 mins between adjuvants and yielded significantly higher mean droplet drying times for 3 µl droplets, between 35-45 mins for different adjuvants with Act B (see Figure 6C). This difference was even more pronounced for the 3 µl droplets of Act C with adjuvants. Droplets took between 24 and 29 mins to reach the same temperature as the leaf as reported by the FLIR system. However, DRTs from the visual observation showed that all droplets were retained on the leaf for at least 40 mins and the maximum adjuvant retention time with Act C was 55 mins (see Figure 6C). The longest time taken for any droplet to reach the temperature of the leaf using the thermal camera was only 42 mins compared to 75 mins, for 5 µl droplets of Act C + adjuvants, when measuring droplet drying time (data not shown).

The rates of droplet temperatures equilibrating with leaf temperature did not correspond to actual droplet lifetimes. The order that adjuvants plus actives increased in temperature did not correspond to the ordered data obtained for DRTs, instead this tended to be random on each repeat, with no clear trends. In Figure 7, as shown using the thermal camera, Adj G reached the leaf surface temperature quicker than Adj H and Adj J with Act B, but mean DRTs as shown in Figure 6C, show that Adj G had one of the highest DRTs with Act B and Adj H and Adj J dried quicker.

Adjuvant droplet areas on *C. communis* leaf surface.

The spreading potential of an adjuvant/active mix on a leaf surface indicates how much is in contact with the surface during droplet drying and for the penetration into a leaf. Droplet areas are also a predicting factor of leaf coverage and retention (adherence rather than losses) during spraying. Droplet areas were therefore measured following application of defined volumes of adjuvant/active combinations to leaves of *C. communis*. It was also expected that droplet wetted areas and evaporation rates would be correlated.

Variation in droplet areas of adjuvants on the surface of *C. communis.*

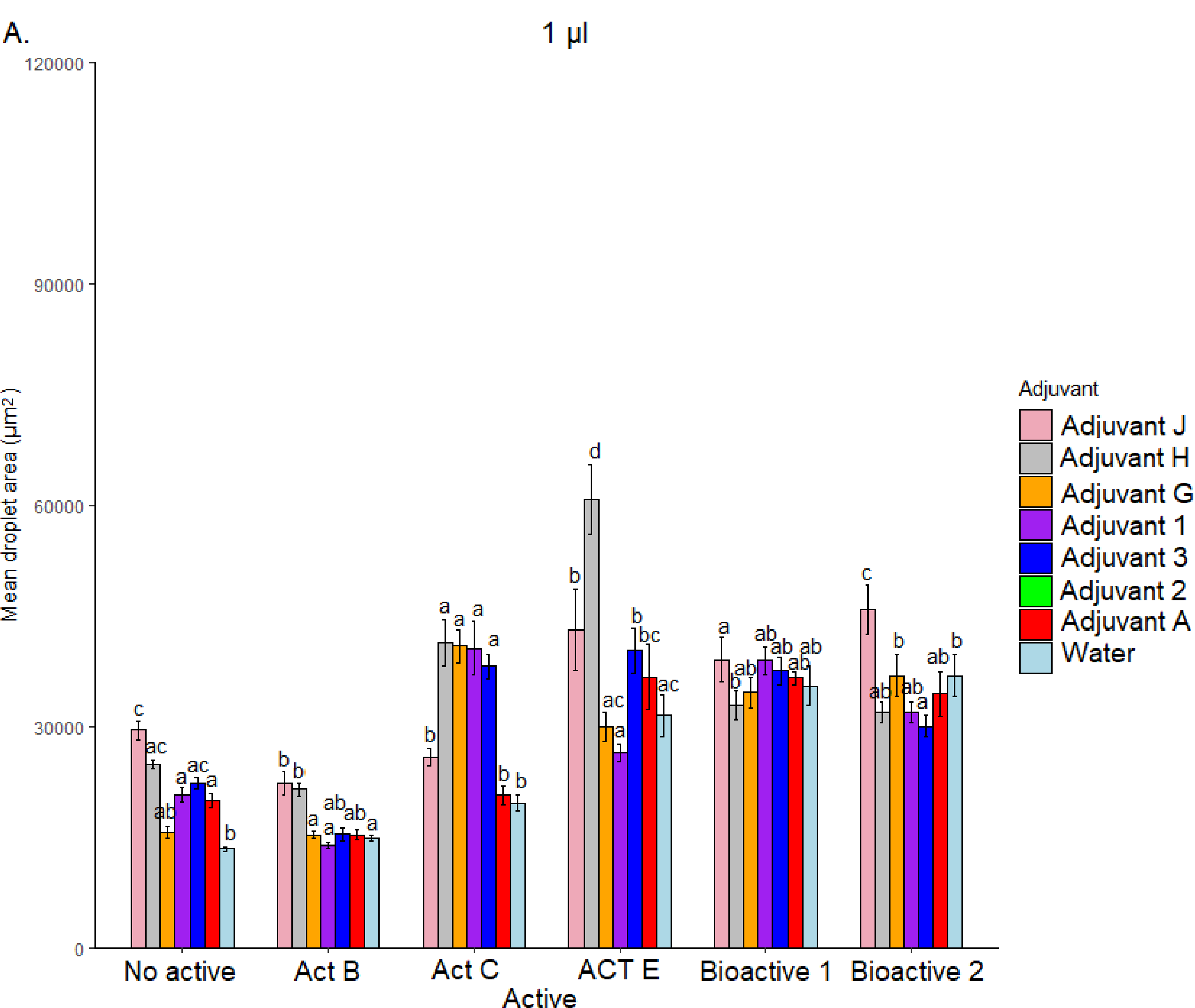
In most cases, adjuvants increased droplet wetted areas (DWAs) relative to water, at all three volumes tested (Figures 8A, 8B, 8C). Adj J had the biggest droplet areas of all adjuvants and had significantly bigger DWAs compared to water at all volumes. For volumes of 1µl, Adj J had significantly greater areas than 3 adjuvants, all adjuvants apart from Adj H at 2 µl and 4 adjuvants at 3 µl.

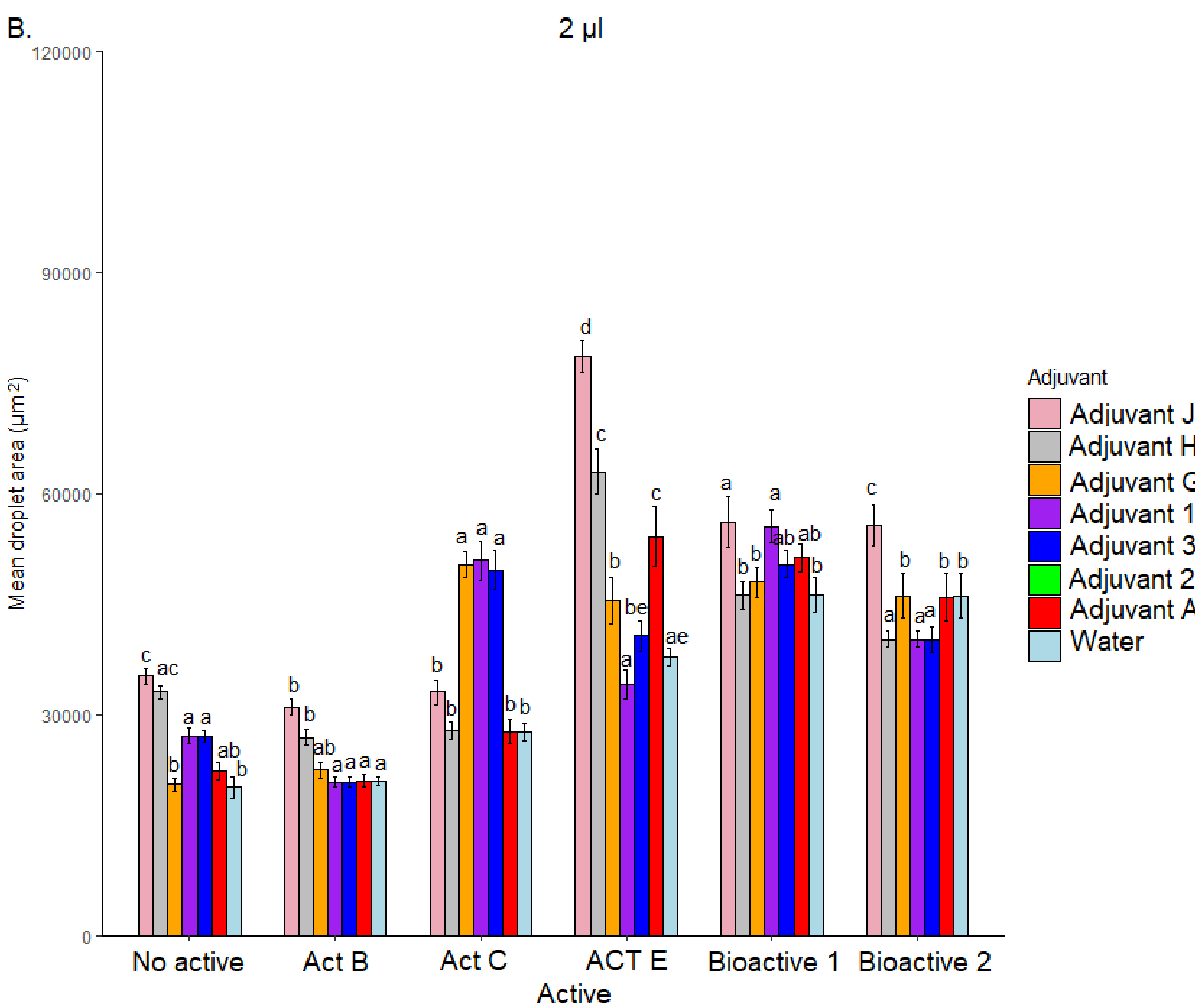
Amongst the different adjuvants, Adj J had the biggest droplet areas overall, independently and with different actives and especially with ACT E at bigger volumes. In contrast, Act C was the only active which when used with Adj J had significantly smaller droplet areas compared to other adjuvants (see Figure 8). For Act C, the Adj 1, Adj 3 and Adj G adjuvants grouped together giving significantly bigger droplet areas than all other adjuvants. At all volumes, for these 3 adjuvants, when combined with Act C, droplet area was significantly bigger than when no active was present in the droplet.

The effect of actives on adjuvant DWAs.

Individual active DWAs with adjuvants can be found in Appendices 6, 7, 8, 9, 10 and 11 which have been combined in Figure 8. Table 8 shows Tukey results for adjuvant and active effects on areas per individual droplet volume. Act B with Adj 1 or Adj 3, gave significantly smaller droplet sizes than when the adjuvants were used without any actives. For other adjuvants, Act B had little effect on mean wetted areas. For the majority of adjuvants, wetted areas with Bioactive 1, 2 and ACT E were similar to each other and had significantly bigger droplet areas than adjuvants combined with either no active or with Act B.

ACT E combined with the two developmental adjuvants had the largest mean DWAs attained of all adjuvant/active combinations (apart from Adj 2). At all volumes with adjuvant Adj H, areas with ACT E were significantly bigger than all other actives combined with Adj H. For 2 µl and 3 µl droplets of Adj J with ACT E, DWAs were significantly bigger than all other droplet areas of Adj J with different actives. In contrast, when ACT E was combined with Adj 1, DWAs were comparable to the standard Adj 1 droplet size without the addition of actives.


A.
1 µl
Mean droplet area (µm²)
120000
90000
60000
30000
0
No active
Act B
Act C
ACT E
Bioactive 1
Bioactive 2
Active
Adjuvant
Adjuvant J
Adjuvant H
Adjuvant G
Adjuvant 1
Adjuvant 3
Adjuvant 2
Adjuvant A
Water


B.
2 µl
Mean droplet area (µm 2)
Active
No active
Act B
Act C
ACT E
Bioactive 1
Bioactive 2
Adjuvant
Adjuvant J
Adjuvant H
Adjuvant G
Adjuvant 1
Adjuvant 3
Adjuvant 2
Adjuvant A
Water
0
30000
60000
90000
120000

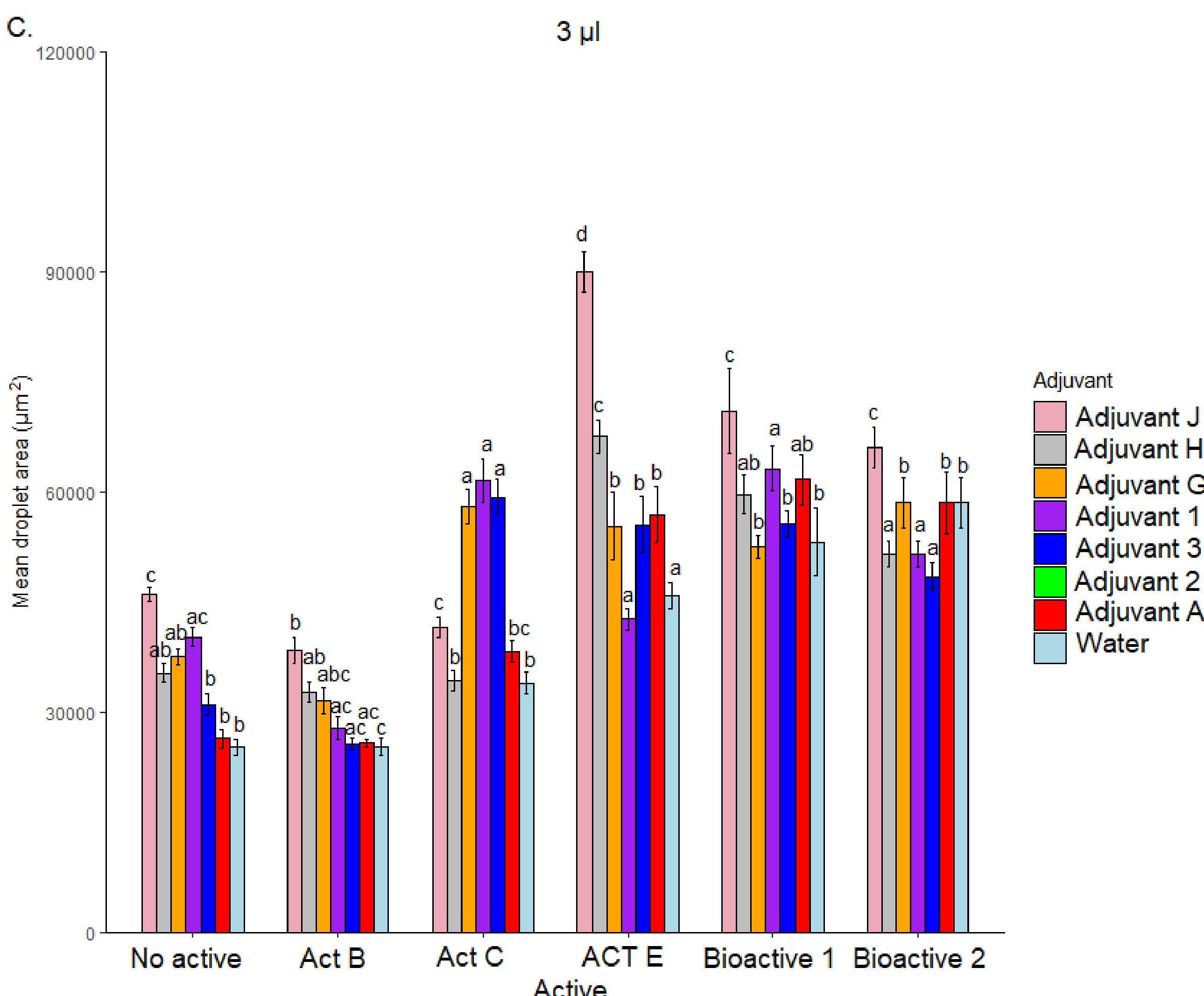

C.
3 µl
Mean droplet area (µm²)
Active
No active
Act B
Act C
ACT E
Bioactive 1
Bioactive 2
Adjuvant
Adjuvant J
Adjuvant H
Adjuvant G
Adjuvant 1
Adjuvant 3
Adjuvant 2
Adjuvant A
Water

Figure 8: Average droplet wetted areas of adjuvants with actives on *C. communis* leaf: *A. 1 µl. B. 2 µl. C. 3 µl. Adjuvants include Adj 1, Adj 3, Adj A, Adj G and two developmental adjuvants Adj H and Adj J plus water. Adj 2 did not behave as a droplet and spread beyond parameters of measurement so this adjuvant was not included in the data set. Actives include: No active (adjuvant used alone in water), Act B fungicide, Act C insecticide, ACT E and two biofungicide preparations* Gliocladium catenulatum *in formulation of Bioactive 1 and Bioactive 2. Error bars represent standard error SE. Letters above bars show statistically significant groupings of adjuvants within active groupings.*

	1 µl		**2 µl**		**3 µl**	
	F- value	*P- value*	*F- value*	*P- value*	*F- value*	*P- value*
Adjuvant	15.81	3.83×10^{-16}	29.94	9.63×10^{-44}	29.70	4.43×10^{-29}
Active	107.77	2.17×10^{-69}	236.21	4.67×10^{-113}	182.45	7.28×10^{-98}
Adjuvant*Active	9.00	9.59×10^{-29}	17.53	4.70×10^{-54}	11.65	1.15×10^{-47}

Table 8: Two-way ANOVA reported significance of droplet areas of adjuvants with actives: *Two-way ANOVA significance considered at 0.05%.*

The correlation between DRTs and DWAs.

To investigate the correlation between droplet area and drying times, mean DWAs was plotted against mean DRTs for each combination of adjuvant and active. Figure 9 shows a linear regression between size of droplet area and retention times of droplets. Statistical analysis indicates a significant correlation ($r^2 = 0.28$, $P = 0.003$). Although this indicates a positive relationship between DWA and DRT, there is high variation within the mid-range of droplet areas (Figure 9). Retention times are more variable for droplets with medium-sized areas between 25,000 μm^2 and 30,000 μm^2, DRTs ranged from 23 mins – 45 mins. Droplets with medium-sized areas between 30,000 μm^2 and 50,000 μm^2 have the biggest range of drying times, varying from 13 mins to 52 mins. The variation in data for droplets with medium-sized areas can partly be explained by the physicochemical differences between different adjuvant:active combinations.

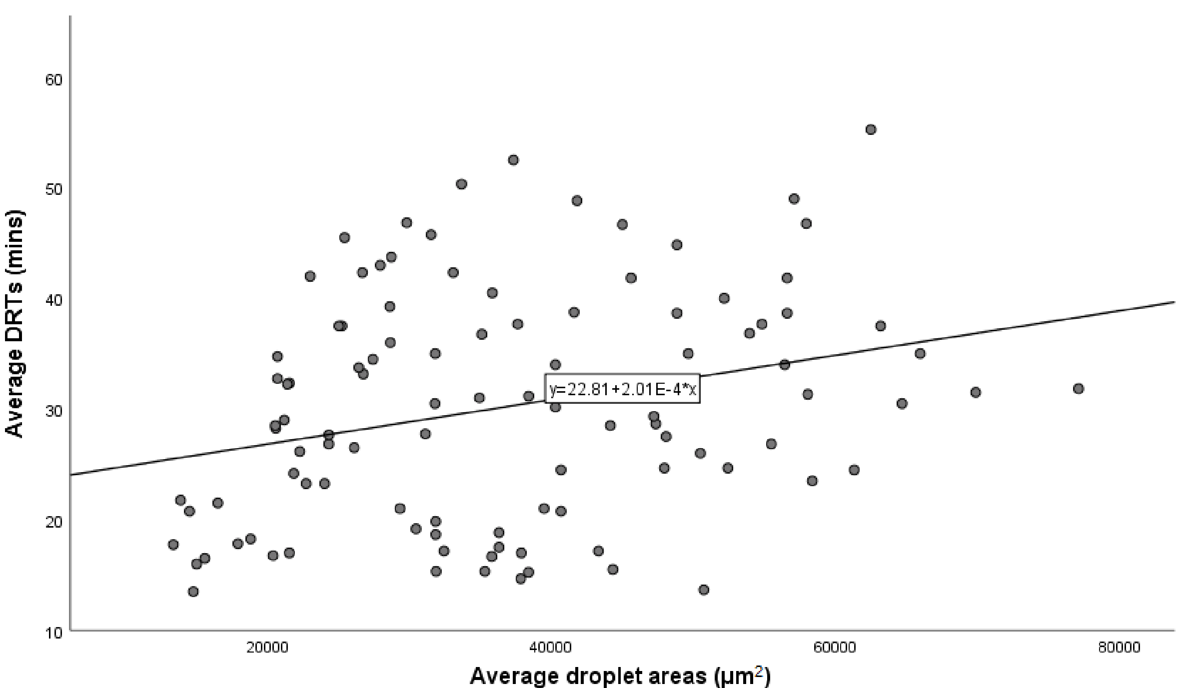


Figure 9: Correlation between average droplet areas and average DRTs: *Individual points represent adjuvant/active combinations at all three volumes total n = 105. Adj 2 adjuvant is not included as areas could not be measured. ACT E is not included as DRTs could not be measured.*

Spray coverage patterns of adjuvants on *C. communis* leaf after spraying.

DRTs could not be determined for the ACT E active due to its highly reflective white nature, meaning it was unclear when droplets had dried. In order to relate droplet areas to retention on the leaf, different adjuvants were mixed with ACT E, sprayed at the leaf and the % coverage of white droplets on the leaf quantified (Figure 10). Adjuvants which give higher coverage over the leaf surface area give better retention during spraying and reduce off-target losses to the environment.

Coverage of ACT E using different adjuvants varied between 3-53%. Adj 2 gave the lowest coverage on the leaf and Adj H gave significantly more coverage than other adjuvants and water.

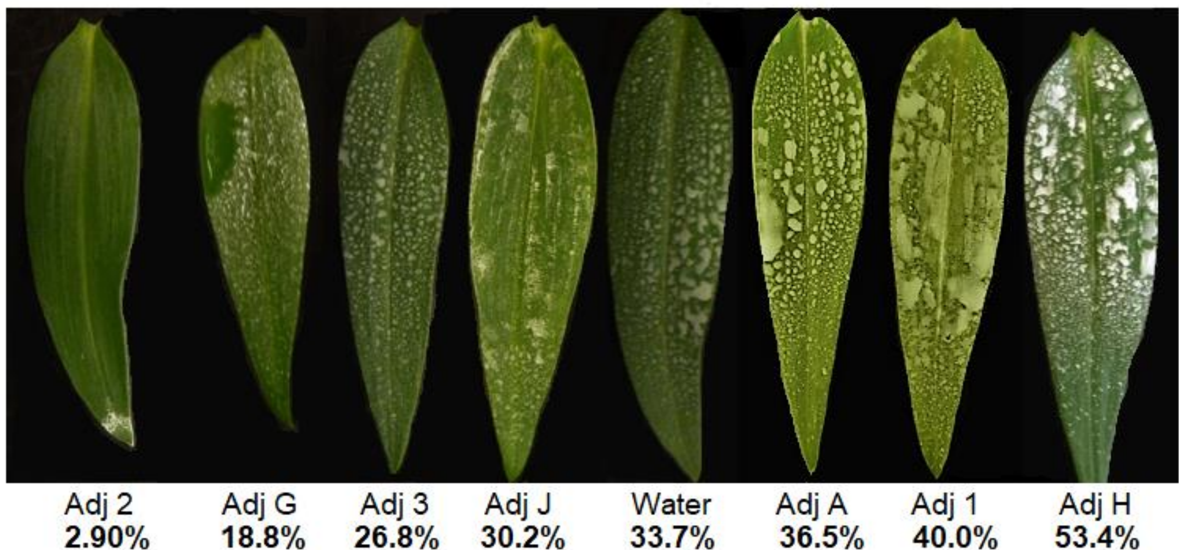


Figure 10: Average percentage leaf coverage of ACT E sprayed with different adjuvants: *Representable leaf from each treatment of ACT E + adjuvants. Adjuvants from left to right: Adj 2, Adj G, Adj 3, Adj J, Water, Adj A, Adj 1 and Adj H and are ordered from least coverage to most coverage. ACT E forms a kaolin hydrophobic film. The percentage coverage averages come from 4 replicate sprays of each spray combination and is shown below each adjuvant.*

Solid residues after drying of adjuvant droplets

Solid residues on the leaf surface after evaporation of water from a droplet indicate different droplet phase dynamics during drying. Different deposit types can be used to show compatibility of components within a mixed droplet, their tendencies to separate and the size and structure of the deposit type left behind may help predict how solids have been distributed in the droplet and how much has been left on the surface or may have penetrated into the leaf during droplet drying.

The effect of surface on droplet residues of adjuvants.

Parafilm was used as a homogeneous hydrophobic model wax layer and different adjuvants were mixed with Act C and their surface droplet residues compared. Figure 11A shows that water left a central solid deposit, adjuvant Adj H left a solid deposit and ring. Adj J looks partly adsorbed but has a central impression on the film. Other adjuvants had smaller ring annuli, some with central partitions suggesting differential separation of bulk flows within mixtures during evaporation. When adjuvants were mixed with Act C and added to a leaf surface in Figure 11B fewer deposits were left behind. Adj J remained damp after drying had stopped, Adj H and Adj 1 left ring residues, but the other adjuvants had no deposits.

The effect of actives on adjuvant droplet residues.

Figures 11B and C show residues on leaves after adjuvants were applied with two conventional pesticides. Adj 3, Adj G and Adj 2 did not leave residues with either Act C or Act B. Adj 1 left a ring with Act C but not with Act B. Adj A left a ring and internal residue which reflects light with Act B, similar to that on Parafilm, but did not leave a deposit with Act C. Both Adj J and Adj H had different residues with different actives. Act C + Adj H left only a ring residue but Act B + Adj H left a central damp deposit similar to that of Act C + Adj J. The damp residue of Adj J with Act C is no longer present when Adj J is mixed with Act B and instead leaves only a partial ring deposit.

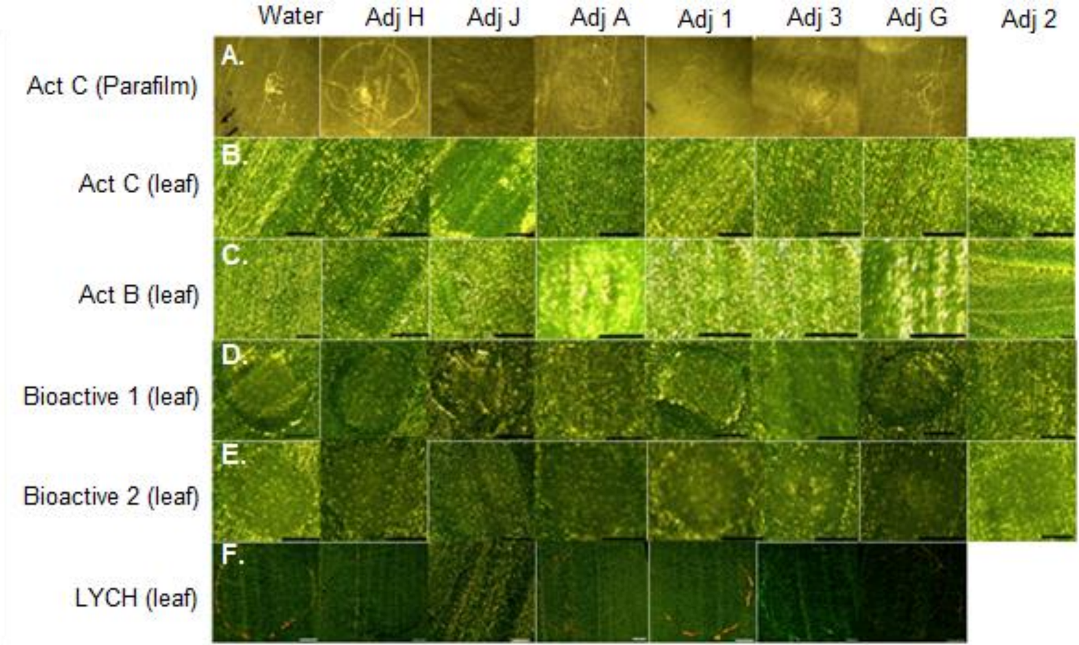


Figure 11: Appearance of residual deposits of actives with adjuvants after drying. ***A.*** *Act C + adjuvants on Parafilm,* ***B.*** *Act C + adjuvants on C. communis adaxial leaf surface.* ***C.*** *Act B + adjuvants on leaf,* ***D.*** *Bioactive 1 + adjuvants on leaf.* ***E.*** *Bioactive 2 + adjuvants on leaf.* ***F.*** *LYCH + adjuvants on leaf. Representative droplet residues from n = 10 repeats for each treatment, apart from on Parafilm which was performed once. Residues for Adj 2 were not performed on Parafilm or with LYCH. All droplets on the leaf surfaces are 3 µl apart from with LYCH which are 30 µl droplets. Droplets on Parafilm were also used as 30 µl droplets.*

Bioactive deposit structures with adjuvants.

Figure 11D and E rows show drying deposits from two formulations of Bioactive with adjuvants on the leaf surface. Bioactive 1 and Bioactive 2 formed deposits with more adjuvants after drying than the conventional pesticides. Both Adj 3 and Adj G left residues with Bioactive 1 and Bioactive 2, as did water. Adj 2 also left a spread deposit with Bioactive 2 but not with any other active. The deposits of adjuvants with Bioactive 1 and Bioactive 2 differed from each other. Adjuvants + Bioactive 2 formed spread out even residues. Bioactive 1 with most adjuvants and water also formed deposits which varied between evenly spread deposits and deposits with thickened dark rings. Water, Adj J, Adj H, Adj 1 and Adj G all left thick ring deposits with an even centre when mixed with Bioactive 1.

LYCH deposit structures with adjuvants.

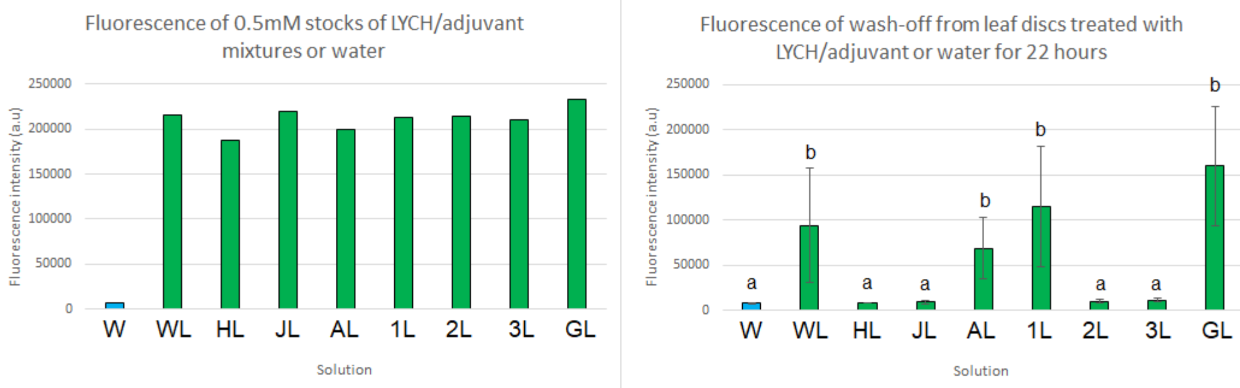
Figure 11F shows LYCH + adjuvants drying deposits. LYCH has been used as a coloured solute whose fate can be visualised by solid deposits after evaporation of the aqueous droplet. With water, full solid ring outlines were orange in colour and predicted to contain the most LYCH as solid residue after drying. Adj 1 with LYCH also gave solid orange deposits but tended to be only on the bottom edge of the droplet rather than around the circumference. LYCH with Adj A or Adj G left varied deposit structures but were characterised by solid ring deposits and sometimes also had marked internal deposits along separation lines, indicating variable flow within droplets. LYCH with Adj H and Adj 3 left faded deposit outlines which did not tend to deposit layers of dye solids. LYCH + Adj J left no observable residues after drying.

3.2. Penetration into leaf tissue.

Adjuvant effect on the amount of LYCH remaining on the leaf surface.

Spectrofluorometry was used to measure the fluorescence of 0.5 mM stocks of LYCH + adjuvant mixes (1/10th dilution of those applied) and water to show potential fluorescence intensity of total applied mixtures (Figure 12A). Adjuvant + LYCH solutions had fluorescence absorbance units (a.us) around 2 x 10^6 but water had low basal fluorescence at the excitation/emission spectra used for LYCH detection. The fluorescence of the solution recovered after 22 hours from the leaf surface from tissue treated with different adjuvants + LYCH or water was measured and is shown in Figure 12B. This may be used as a reference of the amount of applied LYCH which did not enter the leaf disc in the presence of the adjuvant (or water) during the experiment.

The fluorescence was reduced to levels close to background untreated water in solutions recovered from tissue treated with developmental adjuvants Adj H, Adj J, Adj 2 and Adj 3 (Figure 12B), suggesting substantially enhanced uptake of LYCH into the leaf when these adjuvants were present. For LYCH + water or adjuvants Adj A, Adj 1 and Adj G, the amount of LYCH recovered from the leaf surface was variable (Figure 12B) but was significantly higher than background suggesting that from the total applied LYCH was not all taken up into the leaf within 22 hours.



<u>Figure 12: Comparison of fluorescence intensity in stock solutions containing adjuvants + LYCH or water only, with recovered surface liquid from the *C. communis* leaf after treatments of LYCH + adjuvants or water untreated for 22 hours:</u> *A. Fluorescence intensity of water or stock LYCH + adjuvant mixtures at 0.5mM. B. Fluorescence of recovered surface liquid after 22 hours treatment of LYCH + adjuvants or water untreated tissue. Treatment solutions Water (W), Water + LYCH (WL), Adj H + LYCH (HL), Adj J + LYCH (JL), Adj A + LYCH (AL), Adj 1 + LYCH (1L), Adj 2 + LYCH (2L), Adj 3 + LYCH (3L), Adj G + LYCH (GL). For A, the absolute fluorescence intensity has been recorded once per stock. Blue bar is the fluorescence for water only followed by green bars for water + LYCH and adjuvants + LYCH. B. Shows average fluorescence intensities of surface residual fluorescence after 22 hours treatments. Blue bar indicates tissue treated with water only and green bars for water + LYCH and adjuvant + LYCH tissue in the order the treatments were setup. Letters a and b above bars represent statistically significant groups.*

Microscopy to visualise LYCH uptake with adjuvants.

The lower epidermal layer and mesophyll/vasculature are important tissues for the MoA of systemic pesticides, which work either translaminally or via translocation, so both tissue types have been considered to determine the location of LYCH applied with different adjuvants in the scope of this work.

Figures 13 and 14 show peeled epidermis and mesophyll after 1 hour or 22 hours treatment with LYCH + adjuvants respectively. The untreated water control tissue had low fluorescence signal at both time points, compared to LYCH treated tissue. In LYCH treated tissue, signal was greatest in cell walls and around stomatal complexes in the epidermis. LYCH signal was also in the vasculature and found in veins/apoplastic regions in and around the mesophyll. There were differences in fluorescence intensity between tissue treated with different adjuvants, however repetition within times showed signal inconsistencies. Further microscopy with control tissue (as shown in Figure 15) indicate that background autofluorescence was too variable, making it difficult to relate signal strength to penetration ability of adjuvants.

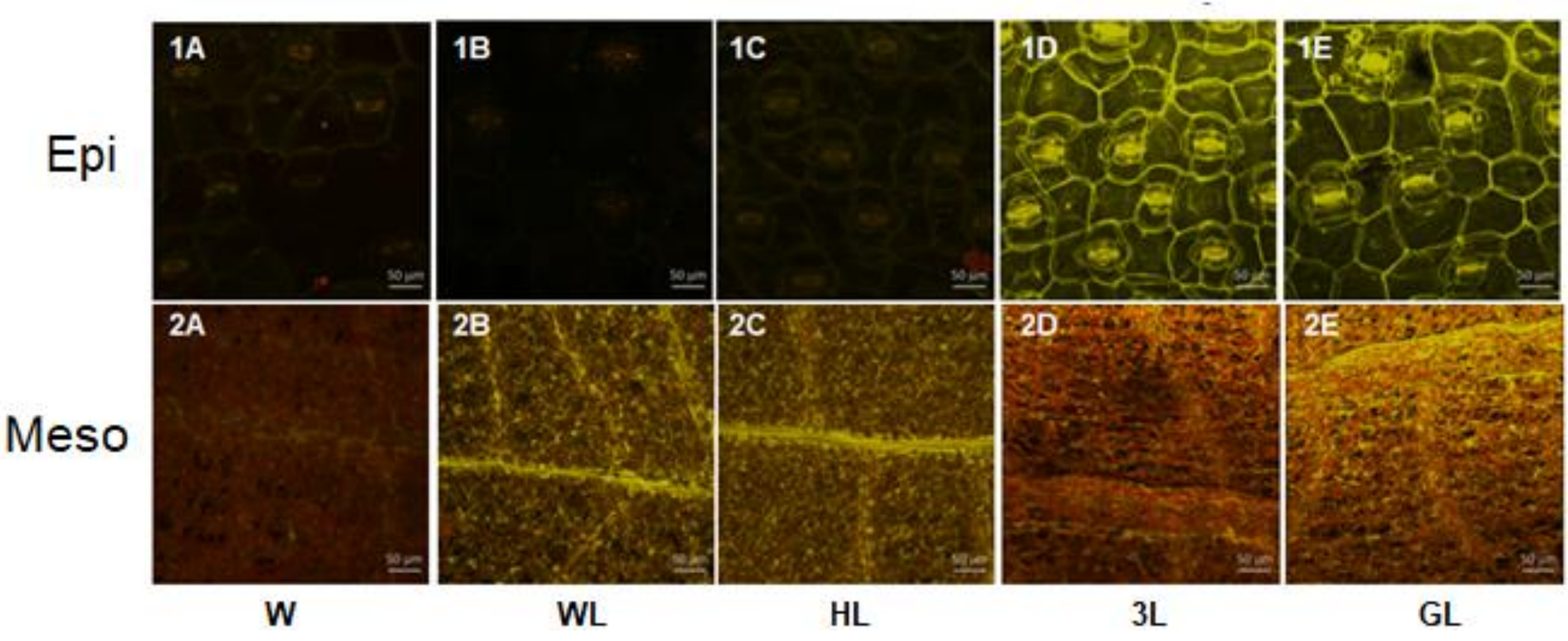


Figure 13: Epidermal and mesophyll tissues of *C. communis* after 1 hour treatment with adjuvants + Lucifer yellow or water: *1. Lower epidermal tissue. 2. Mesophyll tissue. A. Water (W). B. Water + LYCH (WL). C-E. Adjuvants + LYCH treated tissue - C. Adj H + LYCH (HL). D. Adj 3 (3L) + LYCH. E. Adj G + LYCH (GL). Scale bar 50 µm.*

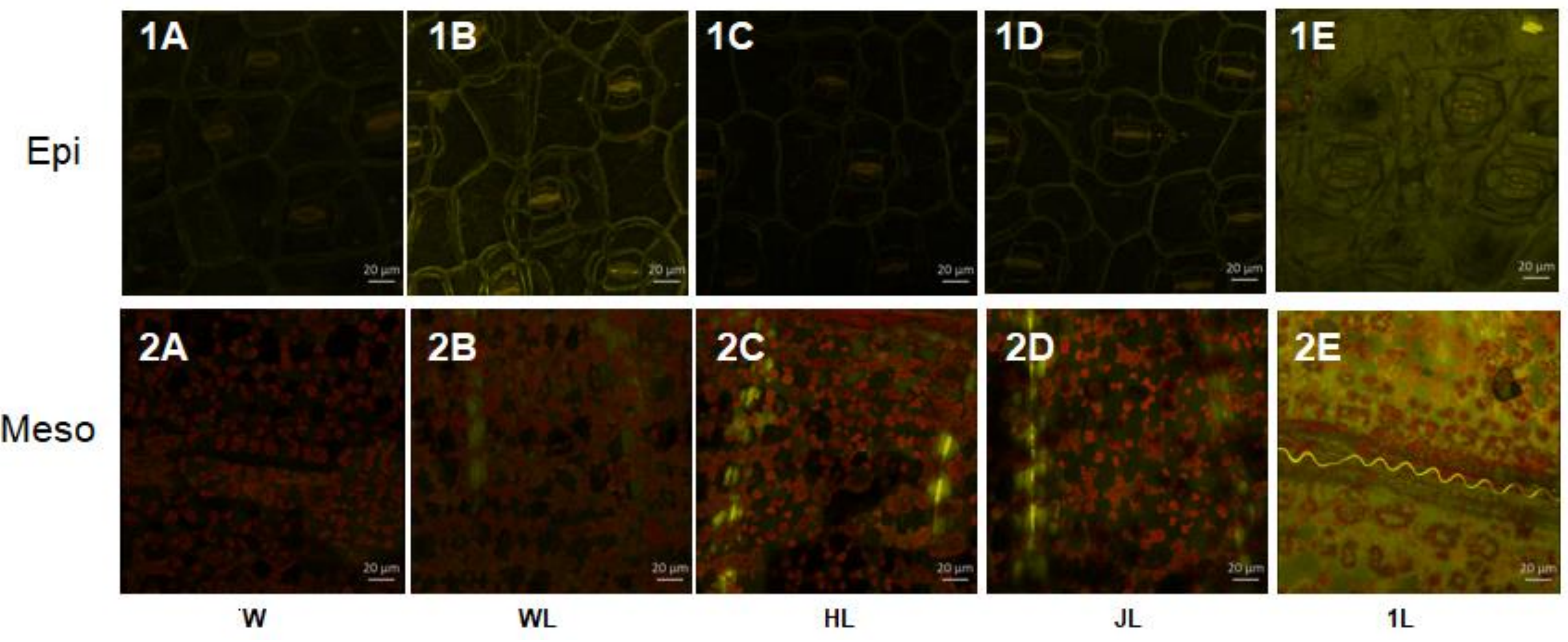


Figure 14: Epidermal and mesophyll tissues of *C. communis* after 22 hours treatment with adjuvants + Lucifer yellow or water: *1. Lower epidermal tissue. 2. Mesophyll tissue. A. Water (W). B. Water + LYCH (WL). C-E. Adjuvants + LYCH. C. Adj H + LYCH (HL). D. Adj J + LYCH (JL). E. Adj 1 + LYCH (1L). Scale bar 20 µm.*

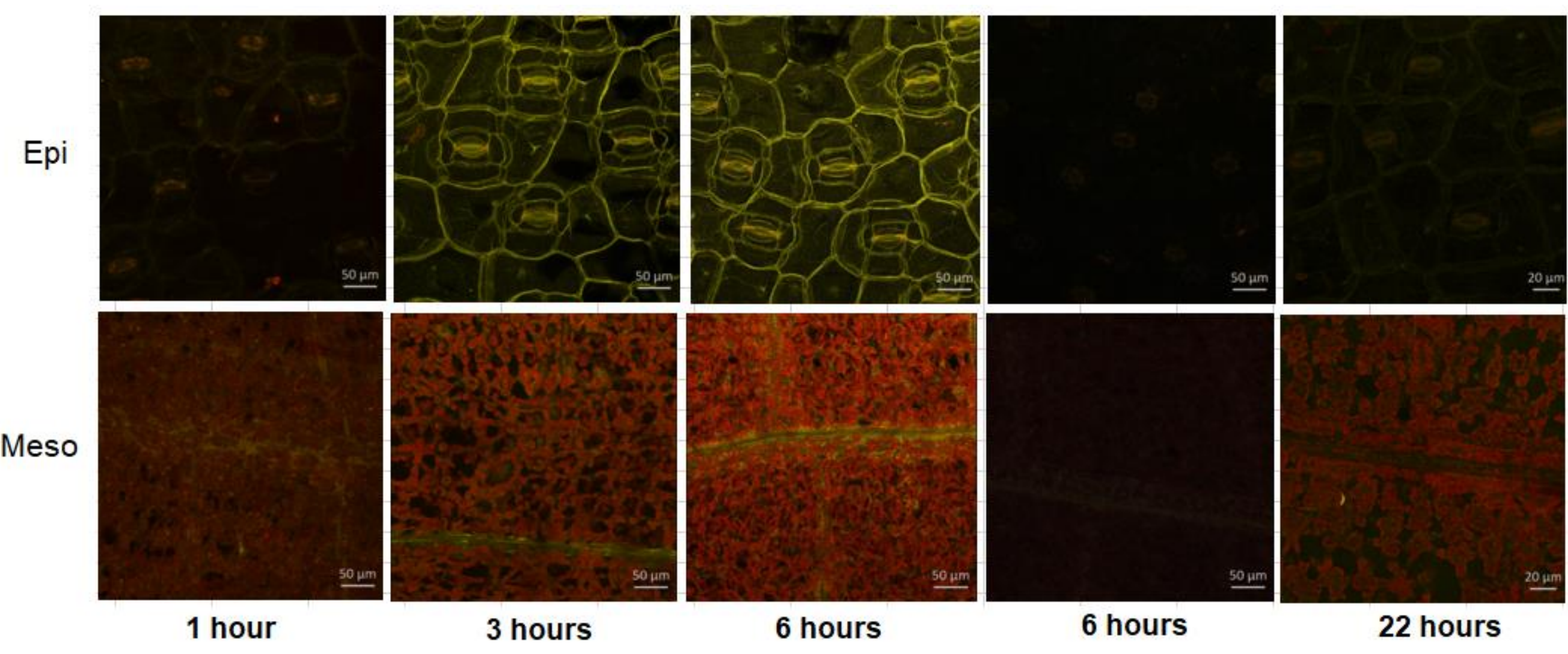


Figure 15: Variation in fluorescence of epidermal and mesophyll tissue of untreated (water-only) *C. communis* between 1 hour and 22 hour time points: *Top row: Lower epidermis. Bottom row: Mesophyll. 1, 3 and 6 hour tissue samples have a scale bar at 50 µm and 22 hour tissue samples have a scale at 20 µm. Two reps from 6 hour treatments with water have been shown to highlight variation in fluorescence between samples from the same batch.*

Spectrofluorometry to determine LYCH uptake into leaf tissue with adjuvants.

As an alternative means to measure LYCH uptake into leaf tissues, fluorescence signals from extracts of whole leaf discs were measured using spectrofluorometry. Figure 16 shows that at all 4 time points tested, tissue that was treated with water only, gave a consistent fluorescence between 9000 and 10000 a.us. Tissue treated with LYCH and different adjuvants had fluorescence intensities between 9000 and 12000 a.us. Therefore, intensity is similar between untreated and treated tissue. There was no obvious time point at which higher signal, possibly attributed to LYCH could be recovered from whole leaf tissue, which suggests that this assay was not sensitive enough to differentiate LYCH fluorescence from tissue autofluorescence.

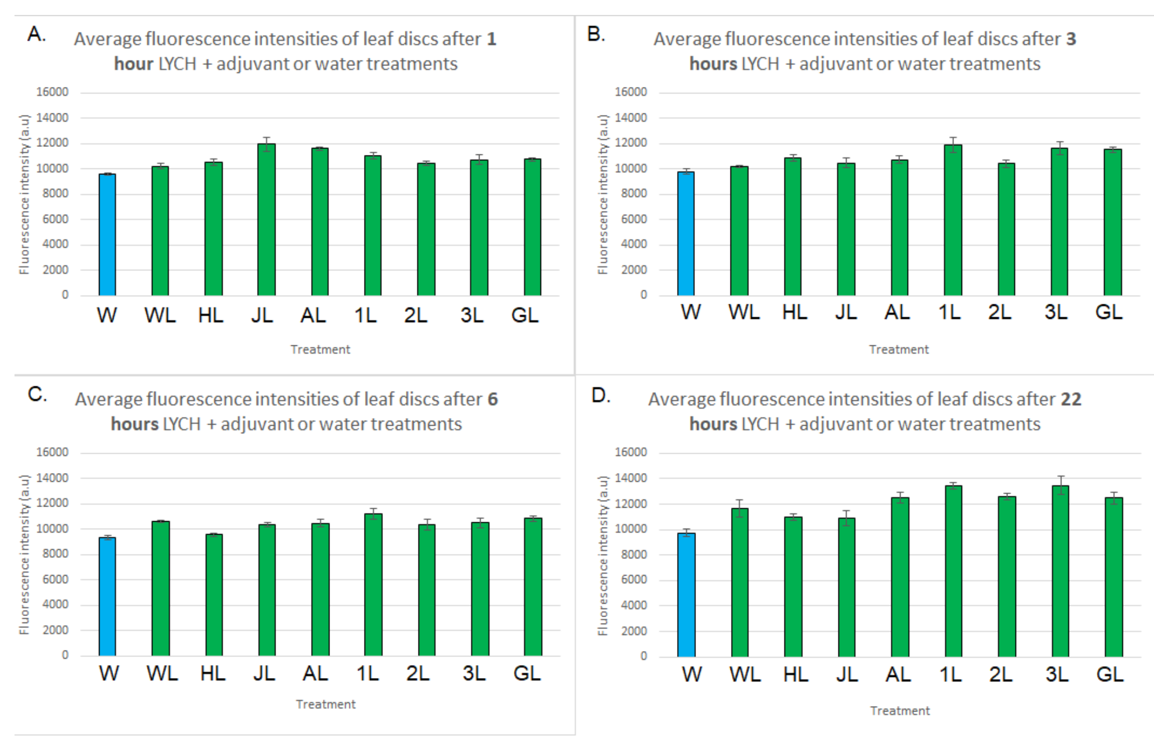


Figure 16: Fluorescence intensities from spectrofluorometric analysis of whole leaf discs treated with LYCH + adjuvants or water only for different time points: *A.1 hour B. 3 hours C. 6 hours D. 22 hours treatment with LYCH + adjuvants or water. Treatments include: Water (W), Water + LYCH (WL), Adj H + LYCH (HL), Adj J + LYCH (JL), Adj A + LYCH (AL), Adj 1 + LYCH (1L), Adj 2 + LYCH (2L), Adj 3 + LYCH (3L), Adj G + LYCH (GL). Average fluorescence intensity in a.us from excitation at 485 nm and emission at 520 nm. Blue bar is water treated negative control (no LYCH) followed by green bars for water + LYCH and adjuvants + LYCH along the X-axis in the order that the treatments were setup. Error bars show SE standard errors n =12 (4 biological reps, 3 technical repeats) for each treatment at each time point.*

Fluorescence techniques to measure pyriofenone uptake with adjuvants.

The pyriofenone active ingredient of Act B is fluorescent so can potentially be used to directly measure adjuvant performance in penetration of a commercial pesticide. However, fluorescence of pyriofenone could not be detected against background autofluorescence using epi-fluorescence/confocal microscopy or using the available settings on the spectrofluorometer (data not shown). This was due to high variability of autofluorescence and the limits of the excitation and emission spectra on different machines which were less then optimum for the detection of pyriofenone. See Appendix 12 for the spectra of pyriofenone which has been labelled with emission and excitation wavelengths used in this study. The NMR of the Act B fungicide confirmed pyriofenone fluorescence (Appendix 13).

Fluorescence lifetime image mapping (FLIM) was used to try to distinguish pyriofenone fluorescence lifetime (period of emission after excitation pulse) from background fluorescence molecules in plant tissues. If this is possible and there were different counts of lifetime corresponding to pyriofenone between adjuvant treated tissues and water treated tissues, it may be possible to compare adjuvant effectiveness in penetration.

Fluorescence lifetime microscopy (FLIM) to detect Pyriofenone uptake into leaf tissues with adjuvants.

Table 9 shows that pyriofenone had two corresponding lifetimes (depending on confirmation in liquid solution). Treated tissue (pyriofenone + water or adjuvant) and untreated tissue had two or three species which fit the best fit curve for each area of interest. All pyriofenone treated tissue samples, irrespective of adjuvant presence or area of the epidermis examined, had three lifetimes averaging around 6.23, 2.98 and 1.15 ns. The two longer lifetimes are within a similar range of that emitted by pyriofenone in solution. Untreated epidermis also yielded 2 or 3 lifetimes with more variation between (probably due to different tissue ages) but the longest two lifetimes averaged at 5.96 and 1.98 ns, only 0.12 and 0.08 ns shorter than pyriofenone lifetimes in solution. This technique was therefore not able to distinguish between autofluorescence and pyriofenone lifetimes. In untreated tissue, the count ratio between the two longer species (6 ns and 2 ns) varied from 1:0.2 to 1:2.3. In tissue treated with adjuvant Adj H, the count ratio was similar to untreated ratios varying between 1:0.5 and 1:1.5. In two of the pyriofenone + water treated samples there was a greater ratio of the two smaller lifetimes 2 ns and 1 ns (7:4 and 4:7). However, it does not seem likely that the higher ratio can be due to pyriofenone presence, as the two lifetimes were seen across all tissue types and the smallest lifetime cannot be trusted due to the possibility of scatter interference from the sample, contributing to the counts attributed to this component. This interpretation unfortunately cannot be confirmed with the use of a positive control, as it is unknown which adjuvant or if water can indeed best improve penetration of pyriofenone.

Pure solution		Lifetimes (ns)			Best fit (n)	Counts (normalised)		
Act B (pyriofenone)		6.08	2.06		2n	1	1.15	
Lower epidermal tissue (treated)	**Area of interest**							
Act B + water 4w old	Cell wall junction	6.46	3.15	1.08	3n	1	1.56	1.21
Act B + water 4w old	Guard cells	6.28	2.72	1.35	3n	1	7.72	4.93
Act B + water 4w old	Epidermal cell (vacuole)	6.47	2.15	0.7	3n	1	4.05	7.56
Act B + Adj H 4w old	Wall junction around guard cells	6.06	3.68	1.19	3n	1	0.54	0.10
Act B + Adj H 4w old	Guard cells	6.24	3.35	1.38	3n	1	1.31	1.35
Act B + Adj H 4w old	Stomatal pore	5.89	2.81	1.2	3n	1	1.57	1.13
Averages		**6.23**	**2.98**	**1.15**				
Lower epidermal tissue (untreated)								
Untreated 4w old	Stomatal pore	7.27	1.5	0.81	3n	1	0.33	0.01
Untreated 7w old	Wall junction around guard cells	4.55	1.68	0.48	3n	1	1.93	1.38
Untreated 4w old	Stomatal pore	5.59	2.08		2n	1	0.23	
Untreated 6w old	Guard cells	6.43	2.66	0.96	3n	1	2.31	1.41
Averages		**5.96**	**1.98**	**0.75**				

Table 9: Lifetimes of pyriofenone and lifetime best fits of different regions of treated and untreated tissue: *Number of species with different lifetimes (n) which best fit each average curve for each area of interest was decided using a Chi-squared of x=1.1 cut-off. Counts were normalised to the longest lifetime (6 ns) and expressed as a ratio. All untreated and treated tissue samples were averaged separately and average lifetimes (ns) provided. Pyriofenone (Act B) was applied with water or Adj H on the* C. communis *adaxial surface for 22 hours.*

3.3. Compatibility of adjuvants with a biofungicide.

The study has so far considered results for adjuvants with conventional pesticides. As modern farming practices are expected to consider organic pest control alternatives and methods of IPM, the compatibility of different adjuvants with a biopesticide was compared. Applications of biopesticides to a leaf surface are limited because of adverse conditions on the leaf and their success (either survival rate or penetration) could be potentially improved with the use of adjuvants. A commercial biofungicide, Bioactive 2 (*Gliocladium catenulatum*) was grown in the presence of adjuvants (and actives) to see if growth was negatively affected.

Bioactive growth with adjuvants and actives.

Inhibition of growth of Bioactive 2 biofungicide was tested on agar plates with various adjuvants and actives included in the agar (Figure 17). All adjuvants and actives had some inhibitory effect on the early growth, relative to Bioactive 2 grown with water. Over the first two days of growth, Bioactive 2 + water had 3x more growth than other treatments. Between 2-5 days there was less of an effect of adjuvants/actives on growth of Bioactive 2. Growth in the presence of adjuvant Adj H or Adj 3 was least effected, whilst Adj A, Adj 1 and the insecticide Act C caused only slight decreases in growth relative to standard agar plates. Act B fungicide showed further inhibition of growth but the Adj G adjuvant had the most serious effect on growth, with 13.7% less growth than the control between 0-2 days and 10.6% of the control between 2-5 days.

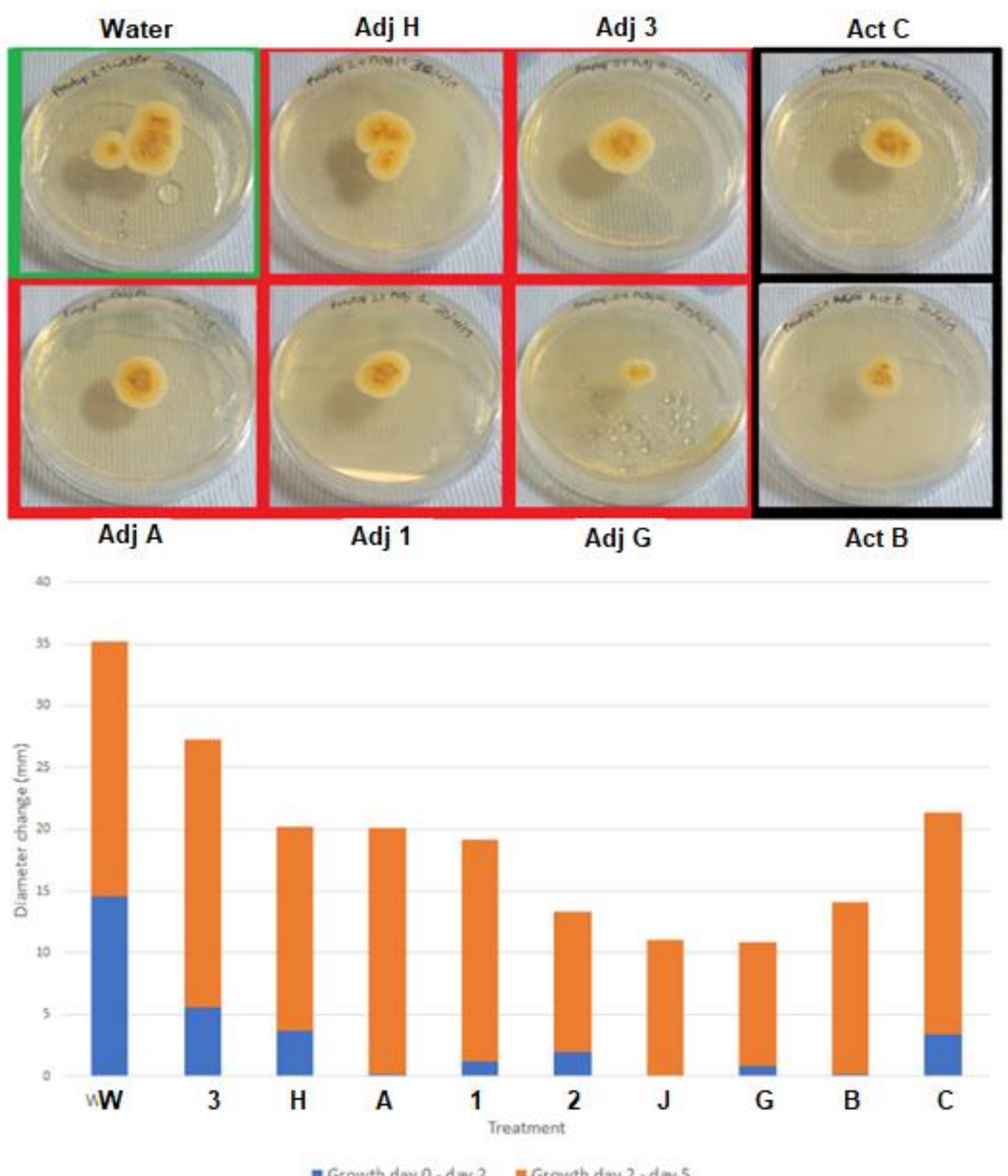


Figure 17: Growth of Bioactive 2 (*Gliocladium catenulatum*) over 5 days (120 hours) with different adjuvants and actives on agar plates: *Bioactive 2 inoculum grown on PDA with water, adjuvants or actives. Top images: Green shading for the water (W) control, red shading for adjuvants used at recommended concentrations and black shading for actives used at recommended concentrations. Adjuvants include Adj H (H), Adj 3 (3), Adj A (A), Adj 1 (1) and Adj G (G). Actives include Act C (C) (insecticide) and Act B (B) (fungicide). Bottom graph: Growth measured by change in inoculum width between 0-2 days (blue) and 2-5 days (orange) for Bioactive 2 grown on adjuvant or active infused agar. From left to right, water control (W), Adjuvants: Adj 3 (3), Adj H (H), Adj A (A), Adj 1 (1), Adj 2 (2), Adj J (J), Adj G (G), Actives: Act B (B), Act C (C).*

As a complementary approach, Bioactive 2 was made up in adjuvant stocks, incubated for either 10 mins or 60 mins and then grown in liquid media for 3 days. Figure 18

shows growth averages after 48 hours. Growth is variable for Bioactive 2 in the untreated water control between both experiments. Similar growth rates to water were observed for Bioactive 2 + Adj H and + Adj 1 but following the trend that more growth was seen after only 10 mins incubation with adjuvants compared to 60 mins. For the Adj A and Adj 2 adjuvants, Bioactive 2 growth was variable but was similar to and in some cases there was more growth than when pre-incubated in water. The 10 min incubation with these adjuvants showed higher growth relative to the water control, however after 1 hour incubation, growth was somewhat lower and more comparable to water.

Triple strength Act B fungicide and 70% EtOH were used as positive controls and Bioactive 2 had limited growth after incubation with these (see Figure 18). Bioactive 2 with Adj G had growth rates comparable to EtOH and Act B after 10 mins incubation and lower than the Act B fungicide after 1 hour incubation with Adj G. The incubation time had little effect on growth of Bioactive 2 with Adj G as inhibition of growth was consistently reduced after both incubations, with an average growth increase of 0.038-0.05 g over 48 hours compared to water, with an average growth increase of 0.08-0.16 g. The growth in liquid media supports the findings in Figure 17 for Bioactive 2 grown on plates in the presence of Adj G.

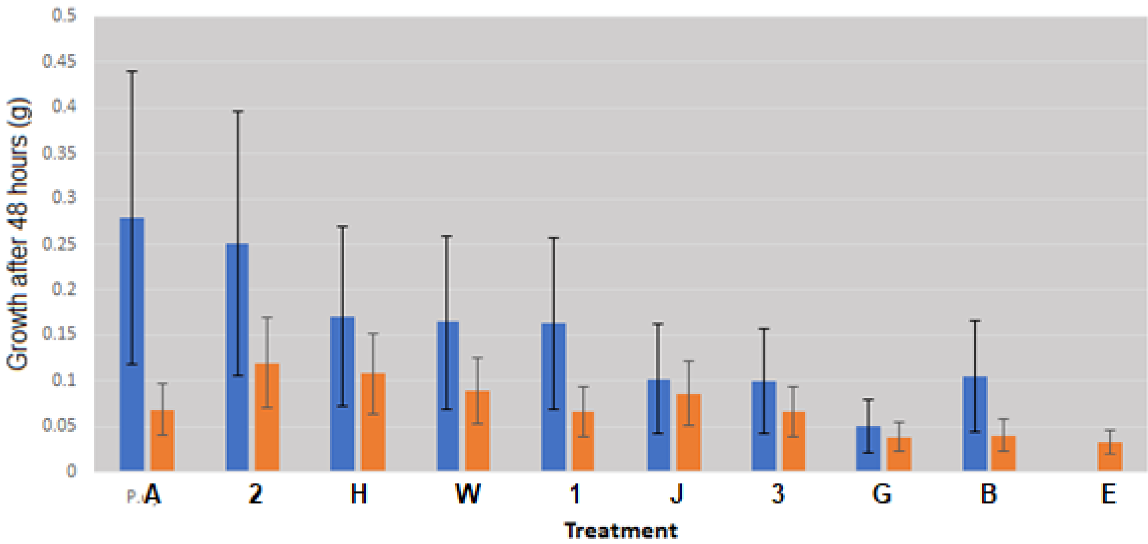


Figure 18: Growth of Bioactive 2 (*Gliocladium catenulatum*) incubated in different adjuvants and grown for 48 hours in liquid culture: *Average growth (measured as weight change in grams) of tubes containing Bioactive 2 pre-treated with adjuvants for 10 mins (blue) n = 3 or 1 hour (orange) n = 6* and then grown in nutrient broth for 48 hours. *Adjuvants include from left to right: Adj A (A), Adj 2 (2), Adj H (H), Water (W), Adj 1 (1), Adj J (J), Adj 3 (3), Adj G (G). Controls include: Act B fungicide (B) (triple strength) and 70% Ethanol (E). Error bars show SE standard errors. There was no data collected for EtOH after 10 mins incubation.*

Chapter 4: Discussion.

4.1 Surface behaviour of adjuvants.

Adjuvant droplet evaporation rates.

Adjuvants are advantageous for the application of agrochemicals because they can lower surface tensions of liquids, increasing spread over the surface to improve contact between pesticides and the leaf. The lowering of surface tension can improve adhesion and coverage on a leaf and modify evaporation rates of a given droplet. Therefore, the DWAs and DRTs are important assessment criteria for an adjuvant. Evaporation rates of droplets depend on the rates of vapour transfer to air as well as losses through diffusion into the plant typically through the plant cuticle. The retention time of a droplet has also been previously implicated in adjuvant ability as a penetrant, as diffusion requires a soluble phase (Ramsey et al., 2005).

DRT dependency on droplet volume.

The drying of sessile droplets on an interface such as a plant leaf is affected by the chemical properties of the solution and the shape of the droplet as a function of surface tension (Brutin and Starov, 2018). For all but one of the adjuvants in this study, DRTs were found to increase linearly as volumes of droplets increased (an increase from 1 – 5 µl). The synthetic organosilicone Adj 2, was the only adjuvant whose aqueous droplets evaporated independent of volume, with a very low average retention time of 13 mins. This is similar to the 13.6 mins reported for Silwett-L77 organosilicone on sour cherry leaves (Knoche and Bukovac, 1992). The low retention behaviour of Adj 2 can be explained by its spreading capability (meaning it is not a sessile droplet), and instead spreads to form a large, thin wetted area. Super-spreading capability of this adjuvant is thought to increase evaporation rate to air as well as lowering surface tension enough to allow diffusion of liquid into the leaf tissue, not only through cuticular penetration but through stomatal penetration as well (Schönherr and Bukovac, 1972, Hall, 1993, Petracek, 1998). Therefore, when considering droplet behaviour at volumes applicable for spraying. *e.g.* 1-3 µl, adjuvant DRTs must be analysed at separate volumes apart from for Adj 2 where retention was more predictable.

Not all the adjuvants in this study were able to increase retention times of water droplets and droplet lifetimes depended strongly on the adjuvant used (see Figure 6). Two adjuvants, namely Adj H and Adj 1, had significantly longer mean DRTs compared to that of a water droplet. In contrast, the Adj A terpene and the synthetic organosilicone Adj 2 had significantly lower drying times compared to water. Adj 1 is a paraffin-wax based adjuvant and Adj H contains 4 mannosylerythritol lipids (MELs) mixed with modified seed oil (MSO). Adj J is similar to Adj H in that it contains MELs but does not contain MSO, which may have been the reason why retention of this adjuvant was more comparable to water. Previous work on a paraffin-wax based adjuvant found evaporation time to be lower than water, but to a lesser extent than other oils and surfactants (Xu et al., 2010b). It is likely that the specific formulations of the oil-based and paraffin-wax adjuvants used in this study are better for droplet retention. Retention data on the *C. communis* leaf surface is however, insufficient to

make assumptions about adjuvant penetration abilities.One possibility is that adjuvants with high retention, Adj H and Adj 1 can prolong droplet lifetimes and promote more penetration into the leaf in the given time. On the other hand, adjuvants which had lower retention than water, Adj 2 and Adj A, may have better penetration into the leaf, accounting for faster droplet losses through diffusion into the leaf. Another possibility is that the presence of adjuvants in dried matrices of deposits, promote continued uptake, so the drying time has little effect on penetration.

Adjuvant DRTs were not correlated with adjuvant chemical properties.

There was no correlation between adjuvant DRTs and molecular mass, viscosity or solubility of adjuvants to differentiate why some adjuvants were better retained on the surface compared to others (see Table 10 for properties). Adj 2 has the lowest surface tension (20.5 mN m^{-1}) of adjuvants followed by Adj H, Adj J and Adj 1 (~30.7 for the MEL-adjuvants and 34.8 mN m^{-1} for paraffin-wax based). There was therefore no correlation between surface tension with droplet lifetimes which suggests drying time is a complex process with more contributing factors than just the area in contact with the leaf. In the controlled lab experiments, humidity and temperature could be reliably controlled, limiting the variability of water evaporation attributed to these environmental factors however air flow was not controlled in the scope of this work. According to Schönherr & Bukovac, few surfactants are able to lower surface tension sufficiently to allow stomatal penetration, and they used the cut off for lowering to less than 30 mN m^{-1} so potentially only the organosilicone adjuvant can achieve stomatal penetration in this study (Schönherr and Bukovac, 1972). A further problem in differentiating between adjuvant retention, is that the estimates shown in Table 10 may not be appropriate because adjuvants tested in this study may also contain several other components, which are kept secret in the commercialisation of these products but which also modify these properties in an unknown way.

Table 10: Chemical properties of adjuvants used in this study:

Trade name	Chemical	Molecular weight g/mol	Log P (K_{ow})	Solubility g/L	Viscosity (25°C mPa.s)	pKa	Surface tension (mN/m)	HLB value	Conc. v/v %
Adj A	di-1-p-menthene	275.0 [1]	4.86 [1]	Insoluble (emulsifiable) [2]	680 [2]	-	-	-	1.25
Adj G	Guar gum	2.5 x 10^6 [21]	-	Soluble 500 [4]	50 [3]	3.68 [22]	50-55 [4]	<8.0	2.5
Adj H	MELs + MSO (modified seed oil)	~634.57 (MEL-C) [8] + (877.7 for rapeseed oil) [9]	-	25 (MEL-D) [6]	Highly viscous	4.7 [8] (rhamnolipids)	~30.7 [8]	8.8 [6]	2.5
Adj J	MELs	~634.57 (MEL-C) [8]	-	25 (MEL-D) [6]	Highly viscous	4.7 [8] (rhamnolipids)	~30.7 [8]	8.8 [6]	5.0
Adj 1	Paraffin wax	196.68 [10]	2.7 – 3.44 [10]	Insoluble 0.13 [10]	Low viscosity	9.37 [10]	34.78 [11]	10 [12]	1.0
Adj 2	Trisiloxane	338.66 [14]	3.28 [13]	Dispersible [13]	20 - 60 [13]	-	<20.5 [15]	10 – 13 [16]	0.5
Adj 3	Triglyceride ethoxylate	933.45 [18] 375.86 [19]	3.01 [19]	Insoluble <1	590 – 710 [17]	0 [20]	40.1 [20]	8 – 11 [17]	1.5

Addition of actives affected DRTs of adjuvants.

When adjuvants had actives present in the droplet, the actives and also the interaction between adjuvant and active influenced DRTs (Table 7). A previous study found adjuvant-specific differences in drying times *i.e.* between non-ionic retardant adjuvant and alkyl polyoxyethylene surfactant abilities to effect drying times of fungicides and insecticides (Yu et al., 2009). However, actives used in their study did not affect DRTs of adjuvants (Yu et al., 2009). This present study used a range of conventional actives and some variations of a biofungicide to test the capability of adjuvants to improve retention of pesticides applicable for both conventional and organic farming. These actives were chosen to consider adjuvant retention capability in the context of penetration of systemic actives where MoAs are inside the leaf and for prolonging moisture within biofungicide droplets to potentially improve germination rates on the surface of a leaf. Generally, the mean DRTs of Bioactive 2 *Gliocladium catenulatum* biofungicide and Act B fungicide droplets dried significantly quicker than Act C insecticide droplets, which had the longest lifetimes with adjuvants, but this depended on droplet volume. Bioactive 1 containing *Gliocladium catanelatum* biofungicide plus milk powder had significantly lower retention than all other actives so this active affected adjuvant retention the most.

One formulation type, containing wettable granules (WG) of the Bioactive 1 biofungicide containing milk powder, had a significant negative effect on evaporation rates and negated adjuvant effects (apart from with the Adj 2 organosilicone where it had no effect). The mean DRTs of adjuvants were lower with Bioactive 1 compared to with other actives and infact water had higher retention times than some of the adjuvants with Bioactive 1 (see Figure 6C). There were clear differences between adjuvant evaporation rates with Bioactive 1 and Bioactive 2 (without the milk powder). It is assumed that the milk powder formulation caused this difference in adjuvant retention because of spatial separation of the biofungicide and the adjuvant. It is expected that the *G. catenulatum* fungus would be closely associated with the milk powder and is not incorporated into an adjuvant carrier system as would be expected with Bioactive 2 (Hunsche and Noga, 2012). Thus, the key message when considering adjuvant application with a particular formulation as an addition to spray or tank mix, one should check that the existing formulation does not inhibit adjuvant effects. This may be a challenge for adjuvant research in the future and may hamper their application as additions to biopesticides in formulation in organic farming.

Some adjuvant-active combinations showed increased droplet retention.

Maximum retention peaks are a result of good interaction between adjuvant and active such that water is included within the droplet for longer. As a result of these data, it has been established which adjuvants give best retention with particular actives. Adj 1 is the adjuvant which has the longest retention of adjuvants in general with combinations of actives so would be recommended as a robust adjuvant for retention with a range of actives. Adj 3 is a good specific adjuvant choice for Act B fungicide and Adj A is a good specific adjuvant choice for Act C insecticide based on retention data (Figure 6). Adj A with Act C is a specific example of a an adjuvant:active

combination which promoted retention. Retention of Adj A droplets alone or with other actives were comparable to water until mixed with Act C, where there was a significant increase in retention, which was even apparent with larger volumes (Figure 6A, 6B compared to 6C). It is unclear why Adj A could prolong lifetimes of droplets with Act C but not with Act B as both actives have similar molecular weights and both have weakly lipophilic Log P values (Act C containing spirotetramat Log P = 2.5 and Act B containing pyriofenone Log P = 3.8, see Table 11). It was suggested that more similar adjuvants and actives will have a better interaction *i.e.* lipophilic with lipophilic would be more closely associated, but this does not explain how water would be physically trapped within a droplet, slowing down the rate of evaporation and it remains unclear why there are volume effects (Hunsche and Noga, 2012). Comparing the Log P of Adj A, which is the highest of all adjuvants with a value of 4.86 (see Table 10) and is therefore the most lipophilic, one would expect that a high Log P in the active would give good interaction and possibly good penetration through the leaf, due to lipophilicity. Adj A has a closer Log P to Act B (containing pyriofenone), than to Act C containing the spirotetramat active ingredient. It is possible that Adj A + Act B dried out quicker because of a higher contribution of loss through penetration into the leaf, but this cannot be measured to confirm this interpretation.

Retention analysis of any study with adjuvants and actives is complicated by unknown factors. Actives are contained in formulations, Act B and Act C are within suspended concentrate formulations, which contain various other unknown compounds such as surfactants, emulsifiers and stabilisers. The presence of unknown chemicals which are kept secret due to trade secrets, means it is unclear whether compatibilities and incompatibilities with tank-mix adjuvants in terms of retention, are as a result of an interaction with the A.I itself or any of the other components in formulation.

Thermal imaging was inaccurate to measure droplet lifetimes.

Monitoring droplet lifetimes of adjuvants with two conventional actives, Act B fungicide and Act C insecticide using the infra-red camera (see Figure 7) provided a different story to that from visually measuring droplet drying times of the actives (see Figure 6). Temperature differences shows that droplets reached the temperature of the leaf within a few minutes of each other at each volume, irrespective of adjuvant and active chosen. Moreover, videos did not show maximum retention times as seen by the visual method. These differences between methods may be because the two methods showed two different phases of drying. The first is characterised by water evaporation to equilibrate the temperature of droplets to the leaf, by evaporative cooling, as seen in Figure 7. It is suggested that the second phase of drying is characterised by the residual presence of adjuvant in the droplet after water has evaporated. This phase was not seen by thermography as the leaf and droplets had reached the same temperature. The overall droplet lifetime, was characterised by the adjuvant in the second phase of drying, giving varied DRTs for different adjuvants with different actives (Figure 6, Table 7).

Adjuvant extended DWAs compared to water.

All adjuvants apart from Adj G were able to significantly increase DWAs compared to water (Figure 8A, 8B). Adj G has different chemistry to the other adjuvants which might explain its inability to increase droplet area significantly. It is composed of a blend of carbohydrates and although its Log P is unknown, it is expected to be the most hydrophilic of adjuvants. Evidence for this comes from the properties in Table 10. Adj G has the highest surface tension of adjuvants (50-55 mN m^{-2}) compared to water which has 72 mN m^{-1} and was the only adjuvant with a hydrophilic-lipophilic balance (HLB) value less than 8, which is the cut-off point for forming oil-in-water emulsions, the expected suspension form for other adjuvants containing oil or wax (Griffin, 1949, Griffin, 1954). Adj G has humectant properties and is more hydrophilic than other adjuvants in the study, which give it a more similar wetting capability of the leaf surface to water droplets.

Adjuvant DWAs affected DRTs.

Adj 1 which had either comparable or in some cases smaller wetted areas than other adjuvants with different actives, had high retention with a range of actives. However, this study scored only wettable areas and did not measure contact angles of droplets on the leaf surface or droplet height or overall shapes of droplets. These factors also effect evaporation rates as they contribute to the perimeter of the droplet experiencing latent heat exchange with the vapour phase. High contact angles, which give rise to ridges between the droplet and leaf surface, have been shown to limit evaporation rates by the trapping of vapour (Zhou et al., 2017). It may be that Adj 1 is retained with different actives due to the presence of ridge structures, but this was not highlighted using the thermal camera and further work measuring contact angles of adjuvants with different actives should be carried out to confirm this interpretation.

The Adj J adjuvant had significantly larger DWAs with actives compared to all other adjuvants, (see Figure 8) apart from the organosilicone spreader Adj 2, which spread outside of measurable parameters. Both of these adjuvants had poor retention with actives. This suggests an inverse relationship between droplet wetted areas and retention times which is supported by previous research. Larger DWAs have both been shown to decrease drying times by increasing area for evaporation (Gimenes et al., 2013) and contribute to more loss through increased absorption into the leaf (de Oliveira et al., 2019). This would be expected for Adj 2 which is known to increase penetration through both the stomatal and cuticular pathways.

It is suggested that Adj J had larger wetted areas because it was used at more than double the concentration of other adjuvants in the study (see Table 10). Gimenes et al., (2013) used a range of adjuvant concentrations and found that droplets containing higher concentrations of adjuvant (up to 2%) increased wetted areas and therefore decreased evaporation time. In droplets with a high concentration of adjuvant it is expected that the majority of droplet space is taken up by adjuvant, resulting in expulsion of water to the edges where it can quickly evaporate (Zhou et al., 2017). It is also implied that adjuvants used at high concentration cannot associate as closely with actives and there is a spatial separation within the droplet which contributes to larger DWAs and quicker evaporation (Hunsche and Noga, 2012). This may be the case for the Adj J MEL adjuvant used at 5%, and potentially also Adj H used at 2.5%

with actives in this study. Although a previous study with bifenthrin found that both petroleum and seed oils increased spreading, retention and binding of droplets on cotton leaves (Mulrooney, 2000), in the example from this experiment, there is a trade-off between high spreading adjuvants and a lowering of droplet lifetimes. Adjuvants Adj H and Adj J would not be suggested for prolonging the liquid phase of systemic actives on the leaf surface based on poor drying times with actives and concentrations tested here, and a suggested lowering of adjuvant concentration may be more effective for this. Whether they are more effective in penetration instead of retention, remains to be seen from penetration studies.

Effects of actives on DWAs.

Different actives have different effects on wetted areas of droplets containing adjuvants (see Table 8). The Act C insecticide increased DWAs of humectants Adj G, Adj 3 and emulsifier Adj 1 compared to droplets without active. The ACT E active containing insoluble kaolite had the largest droplet wetted areas of all other active types, but these peaks were only with MEL adjuvants Adj H and Adj J and were volume specific (Figure 8). The differences in droplet areas between adjuvant-active combinations are indicators of both spreading potential and degree of association. In the case of Act C and Adj A the droplet areas were small, suggesting a high level of interaction, but droplet mean lifetimes were high. Conversely Act C extended DWAs of humectants and emulsifying adjuvants, but retention times were also high. This suggests both small and large DWAs or associations can promote retention and this depends on the complex interaction between combination of adjuvant and active and their formulation components respectively.

These studies suggest that a number of criteria are important for determining optimum retention, including adjuvant concentration, droplet area (or volume applied). Not all adjuvants and actives are compatible and not all adjuvants are effective for every application limitation and must be tested in the presence of actives before making a recommendation.

Spray coverage of ACT E + adjuvants on target leaf surface were variable

Adjuvants were used in spray application of ACT E pesticide, where a high uniform coverage is suggested to be ideal as both a heat protectant and a contact barrier for insect-host recognition (Glenn et al., 1999, Liang, 2002, Peng, 2011). Adjuvant Adj H had the highest coverage with ACT E, 53% coverage compared to 3% by the organosilicone wetter which had the least (see Figure 10). A study which sprayed adjuvants mixed with a UV-dye showed that two organosilicone surfactants gave the best coverage of all adjuvants, 87% and 83% and their reported values for water and an MSO adjuvant were 46% and 54% coverage (Gent, 2003). The percentage coverage for organosilicone surfactants was remarkably different to Adj 2 used in this study, the water values slightly different, but the Adj H adjuvant, containing MSO corresponds very well to this data.

DWAs of adjuvants with ACT E did not correlate with good coverage.

Small-sized droplets (1.2 µm) are predicted to result in high off-target losses during spraying run-off. Medium-sized droplets (2.5 µm) are considered optimum for spraying (Akesson et al., 1994, de Oliveira et al., 2019). Adj H with ACT E had significantly higher coverage than all other adjuvants and water after spray application. The DWAs of Adj H with ACT E tended to be larger than any other adjuvant with ACT E when used at 1 µl (see Figure 8A), therefore the good coverage performance of Adj H is not related to it having medium-sized droplets and is more likely related to physicochemical characteristics during spraying. Adj 1 and water have the two smallest DWAs when combined with ACT E in 1 µl-3 µl droplet volumes (see Figure 8) but Adj 1 and water % coverages came in the top 2/8 and 4/8 of all spray combinations respectively. However, loss through drift would not be a significant problem in controlled laboratory conditions where weather is not a factor and spray distance from nozzle to leaf is small. It was not possible to measure DRTs of adjuvants with ACT E as sessile droplets due to its reflective white nature, as it was not possible to confirm when droplets were dry. However, interestingly, Adj H and Adj 1 adjuvants, which gave the best coverage with ACT E, also gave the longest droplet lifetimes when used as adjuvant-only sessile droplets on the leaf surface (see Figure 6). DRTs of ACT E with adjuvants would be a useful parameter to measure to see whether Adj 1 and Adj H also increase DRTs with the insoluble ACT E active. However, in the case of ACT E, adjuvants which promote film-forming rather than enhancing penetration are desirable.

Adj J was used at the highest concentration of adjuvants of all droplets and also had the largest wetted areas and highest viscosity (see Table 10 and Figure 8) but it did not have the highest levels of coverage on the *C. communis* leaf (Figure 10). It has been shown that increasing adjuvant concentration from 0.01% to 0.11% significantly increased coverage on three leaf types (Januskiewitz, 2019) which suggests high concentration improves spreading and coverage. High viscosity is typically associated with 'sticking' adjuvants which maximise adhesion and spreading. The spray coverage produced with Adj J had thick layers around the leaf edges and more gaps on the leaf (Figure 10), which is a pattern that has been described when the distribution of large, viscous droplets leads to coalescence and run-off from the edge of the leaf (Faers and Pontzen, 2008). An experiment which varied concentrations of different adjuvants and spray volumes found this pattern tended to occur when droplets were large, too concentrated and the spray volume too high (Faers and Pontzen, 2008). This also helps to explain why the high-spreading Adj 2 adjuvant only resulted in 3% coverage concentrated at the leaf tip. High wetting organosilicone surfactants, are dispersible in water and low surface tension allows spread across the surface, they therefore have a high loss rate through run-off when spray volumes and concentration of adjuvant are not optimised. This experiment is further evidence for the potential losses to the environment from using an organosilicone incorrectly in a spray application.

Emulsions have been described as the preferential liquid of choice for spraying (Hilz and Vermeer, 2013, Vernay et al., 2016) especially when using kaolin particles, which are insoluble, hydrophobic and may be best retained in oil-water type emulsions. In studying droplet drying behaviour of adjuvants with actives, only adjuvant Adj H (not Adj J) was seen to form a characteristic dome at the top edge of droplets, a

characteristic of emulsion behaviour (Wang et al., 2018). Adj H formed a dome with all actives (apart from Bioactive 1) and consistently developed central domes within droplets within 10 mins of droplets starting to dry (data not shown). Therefore, it is thought that the addition of MSO gave Adj H emulsion properties. Liquid behaviour during atomisation, impaction and drying of Adj H with ACT E kaolin particles may be more optimum for maintaining solubility and heterogeneity of mixtures containing hydrophobic particles. This explains why Adj H and Adj 1 emulsions may have the highest level of coverage with ACT E during spray application but does not explain why Adj G and Adj 3 emulsions had low coverage (18% and 26% respectively). These two humectant adjuvants had the smallest droplet sizes on the *C. communis* leaf surface during spraying but not when combined with ACT E as sessile droplets. Adj G is hydrophilic and has an HLB value below 8 so is not predicted to form oil-in-water emulsions with ACT E. It is suggested that Adj G and Adj 3 adjuvants may perform better if sprayed at higher concentrations or with a coarser nozzle type, or it may be that these humectant type adjuvants may be incompatible with insoluble kaolin.

Deposition after droplet evaporation.

Solid deposits on the leaf surface after drying of sessile droplets have been used because it has not possible within this study to directly measure the interaction of adjuvants and actives within the droplet. Droplet sizes and retention data, as well as residual deposit types are used as indicators of compatibility. In the case of using a Parafilm surface, solid deposits can be seen deposited on the film and shows localisation of actives after droplets containing adjuvants or water have dried.

Adjuvants left different deposit structures on Parafilm.

The deposit from Adj H + Act C showed drying occurred from outside-in and there was separation of phases during drying, probably due to the complex mixture of MSO, MELs and Act C. The emulsion properties of Adj H also gave rise to the central A.I deposit which was deposited after flattening of the central emulsion dome structure, originally present at the top edge, which falls down under gravity and is deposited on the leaf surface. On Parafilm at least with Act C, it is expected that Adj H concentrates most of the A.I within the emulsion dome.

Adjuvant Adj J spread the most on Parafilm, also dried from outside-in and after drying, still appears wet (see Figure 11A). The large, flat droplet formed by Adj J shows adsorption onto the Parafilm surface due to low surface tension, high lipophilicity and its high concentration, which maximises spreading. Adsorption effects advancing and retracting of droplets during drying which also effects deposit residues after drying (Knoche and Bukovac, 1992). Adsorption of the densely concentrated MEL adjuvant Adj J to both Parafilm and leaf may help explain why the droplet with Act C on the *C. communis* leaf also did not 'look dry' after there was no more flattening of droplet (see Figure 11). After a droplet spreads and loses its droplet height, retraction is the last part of the evaporation process. High viscosity due to the high concentration of adjuvant limits recession of droplets as concentration of adjuvant becomes even greater during drying, as water evaporates from droplets

(Deegan et al., 1997, Hunsche and Noga, 2012). The unequal drying from outside-in on Parafilm does suggest that in the case of Adj J, recession was limited, but whether this type of deposit has any effect on penetration remains to be seen when linking deposits to penetration capability of adjuvants.

Adj 1, Adj 3 and Adj G had ring deposits with Act C on Parafilm showing partitioning of phases within droplets but with smaller deposit diameters than Adj J, Adj H and Adj A (see Figure 11A). In the Parafilm experiment, with 30 µl droplets, these three adjuvants had the longest drying times. This may mean that for these adjuvant types, a longer drying period is characterised by droplets which experience shrinkage during drying, giving rise to the smaller deposit outlines after drying. These adjuvants are used at lower concentrations than the MEL adjuvants and are not as viscous (see Table 10) so would not be expected to have as much resistance to retraction of the droplets during drying. Both Adj 3 and Adj G have humectant properties so have the potential to take on water during drying, slowing the overall process of water evaporation from droplets. The DRTs for 30 µl droplets of Act C with adjuvants on Parafilm did not correspond to the order of DRTs for 3 µl droplets as shown in Figure 6. Both surface and volume effects are expected to account for this difference.

Bioactive formulations formed different deposits with adjuvants on the *C.communis* leaf.

Both Bioactive formulations left more deposits with adjuvants than the two conventional synthetic actives (compare Figure 11B and C with Figure 11D and E). Bioactive 1 mixed with different adjuvants tended to form thick, dense ring deposits and only two adjuvants left dried dispersed deposits. This is different to deposits left by adjuvants with Bioactive 2, where all adjuvants left an evenly dispersed deposit. The presence of milk powder in the Bioactive 1 formulation appears to have caused separation of adjuvants from the biofungicide active, giving rise to o-rings which may be thickened because of the sedimentation of the higher solid content of both milk powder and fungus on the outside of drying droplets. It cannot be confirmed that adjuvants are present only in the centre of deposits, however thicker o-rings have also been implicated in low association between adjuvant and active causing separation of phases (Deegan et al., 1997, Faers and Pontzen, 2008, Taylor, 2011). These ring deposit structures may be further support for low mean drying times for droplets containing Bioactive 1 and the negated adjuvant effects on retention. Some studies have attempted to use novel methods of detecting adjuvants and active distributions separately within drying droplets and have attempted to link this to uptake (Perkins et al., 2008, Hunsche and Noga, 2012, Kraemer et al., 2009). However, the links between behaviour of actives and adjuvants within the droplet, deposit type and uptake are still not known. An added complexity of droplet behaviour comes when one uses live organisms such as a bacteria or fungi as their biological activities and motility within liquids can affect drying, partitioning and formation of ring droplets (Callegari et al., 2018). To further extend the applicability of these deposit results for Bioactive, it would be useful to assess coverage after spray application with different adjuvants over a leaf, similar to the work done with the conventional active, ACT E.

As seen from Figure 11E, Adj 2 left a spread deposit with Bioactive 2. Bioactive 2 had some effects on spreading and deposition behaviour of Adj 2. The addition of the Bioactive 2 active is suggested to affect vesicle formation by organosilicone head groups, effecting ability to spread, resulting in thicker droplets which increases DRTs and leave a spread deposit with Bioactive 2 after drying. However, it is not clear why the higher solid content within Bioactive 1, would not have the same effect on vesicular formation over the surface with the super-spreader Adj 2.

The quantity of actives and distribution of adjuvants in the dried deposit matrices could not be determined on the leaf in this study. It was predicted that because Adj A + Act C had very small droplet sizes and high retention times that this was a highly compatible active and adjuvant which also shows no residue on the leaf, suggesting equal drying throughout the droplet is the most optimum. However, equal drying of droplets resulting in no visible residue cannot be directly correlated with higher uptake but higher retention gives more time for uptake to occur in the liquid phase (Liu, 2004, Faers and Pontzen, 2008, Xu et al., 2010a), Adj A + Act B were expected to have a good interaction and penetration potential from the Log P values but the solubility of the active is not known. Act B + Adj A did not show characteristics of good compatibility from retention times and formed a light-casting deposit on the leaf. This could be indicative of even spread of active throughout the droplet or even damage to the cuticle caused by high penetration of the Act B + Adj A combination (Knoche and Bukovac, 1992, Steurbaut, 1993, Gent, 2003, Orbovic et al., 2007). Using deposit structures to make inferences about penetration of actives in each adjuvant system is incomplete without more detailed studies quantifying actives within the leaf tissue.

The incorporation of LYCH fluorescent dye within drying droplets can be used as an indicator of the deposition of actives at the end of the evaporation process. Pinning of droplets to the leaf surface must occur for annuli (coffee rings) to form and this is based on the sedimentation of solids around the perimeter of droplets and the leaf, which is also based on adjuvant type, concentration of active and surface tension of the droplet (Hunsche and Noga, 2012). In contrast to water and other adjuvants, no residue was formed for Adj J or Adj H which may be because they have the lowest surface tension and are able to wet the surface to a greater extent (bigger DWAs). The lack of deposits for these adjuvants suggest that LYCH was better dispersed within the droplet and lack of aggregated solid deposits may lead to the presumption of more LYCH uptake relative to other adjuvants. However, inconsistencies in uptake experiments hours after droplet dry down suggest that uptake can still occur from deposits (Liu et al., 2004, Liu, 2004, Forster and Kimberley, 2015). The re-solubilisation of deposit matrices is one of the functions of adjuvants and presuming there is a high association of adjuvants:LYCH still within deposits, adjuvants have the potential to prolong uptake even after droplet drying (Perkins et al., 2008). Unfortunately, adjuvant quantities or location within deposits is not known but if penetration continues after drying, DRTs of adjuvants may not be linked to uptake of LYCH. Results from re-wetting studies such as those with uranine deposits on leaves suggest the re-addition of water can increase uptake of solids through stomata (Eichert and Burkhardt, 2001). It is expected that humectants may have this advantage in conditions of high humidity with a hydrophilic dye such as LYCH.

4.2. Penetration into the leaf.

Adjuvant penetration could not be quantified using Lucifer yellow (LYCH).

Previous methods have used radiolabelled chemicals tagged onto actives or used fluorescent dyes to analyse recovery of fluorescence from the tissue surface after washing, and visualisation within tissue using confocal laser scanning microscopy over time. (Omokawa et al., 1989, Gent, 2003, Liu, 2004, Liu et al., 2004, Nauen, 2007, Maschhoff, 2009, Forster and Kimberley, 2015, Etxeberria et al., 2016, Cardoso-Gustavson et al., 2018). The aim of using penetration assays was to compare adjuvant penetration abilities with LYCH and potentially also of commercially important lipophilic pesticides. Localisation in tissue layers was considered for translaminar and translocating actives, using the LYCH dye. It was also hoped that penetration work would aid to a better understanding of surface behaviour of adjuvants: processes of evaporation, spreading and deposition through to penetration properties of adjuvants. However, it has not been possible in this study to measure penetration reliably using (i) microscopy or (ii) spectrofluorometry.

Assessment of unabsorbed LYCH after 22 hours was compared after different adjuvant and water treatments. Fluorescence of Adj H, Adj J, Adj 3 and Adj 2-treated tissue was comparative to untreated tissue (see Figure 12) highlighting that these adjuvants maximised uptake of total applied LYCH over 22 hours. In contrast, LYCH applied with water or with adjuvants Adj A, Adj 1 or Adj G had variable residual LYCH surface fluorescence. This data supports the qualitative solid residue deposits seen for adjuvants with LYCH after drying (Figure 11), whereby Adj J, Adj H, Adj 3 and Adj 2 formed evenly spread or no surface deposits. Solid ring deposits from pinned droplets correlated with the adjuvants which did not enhance uptake of total applied LYCH after 22 hours. It is expected that the organosilicone Adj 2 would be a positive control and would enhance uptake through both stomatal and cuticular pathways. Oil-based adjuvants, those based on MELs and ethoxylated seed oil adjuvants have enhanced uptake of LYCH through the cuticle, however the small lipophilic resin Adj A, paraffin-wax based Adj 1 and hydrophilic Adj G with humectant properties have not enhanced uptake.

Levels of autofluorescence were very high and variable in *C. communis* epidermal and mesophyll tissue despite growth in consistent conditions meaning that this observation could not be confirmed in planta. The extent of fluorescence variation in tissues may include, but is not limited to, cell wall components which could be attributed to: pectins in epidermal cell walls, arabinan epitopes and feruloyl esters in walls of guard cells and subsidiary cells (Parker et al., 2000, Jones et al., 2003), lignin in ventral walls of stomatal pores and inner walls of guard cells in *C. communis* (Shtein et al., 2017). Emission from chlorophyll is at >650 nm and varied in intensity. Even though previous research has been able to achieve complete separation of chloroplast signal from those of LYCH and other dyes, (Liu et al., 2004, Botha et al., 2008, Burrows et al., 2013) it was not possible in the present study.

The effect of different adjuvants at different time points on LYCH uptake could not be established, as untreated tissue also gave the same fluorescence around stomata between repeats (see Figures 13, 14 and 15). Studies in *Solanum elaegnifolium*

(silverleaf nightshade) and *Glycine max* (soybean) visualised LYCH in the veins between mesophyll cells and a study in *C. communis* showed fluorescence around stomatal regions in patterns similar to that found here (Hillmer et al., 1990, Burrows et al., 2013, Cardoso-Gustavson et al., 2018). However, some of these studies can be criticized for not showing untreated control tissue. In this present study for each adjuvant-treated tissue, fluorescence pattern repeatability was typically only 1 out of 4 times, making it difficult to establish if uptake, the assay or adjuvant effect on LYCH is the biggest variable. Previous studies with LYCH suggested that uptake occurs through stomatal pores. However, as the experiments reported here were done in the dark, stomatal penetration would be expected to be minimal and polar penetration through the cuticle is more likely to be the predominant pathway into the leaf. As the mesophyll and epidermis were both peeled and the adaxial surface not visualised using microscopy, any LYCH still present in either the cuticle or upper layers, has not been analysed in the context of different adjuvants. Previous work has indeed shown that dyes can be retained within in the cuticle (Liu et al., 2004, Cardoso-Gustavson et al., 2018). The permanency and frequency of cuticle-trapping is an important factor to research in the future, if adjuvants are to be recommended for use with synthetic compounds entering the leaf for their MoAs either in the vasculature or at the opposite side of the leaf.

One study found variation in uptake between 6.7 to 442 pmol/mm^2/hr of isolated adaxial cuticles of *C. communis* when they measured penetration of propanil (a lipophilic compound) without surfactants (Omokawa et al., 1989). This suggests that *C. communis* adaxial leaf surface may be inherently variable in uptake of lipophilic compounds over time across the surface. In those experiments, uptake of propanil was linear over time and all adjuvants tested had a positive effect on uptake within 3 hours. In this study, the use of spectrofluorometry for LYCH recovery did not produce increasing fluorescence intensity patterns for LYCH between 1, 3 and 6 hours (see Figure 16) as suggested with uptake of propanil and benzyladenine in previous studies (Omokawa et al., 1989, Knoche and Bukovac, 1992, Petracek, 1998). The fluorescence was measured for the whole leaf disc, which includes the upper epidermis and cuticle, which would be expected to show fluorescence if any LYCH is trapped in the upper layers. This study measured uptake for up to 22 hours and did not see a characteristic increase in uptake with any adjuvant at any time point. It cannot be concluded from this assay that adjuvants influenced uptake within the timescale measured.

The use of LYCH dye is predicted to have enhanced uptake effects with adjuvants because uptake of hydrophilic compounds is comparably slower across the cuticle than lipophilic compounds (Mercer, 2007, Forster and Kimberley, 2015). The adjuvant-treated tissue giving the highest signal between 1-3 hours included Adj 3 and Adj G (see Figure 13). These adjuvants both have humectant properties as Adj 3 contains ethylene oxides (EOs) and Adj G contains guar gums and glycerol. Although humectants have been shown to be effective at high humidity for maintaining solubility in droplets and suggested to maintain diffusion, droplets containing LYCH did not dry in lab conditions until between 2-3 hours so it is unclear how humectancy would enhance diffusion compared to other adjuvants at this time point. It has been predicted that humectants with high EO values can enhance uptake of hydrophilics

such as glyphosate but an ionic weak acid such as 2-4-D had better uptake with lower EO values (Liu, 2004). Lipophilic molecules are predicted to have better uptake with lower EO values which supposedly increase fluidity of the cuticle but work with epoxiconazole found differences in EO content of adjuvants was not significant to explain variations in uptake (Forster and Kimberley, 2015). It is unknown to what extent both Adj 2 and Adj 3 adjuvants in this study are ethoxylated but surface residual fluorescence indicated that they both could enhance uptake of LYCH (water-soluble ionic salt). For the case of humectants, Adj 3 was shown to improve uptake from the surface residues but Adj G was not. Unfortunately, uptake could not be reliably confirmed *in planta* after 22 hours for these adjuvants and it is not possible to infer penetration behaviour with lipophilic actives in this study as LYCH has different properties (see Table 11).

Table 11: Chemical properties of systemic actives and LYCH dye used in this study:

	Structure	Molecular weight g/mol	Log P (K_{ow})	Excitation/emission nm
Lucifer yellow (LYCH)	Lucifer yellow CH (dipotassium salt)	521.57	?	428 / 536
Spirotetramat (Act C)	Spirotetramat	373.45	2.51	-
Pyriofenone (Act B)	Pyriofenone	365.81	3.8	355 / 485

In this study, Adj 2 and Adj J had the largest DWAs but Adj 1 had best overall retention times. An increase in DWAs of different actives has been both argued for and against the case of adjuvants in improving uptake of systemic pesticides (Liu, 2004, Mercer, 2007). Long retention times as aqueous droplets on the leaf have also been implicated in promoting penetration. From the surface fluorescence data, low retention and high spreading of Adj J and Adj 2 seems to be most optimal for uptake of LYCH. The high retention with Adj 1 did not seem to enhance LYCH uptake after 22 hours, but it is unknown if there would be any further adjuvant uptake effects using

a longer treatment time. The use of fluorescence dyes and retention data are not enough to recommend adjuvants for penetration ability for either pesticides which have functions at the opposite side of the leaf (translaminar), or which require translocation into vascular tissues.

Uptake of pyriofenone using adjuvants could not be quantified.

There is little research on the effect of translocation of lipophilic systemic insecticides and fungicides such as those used in this study, as previous research has tended to use hydrophilic herbicides such as glyphosate (Augusto and Brenneman, 2012, Melo et al., 2015). Research is made more complicated because pesticides are typically not fluorescent and cannot be traced easily within tissue. Pyriofenone, the active ingredient of Act B SC 300 fungicide is fluorescent, and its properties were characterised as part of this study (see Table 11). However, due to limitations of wavelength filters and low intensity of signal for pyriofenone (Table 9) it could not be detected using fluorescence techniques.

Until uptake of actives (and ideally adjuvants as well) can be accurately measured in a range of crop species following droplet dry down, through deposition, through penetration, to translocation around the plant to target site, the efficiency of adjuvants is difficult to measure. The effect of translocation on bioactivity of compounds requires application in field trials.

4.3. Compatibility of adjuvants with a biofungicide.

The limitations in foliar application of bio-pesticides such as bio-nematicides or biofungicides such as *G. catenulatum* include UV degradation and moisture loss (Shapiro-Ilan, 2006, Bailey et al., 2007). Using formulations, and adjuvants in particular, has been suggested to improve their overall field efficacy both through improving moisture retention and modifying droplet spectra during spraying to reduce fine droplets and increase coverage and colonisation over a target (Gasic, 2013). Adjuvants with UV protection properties (*i.e.* Adj A) or humectancy properties such as Adj G and Adj 3 may be expected to prolong survival through better retention on the leaf, especially in high humidity conditions (Lyn, 2010). However, none of the adjuvants significantly improved retention of Bioactive 2 droplets compared to water. Adjuvant Adj J droplets dried out significantly quicker than all other adjuvants and water so would not be a good adjuvant choice for Bioactive 2 as CFUs in droplets may lose moisture before germination and growth can occur. If an adjuvant is to be compatible for organic farming, it must itself be organic as well as both improve retention and not be severely toxic. To test adjuvant compatibility, *G. catenulatum* (Bioactive 2) was grown in the presence of adjuvants, either for a limited time using an incubation period or with prolonged presence of adjuvants in adjusted-agar during the growth phase.

The biofungicide Bioactive was not compatible with adjuvants.

Although there was variation between experiments, the general finding throughout was that Adj G had some toxic effects on the growth of *G. catenulatum* both on agar plates and in liquid culture (see Figures 17 and 18). A previous study has highlighted the potential of adjuvants to have fungi-static or fungi-toxic properties (Steurbaut, 1993) which did seem to be the case for *G. catenulatum* with Adj G. One study used a range of adjuvants and found that some humectants were detrimental for growth of the *M. ocharea* biofungicide, but others were beneficial, so the effect must be adjuvant- and species-specific (Bailey et al., 2007).

The *in-vitro* assays used in this study have limitations because other than the presence of the adjuvant, the biofungicide is given ideal growth conditions that would not be the case if present on the leaf surface. Previous work using different assays with biofungicides showed that germination, mycelial and conidial growth were affected by varying adjuvant concentrations (Fravel et al., 2005, Bailey et al., 2007). The possible growth benefits seen with Adj A and Adj 2 adjuvants in the tube assay (see Figure 18) may be limited as *G. catenulatum* was only exposed to adjuvants for either 10 mins or 1 hour. This may not have been enough time to see a significant effect on growth and in practice; the biofungicide may be in contact with adjuvants for a much longer time span at conditions much less ideal for growth. There is some evidence from this study and others that longer contact time with fungicides and surfactants may decrease survival rates (Schroeder and Sieburth, 1997, Guijarro, 2018). Previous studies with surfactants similar to the organosilicone Adj 2, tend to report negative effects on organisms. Work with the biofungicide *M. ocharea* showed that spreader and surfactants similar to Adj 2, limited colonisation during spraying due to increased evaporation rates. Therefore, high growth does not necessarily mean good

colonisation or biocontrol after spraying. Generally, organosilicone surfactants such as Adj 2 are considered to move readily across membranes and have plasticisation effects on membrane lipids which is attributed to increased leakage of membranes, causing toxicity to organisms, especially aquatics (Mojsiewicz-Pieńkowska et al., 2016, Mullin et al., 2016). The Silwett-L77 organosilicone applied with *M. ocharea* biofungicide was previously found to slow mycelial growth and has been previously found to have some toxicity to both pest insects and pollinators including aphids and bees (Bailey et al., 2007, Ciarlo et al., 2012, Ganchev and Atanasova, 2018).

From the results of this study, adjuvants which have both high DRTs and moderate effects on growth include Adj 1 and Adj A. Further research is needed to see whether tank-mixed adjuvants can improve biocontrol efficacy of *G. catenulatum* with *Botrytis cinerea* or *Fusarium spp*. Assessment of spray application with adjuvants should also be carried out through measuring germination rates and CFUs per leaf area (coverage). It is hoped that through the continuation of screening trials for adjuvants, organic adjuvants which can benefit efficiency of Bioactive and other biopesticides can be applied to the organic farming market.

Bioactive had varied growth effects in the presence of actives.

Finally, *G. catenulatum* was grown in the presence of triple strength fungicide Act B and Act C insecticide to see whether these chemicals can be suggested for use as part of an IPM practice. Previous research has shown that *G. catenulatum* is robust in the presence of some fungicides *e.g.* furalaxyl, propamocarb HCl and fosetyl-aluminium had minimal effect on mycelial growth (McQuilken, 2008). In this study, Act B (pyriofenone) did reduce the growth of the biofungicide significantly but did not completely kill the biofungicide even after incubation at triple strength for 1 hour. The insecticide Act C had moderate effect on growth of *G. catenulatum* on agar plates for 5 days so potentially can be trialled as part of an IPM programme.

Chapter 5: Summary.

This study aimed to use a range of methods to screen adjuvant effectiveness using a model leaf from the monocot *Commelina communis*. Bio-adjuvants were compared with an organosilicone surfactant as an industry standard adjuvant.

This study looked at droplet retention times (DRTs), the evaporation rate of droplets on the adaxial leaf surface at 24°C with an RH 40%. Droplet lifetimes on a leaf surface were used to compare adjuvant ability to promote retention. Two adjuvants, Adj H and Adj 1 were shown to significantly enhance retention and the synthetic adjuvant Adj 2 had low retention. Adjuvants were also mixed with conventional and biological actives to see adjuvant performance compared to each other and assess how actives modified adjuvant retention. Adj 1 had the best retention with a range of actives whilst the two developmental adjuvants Adj H and Adj J could not extend active retention times on the leaf surface. Actives differed in their retention potential with adjuvants, Act C insecticide had longer lifetimes with adjuvants than any other actives. Two different formulations of a biofungicide containing *Gliocladium catenulatum* had different retention profiles with adjuvants. The Bioactive 1 formulation was incompatible and negated adjuvant retention effects, highlighting a need for studying compatibility between active formulations and adjuvants before recommendations can be made to organic growers. Maximum and minimum retention was compared across adjuvants and actives to assess compatibility. Act C insecticide with the Adj A adjuvant show good retention properties and now need to be further assessed for compatibility in studies which measure active function in pest control. The study used two methods to compare droplet drying times which included observation and quantification using a microscope affixed with camera and as a complimentary approach, analysis of the same droplet combinations using a thermography camera. The thermography camera highlighted the first phase of drying, characterised by water evaporation, whilst the second method showed adjuvant ability to prolong droplet lifetimes after water had evaporated.

The DWAs or the degree of adjuvant spreading potential over the surface was compared for different adjuvants with different actives and was correlated with evaporation times to see how adjuvants affected both properties. Adj 2 and Adj J had the largest droplet areas with actives and also had poor retention, Adj 1 formed smaller wetted areas with actives and had high retention. The resulting deposits after water evaporation from mixtures of adjuvants with different actives on the adaxial *C. communis* leaf surface and a model hydrophobic surface, Parafilm were compared to further gage compatibility of adjuvants and actives. Adjuvants formed different deposits with different actives and different deposits depending on surface type and droplet volumes used.

The use of a fluorescent tracer dye, Lucifer yellow (LYCH) was used to visually assess penetration ability of adjuvants compared to penetration of the dye with water over the same given time period. The solid deposits and residual fluorescence in total

unabsorbed surface liquid has been used to determine the amount of fluorescent dye not penetrated in the leaf in each adjuvant system. Bio-adjuvants Adj J, Adj H, Adj 3 and synthetic adjuvant Adj 2 had no detectable surface LYCH fluorescence, suggesting they had the best penetration potential with LYCH. The location of LYCH within the mesophyll and lower epidermis using confocal microscopy was not consistent between repeats of adjuvant-treated tissue. Recovery of LYCH fluorescence from whole leaf discs by spectrofluorometry was carried out at different time points but LYCH was not detectable above background autofluorescence at any time point. Direct assessment of a fluorescent active ingredient from Act B fungicide, namely pyriofenone was measured after treatment with adjuvants or water. Pyriofenone was not detectable above background fluorescence using FLIM, confocal microscopy or spectrofluorometry. Therefore, these technologies were not able to successfully translate observations from surface behaviours: evaporation, DWAs and deposition structures to rationalise adjuvant penetration of fluorescent compounds.

Finally, the study compared toxicity of adjuvants with a biofungicide Bioactive, containing bio-active *G. catenulatum*, to see whether adjuvants had negative effects on the growth of the fungus and to see whether they could be potentially applied in organic farming as potential ‘greener’ replacements of traditional surfactants. Not all adjuvants were found to be suitable for application with *G. catenulatum* and ones which showed potential in maximising both retention of droplets and growth of the fungus, should now be included in field trials to measure biocontrol success of *G. catenulatum* applied with adjuvants.

Chapter 6: References.

AKESSON, N. B. & GIBBS, R. E. 1990. Pesticide drop size as a function of spray atomizers and liquid formulations. *Pesticide Formulations and Application Systems: 10th Volume,* 170-183. BODE, L. E., HAZEN, J. L. & CHASIN, D. G. (eds.). West Conshohocken, PA: ASTM International.

AKESSON, N. B., STEINKE, W. E. YATES, W. E. 1994. Spray atomization characteristics as a function of pesticide formulations and atomizer design. *Journal of Environmental Science and Health, Part B,* 29**,** 785-814.

ARIAS-ESTÉVEZ, M., LÓPEZ-PERIAGO, E., MARTÍNEZ-CARBALLO, E., SIMAL-GÁNDARA, J., MEJUTO, J.C. & GARCÍA-RÍO, L. 2008. The mobility and degradation of pesticides in soils and the pollution of groundwater resources. *Agriculture, Ecosystems & Environment,* 123**,** 247-260.

ASMUS, E. 2016. Mode of action of adjuvants for foliar application. *PhD thesis.* University of Wuerzberg.

ATKINSON C.J., MANSFIELD T.A., McAINSH, M.R., BROWNLEE, C., HETHERINGTON, A.M. 1990. Interactions of calcium with abscisic acid in the control of stomatal aperture. *Biochemistry, Physiology of Pflanzen*, 186, 333 - 339.

AUGUSTO, J. & BRENNEMAN, T. 2012. Assessing systemicity of peanut fungicides through bioassay of plant tissues with *Sclerotium rolfsii. Plant Disease,* 96**,** 330-337.

AVEYARD, R., BINKS, B. P. & CLINT, J. H. 2003. Emulsions stabilised solely by colloidal particles. *Advances in Colloid and Interface Science,* 100-102**,** 503-546.

H. BAHROUNI, H., SINFORT,C., HAMZA, E. 2008. Evaluation of pesticide losses during cereal crop spraying in Tunisian conditions. 1*0th International Congress on Mechanization and Energy in Agriculture*, Oct 2008, Antalya, Turkey. 8 p.

BAILEY, K., CARISSE, O., LEGGETT, M., HOLLOWAY, G., LEGGETT, F., M. WOLF, T., SHIVPURI, A., DERBY, J., CALDWELL, B. & GEISSLER, H. 2007. Effect of spraying adjuvants with the biocontrol fungus *Microsphaeropsis ochracea* at different water volumes on the colonization of apple leaves. *Biocontrol Science and Technology,* 17**,** 1021-1036.

BARNES J. 1994. Effects of UV-B Radiation on wax biosynthesis. In: *Air pollutants and the leaf cuticle.* Percy K.E., Cape J.N., Jagels R., Simpson C.J. (eds) NATO ASI Series (Series G: Ecological Sciences), 36. Springer, Berlin, Heidelberg

BEATTIE, G. A. & MARCELL, L. M. 2002. Effect of alterations in cuticular wax biosynthesis on the physicochemical properties and topography of maize leaf surfaces. *Plant, Cell & Environment*, 25**,** 1-16.

BERNARD, T., CHANTELOT, E. & OGAWA, M. 2012. Pyriofenone: a novel powdery mildew fungicide for grapevine. Alfortville: Association Française de Protection des Plantes (AFPP).

BISHOP, C.A. 2015. Chapter 5 - Process Diagnostics and Coating Characteristics, V*acuum deposition onto webs, films and foils* (third edition), William Andrew Publishing, 85-128

BLACKMAN, P.G., DAVIES, W.J. 1983. The effect of cytokinins and ABA on stomatal behaviour of maize and Commelina. *Journal of Experimental Botany*, 34, 149, 1619 - 1626.

BOTHA, C. E., AOKI, N., SCOFIELD, G. N., LIU, L., FURBANK, R. T. & WHITE, R. G. 2008. A xylem sap retrieval pathway in rice leaf blades: evidence of a role for endocytosis? *Journal of Experimental Botany,* 59**,** 2945-54.
BRUTIN, D. & STAROV, V. 2018. Recent advances in droplet wetting and evaporation. *Chemical Society Reviews,* 47**,** 558-585.
BULLION, A. 2017. US still leads the way in biopesticides, as EU rules remain complex [Online]. Informa. Available: https://iegpolicy.agribusinessintelligence.informa.com/PL214649/US-still-leads-the-way-in-biopesticides-as-EU-rules-remain-complex [Accessed 08/08/2019].
BURROWS, G. E., WHITE, R. G., HARPER, J. D., HEADY, R. D., STANTON, R. A., ZHU, X., WU, H. & LEMERLE, D. 2013. Intrusive trichome bases in the leaves of silverleaf nightshade (*Solanum elaeagnifolium*; Solanaceae) do not facilitate fluorescent tracer uptake. *American Journal of Botany,* 100**,** 2307-17.
BUSCHHAUS, C., HERZ, H. & JETTER, R. 2007. Chemical composition of the epicuticular and intracuticular wax layers on adaxial sides of *Rosa canina* leaves. *Annals of botany,* 100**,** 1557-1564.
CALLEGARI, A., ANDAC, T., WEIGMANN, P., VELU, S. K. P., PINCE, E., VOLPE, G. & VOLPE, G. Active matter alters the growth dynamics of coffee rings. Latin America Optics and Photonics Conference, 2018/11/12 2018 Lima. Optical Society of America, W2A.4.
CARDOSO-GUSTAVSON, P., DIAS, M. G., COSTA, F. O. B., DE MOURA LEITE CAMARGOS, G. & DA CRUZ CENTENO, D. 2018. Imaging of glyphosate uptake and identification of early microscopic markers in leaves of C3 and C4 glyphosate-resistant and -susceptible species. *Ecotoxicology & Environmental Safety,* 163**,** 502-513.
CARY, D. The maturing biocontrol industry and it's role in true Green Revolution. *Biopesticide summit 2019, 2019 Swansea*. IBMA.
CHANDLER, D. B., A.S.; TATCHELL, G.M.; DAVIDSON, G.;L GREAVES, J.; GRANT, WP. 2011. The development, regulation and use of biopesticides for integrated pest management. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences,* 366**,** 1987-1998.
CHATTERTON, S., JAYARAMAN, J. & PUNJA, Z. K. 2008. Colonization of cucumber plants by the biocontrol fungus *Clonostachys rosea f. catenulata. Biological Control,* 46**,** 267-278.
CHATTERTON, S. & PUNJA, Z. 2009. Chitinase and β-1,3-glucanase enzyme production by the mycoparasite *Clonostachys rosea f. catenulata* against fungal plant pathogens. *Canadian Journal of Microbiology,* 55**,** 356-67.
CHATTERTON, S. & PUNJA, Z. 2011. Colonization of geranium foliage by *Clonostachys rosea f. catenulata*, a biological control agent of botrytis grey mould. *Botany,* 90**,** 1-10.
CHUNG, H. S., KABIR, M. H., ABD ELATY, A. M., LEE, H. S., RAHMAN, M. M., CHANG, B.J., SHIN, H.C. & SHIM, J.H. 2017. Dissipation kinetics and pre-harvest residue limit of pyriofenone in oriental melon (*Cucumis melo Var. makuwa*) grown under regulated climatic conditions. *Biomedical Chromatography,* 31**,** e3965.
CIARLO, T. J., MULLIN, C. A., FRAZIER, J. L. & SCHMEHL, D. R. 2012. Learning impairment in honey bees caused by agricultural spray adjuvants. *PLOS ONE,* 7**,** e40848.

CROSS, J., WALKLATE, P., MURRAY, R. A. & RICHARDSON, G. M. 2001. Spray deposits and losses in different sized apple trees from an axial fan orchard sprayer: 2. Effects of spray quality. *Crop Protection,* 20**,** 333-343.
DE COCK, N., MASSINON, M., SALAH, S. O. T. & LEBEAU, F. 2017. Investigation on optimal spray properties for ground based agricultural applications using deposition and retention models. *Biosystems Engineering,* 162**,** 99-111.
DE OLIVEIRA, R. B., BONADIO PRECIPITO, L. M., GANDOLFO, M. A., DE OLIVEIRA, J. V. & LUCIO, F. R. 2019. Effect of droplet size and leaf surface on retention of 2,4-D formulations. *Crop Protection,* 119**,** 97-101.
DEEGAN, R. D., BAKAJIN, O., DUPONT, T. F., HUBER, G., NAGEL, S. R. & WITTEN, T. A. 1997. Capillary flow as the cause of ring stains from dried liquid drops. *Nature,* 389**,** 827-829.
DESNEUX, N., DECOURTYE, A. & DELPUECH, J.-M. 2007. The sublethal effects of pesticides on beneficial arthropods. *Annual Review of Entomology,* 52**,** 81-106.
DOMÍNGUEZ, E., HEREDIA-GUERRERO, J. A. & HEREDIA, A. 2017. The plant cuticle: old challenges, new perspectives. *Journal of Experimental Botany,* 68**,** 5251-5255.
DONG, X., ZHU, H. & YANG, X. 2015. Characterization of droplet impact and deposit formation on leaf surfaces. *Pest Management Science,* 71**,** 302-308.
EBERT, T., TAYLOR, R., DOWNER, R. A. & R HALL, F. 1999. Deposit structure and efficacy of pesticide application. 1: Interactions between deposit size, toxicant concentration and deposit number. *Pesticide Science,* 55**,** 783-792.
EFSA 2015. Reasoned opinion on the modification of the existing maximum residue level (MRL) for pyriofenone in table grapes. *EFSA Journal,* 13**,** 4071.
EGLEY, G. H., HANKS, J. E. & BOYETTE, C. D. 1993. Invert emulsion droplet size and mycoherbicidal activity of *Colletotrichum truncatum*. *Weed Technology,* 7**,** 417-424.
EICHERT, T. & BURKHARDT, J. 2001. Quantification of stomatal uptake of ionic solutes using a new model system. *Journal of Experimental Botany,* 52**,** 771-781.
ETXEBERRIA, E., GONZALEZ, P., FANTON BORGES, A. & BRODERSEN, C. 2016. The use of laser light to enhance the uptake of foliar-applied substances into citrus (*Citrus sinensis*) leaves. *Applications in Plant Sciences,* 4.
FAERS, M. A. & PONTZEN, R. 2008. Factors influencing the association between active ingredient and adjuvant in the leaf deposit of adjuvant-containing suspoemulsion formulations. *Pest Management Science,* 64**,** 820-33.
FALK, R. H. Influence of formulation and adjuvants on the foliar location and physical form of the active ingredient. *In: Interactions between adjuvants, agrochemicals and target organisms*. HOLLOWAY, P. J., REES, R. T. & STOCK, D., eds., 1994// 1994 Berlin, Heidelberg. Springer Berlin Heidelberg, 53-82.
FAO. 2012. *FAOSTAT homepage.* [Online]. Available: http://faostat3.fao.org/home [Accessed 08/08/2019].
FERNÁNDEZ, V. & EICHERT, T. 2009. Uptake of hydrophilic solutes through plant leaves: Current state of knowledge and perspectives of foliar fertilization. *Critical Reviews in Plant Sciences,* 28**,** 36-68.

FERNANDEZ, V., GUZMAN-DELGADO, P., GRACA, J., SANTOS, S. & GIL, L. 2016. Cuticle structure in relation to chemical composition: Re-assessing the prevailing model. *Frontiers in Plant Science,* 7**,** 427.
FORSTER, W. A. & KIMBERLEY, M. O. 2015. The contribution of spray formulation component variables to foliar uptake of agrichemicals. *Pest Management Science,* 71**,** 1324-34.
FORSTER, W. A., KIMBERLEY, M. O. & ZABKIEWICZ, J. A. 2005. A universal spray droplet adhesion model. *Transactions of the ASAE,* 48**,** 1321-1330.
FOY, C. L. 2017. Adjuvants and agrochemicals volume 1: Mode of action and physiological activity (Chapter 1 Adjuvants terminology classification and mode of action). Chapman and Hall/CRC, 2017*,* 1.
FRAC 2019. FRAC code list [Online] https://www.frac.info/docs/default-source/publications/frac-code-list/frac-code-list-2019.pdf [Accessed 06/07/2019].
FRAVEL, D. R., DEAHL, K. L. & STOMMEL, J. R. 2005. Compatibility of the biocontrol fungus *Fusarium oxysporum* strain CS-20 with selected fungicides. *Biological Control,* 34**,** 165-169.
GANCHEV, D. & ATANASOVA, D. 2018. Insecticidal actionof silwet L-77 towards some aphid species.
GASIC, S. Brankica., T. 2013. Biopesticide formulations, possibility of application and future trends. *Pesticidi i fitomedicina,* 28**,** 97-102.
GENT, H. Schwarz. D. H. Nissen., S. 2003. Effect of commercial adjuvants on vegetable crop fungicide coverage, absorption, and efficacy. *Plant Disease,* 87**,** 591-597.
GIMENES, M. J., ZHU, H., RAETANO, C. G. & OLIVEIRA, R. B. 2013. Dispersion and evaporation of droplets amended with adjuvants on soybeans. *Crop Protection,* 44**,** 84-90.
GLENN, D. M., PUTERKA, G., VANDERZWET, T., BYERS, R. E. & FELDHAKE, C. 1999. Hydrophobic particle films: A new paradigm for suppression of arthropod pests and plant diseases. *Journal of Economic Entomology,* 92**,** 759-771.
GORB, E. V. & GORB, S. N. 2017. Anti-adhesive effects of plant wax coverage on insect attachment. *Journal of Experimental Botany,* 68**,** 5323-5337.
GRIFFIN, W. C. 1949. Classification of surface-active gents by “HLB”. *Journal of Cosmetic Science,* 1**,** 311-326.
GRIFFIN, W. C. 1954. Calculation of HLB valuess of nonionic surfactants. *Journal of the Society ofCosmetic Chemists,* 5**,** 249-256.
GUEST, D. 2008. Black pod: diverse pathogens with a global impact on cocoa yield. *Phytopathology,* 97**,** 1650-3.
GUIJARRO, B. L., I.; MELGAREJO, P.; DE CAL, A. 2018. Surfactant effects on wettability of *Penicillium frequentans* formulations to improve brown rot biocontrol. *Journal of the Science of Food and Agriculture,* 98**,** 5832-5840.
HALL, F.R. CHAPPLE. A.C.; DOWNER R.A, KIRCHNER L.M, THACKER R.M. 1993. Pesticide application as affected by spray modifiers. *Pesticide Science,* 38**,** 123-133.
HAZEN, J. 2000. Adjuvants terminology classification and chemistry. *Weed Technology,* 14**,** 773-784.
HE, Y., XIAO, S., WU, J. & FANG, H. 2019. Influence of multiple factors on the wettability and surface free energy of leaf surface. *Applied Sciences,* 9.

HILLMER, S., HEDRICH, R., ROBERT-NICOUD, M. & ROBINSON, D. G. 1990. Uptake of Lucifer yellow CH in leaves of *Commelina communis* is mediated by endocytosis. *Protoplasma,* 158**,** 142-148.
HILZ, E. & VERMEER, A. W. P. 2013. Spray drift review: The extent to which a formulation can contribute to spray drift reduction. *Crop Protection,* 44**,** 75-83.
HUANG, H. C. 1978. *Gliocladium catenulatum*: Hyperparasite of *Sclerotinia sderotiorum* and Fusarium species. *Canadian Journal of Botany,* 56**,** 2243-2246.
HUNSCHE, M. & NOGA, G. 2012. Effects of relative humidity and substrate on the spatial association between glyphosate and ethoxylated seed oil adjuvants in the dried deposits of sessile droplets. *Pest Management Science,* 68**,** 231-9.
JANKU, J., BARTOVSKA, L., SOULKUP J., JURSIK M., HAMOUZOVA K. 2012. Density and surface tension of aqueous solutions of adjuvants used for tank-mixes with pesticides. *Plant Soil and Environment*, 58: 568-572.
JANUSZKIEWICZ, K., MROZEK-NIEĆKO, A. & RÓŻAŃSKI, J. 2018. Effect of surfactants and leaf surface morphology on the evaporation time and coverage area of ZnIDHA droplets. *Plant and Soil,* 434**,** 93-105.
JETTER, R., KUNST, L. SAMUELS A.L. 2006. Composition of plant cuticular waxes. *Annual Plant Reviews 23: Biology of the Plant Cuticle.*
JETTER, R. & RIEDERER, M. 2015. Localization of the transpiration barrier in the epi- and intracuticular waxes of eight plant species: Water transport resistances are associated with fatty acyl rather than alicyclic components. *Plant Physiology,* 170, 921-934.
JETTER, R., SCHÄFFER, S. & RIEDERER, M. 2000. Leaf cuticular waxes are arranged in chemically and mechanically distinct layers: evidence from *Prunus laurocerasus* L. *Plant, Cell & Environment,* 23**,** 619-628.
JONES, L., MILNE, J., ASHFORD, D. & J MCQUEEN-MASON, S. 2003. Cell wall arabinan is essential for guard cell function. *Proceedings of the National Academy of Sciences of the United States of America,* 100**,** 11783-8.
JUNIPER, B.E, Jeffree, C.E. 1983. *Plant surfaces*. London, UK: Edward Arnold.
KATHAGE, J. C., P.; ALONSO-PRADOS, J.L; GÓMEZ-BARBERO, M.; RODRÍGUEZ-CEREZO, E.; 2018. The impact of restrictions on neonicotinoid and fipronil insecticides on pest management in maize, oilseed rape and sunflower in eight European Union regions. *Pest Management Science*, 74**,** 88-99.
KERSTIENS, G. 1996. *Plant cuticle: an integrated functional approach.*, BIOS Scientific Publishers Ltd, Oxford.
KERSTIENS, G. 1996. Cuticular water permeability and its physiological significance. *Journal of Experimental Botany* 47; 1813 - 1832.
KESSEL, G. J. T., DE HAAS, B. H., VAN DER WERF, W. & KÖHL, J. 2002. Competitive substrate colonisation by *Botrytis cinerea* and *Ulocladium atrum* in relation to biological control of *B. cinerea* in cyclamen. *Mycological Research,* 106**,** 716-728.
KNOCHE, M. & BUKOVAC, M. 1992. Surfactants influence foliar absorption of gibberellic acid by sour cherry leaves. *Journal of the American Society for Horticultural Science.* 117.
KOCH, K., BHUSHAN, B. & BARTHLOTT, W. 2008. Diversity of structure, morphology and wetting of plant surfaces. *Soft Matter,* 4**,** 1943-1963.

KÖHL, J., BÉLANGER, R. R. & FOKKEMA, N. J. 1997. Interaction of four antagonistic fungi with *Botrytis aclada* in dead onion leaves: A comparative microscopic and ultrastructural study. *Phytopathology,* 87**,** 634-642.
KOLATTUKUDY, P. 1980. Bio-polyester membranes of plants - cutin and subrerin. *Science*, 208, 990 - 1000
KRAEMER, T., HUNSCHE, M. & NOGA, G. 2009. Surfactant-induced deposit structures in relation to the biological efficacy of glyphosate on easy- and difficult-to-wet weed species. *Pest Management Science,* 65**,** 844-50.
LABANDEIRA, C. 2013. A paleobiologic perspective on plant–insect interactions. *Current Opinion in Plant Biology,* 16.
LACEY, L. A. G., R. 2012. Entomopathogenic nematodes for control of insect pests above and below ground with comments on commercial production. *Journal of nematology,* 44**,** 218-225.
LACEY, L. S.-I., D. 2003. The potential role for microbial control of orchard insect pests in sustainable agriculture. *Journal of Food, Agriculture and Environment.,* 1.
LIANG, G. L., T. X. 2002. Repellency of a kaolin particle Film, Act E, and a mineral oil, sunspray oil, to Silverleaf Whitefly (Homoptera: Aleyrodidae) on Melon in the Laboratory. *Journal of Economic Entomology,* 95**,** 317-24.
LIN, H., ZHOU, H., XU, L., ZHU, H. & HUANG, H. 2016. Effect of surfactant concentration on the spreading properties of pesticide droplets on Eucalyptus leaves. *Biosystems Engineering,* 143**,** 42-49.
LIU, H., SHAO, B., LONG, X., YAO, Y. & MENG, Q. 2016. Foliar penetration enhanced by biosurfactant rhamnolipid. *Colloids and Surfaces B: Biointerfaces,* 145**,** 548-554.
LIU, Z. 2004. Effects of surfactants on foliar uptake of herbicides - a complex scenario. *Colloids and Surfaces B: Biointerfaces,* 35**,** 149-53.
LIU, Z. Q., GASKIN, R. E. & ZABKIWICZ, J. A. 2004. Visualization of the effect of a surfactant on the uptake of xenobiotics into plant foliage by confocal laser scanning microscopy. *Weed Research,* 44**,** 237-243.
LIZAMORE, D. & WINEFIELD, C. 2014. The addition of an organosilicone surfactant to Agrobacterium suspensions enables efficient transient transformation of *in vitro* grapevine leaf tissue at ambient pressure. *Plant Cell Tissue and Organ Culture,* 120.
LYN, M. B., D.; GARCIA, A.; GRAY, R. 2010. Interaction of water with three granular biopesticide formulations. *Journal of agricultural and food chemistry,* 58**,** 1804-14.
MANSFIELD T.A., JONES R.J. 1971. Effects of abscisic acid on potassium uptake and starch content of stomatal guard cells. *Planta*. 101 (2), 147-158
MASCHHOFF, J. H., E.S.; BALDWIN L.J 2009. Effect of ammonium sulfate on the efficacy, absorption, and translocation of glufosinate. *Weed Science,* 48**,** 2-6.
MCQUILKEN, M. G., L. 2008. *Gliocladium catenulatum* as a potential biological control agent of damping-off in bedding plants. *Journal of Phytopathology,* 149**,** 171-178.
MELO, A. A., USANO-ALEMANY, J., GUEDES, J. V. C. & HUNSCHE, M. 2015. Impact of tank-mix adjuvants on deposit formation, cuticular penetration and rain-induced removal of chlorantraniliprole. *Crop Protection,* 78**,** 253-262.
MERCER, G. N. 2007. A simple diffusion model of the effect of droplet size and spread area on foliar uptake of hydrophilic compounds. *Pesticide Biochemistry and Physiology,* 88**,** 128-133.

MOJSIEWICZ-PIEŃKOWSKA, K., JAMRÓGIEWICZ, M., SZYMKOWSKA, K. & KRENCZKOWSKA, D. 2016. Direct human contact with siloxanes (silicones) - Safety or risk part 1. Characteristics of siloxanes (silicones). *Frontiers in Pharmacology,* 7**,** 132-132.

MOLE, N. Rethinking our approach to pesticides. PAN UK. *Biopesticide Summit, June 2019, 2019 Swansea University.*

MORITA, T., FUKUOKA, T., IMURA, T. & KITAMOTO, D. 2015. Mannosylerythritol lipids: Production and applications. *Journal of Oleo Science,* 64**,** 133-141.

MORITA, T., ISHIBASHI, Y., FUKUOKA, T., IMURA, T., SAKAI, H., ABE, M. & KITAMOTO, D. 2009. Production of glycolipid biosurfactants, mannosylerythritol lipids, using sucrose by fungal and yeast strains, and their interfacial properties. *Bioscience, Biotechnology, and Biochemistry,* 73**,** 2352-2355.

MULLIN, C. A., FINE, J. D., REYNOLDS, R. D. & FRAZIER, M. T. 2016. Toxicological risks of agrochemical spray adjuvants: Organosilicone surfactants may not be safe. *Frontiers in Public Health,* 4**,** 92-92.

MULROONEY, J. E., ELMORE, C.D. 2000. Rainfastening of bifenthrin to cotton leaves with adjuvants. *Journal of Environmental Quality,* 29.

MUSIU, E. M., QI, L. & WU, Y. 2019. Spray deposition and distribution on the targets and losses to the ground as affected by application volume rate, airflow rate and target position. *Crop Protection,* 116**,** 170-180.

NAUEN, R. R., U.; THOMZIK, J.; THIELERT, W. 2007. Biological profile of spirotetramat (Act C)—a new two way systemic (amimobile) insecticide against sucking pests. *Bayer Crop Science Journal,* 61.

OERKE, E. C. 2006. Crop losses to pests. *The Journal of Agricultural Science,* 144**,** 31-43.

OMOKAWA, H., NAKAYAMA, H. & KONNAI, M. 1989. Effects of surfactants on propanil penetration through adaxial epidermis of *Commelina communis*. *Journal of Pesticide Science,* 14**,** 301-306.

ORBOVIC, V., ACHOR, D. & P. SYVERTSEN, J. 2007. Adjuvants affect penetration of copper through isolated cuticles of citrus leaves and fruit. *HortScience,* 42.

P.C.H. MILLER, M. C. BUTLER-ELLIS. 2000. Effects of formulation on spray nozzle performance for ground-based boom sprayers. *Crop Protection,* 19**,** 609-615.

PARK, N. I., YONG I.Y, KWON O.S, PARK J.E, KIM S.E, CHUN, JC 2004. Tolerant mode of Dayflower (*Commelina communis* L.) to glyphosate. *Korean Journal for Weed Science,* 24**,** 230-237.

PARKER, M. L., NG, A., SMITH, A. C. & WALDRON, K. W. 2000. Esterified phenolics of the cell walls of chufa (*Cyperus esculentus* L.) tubers and their role in texture. *Journal of Agriculture and Food Chemistry,* 48**,** 6284-91.

PENG, L. TRUMBLE., J.; MUNYANEZA, J.; LIU, T. X. 2011. Repellency of a kaolin particle film to potato psyllid, *Bactericera cockerelli (*Hemiptera: Psyllidae), on tomato under laboratory and field conditions. *Pest Management Science,* 67**,** 815-24.

PERCY K.E., McQUATTIE C.J., REBBECK J.A. 1994. Effects of air pollutants on epicuticular wax chemical composition. In: *Air pollutants and the leaf cuticle*. Percy K.E., Cape J.N., Jagels R., Simpson C.J. (eds) NATO ASI Series (Series G: Ecological Sciences), 36. Springer, Berlin, Heidelberg.

PERKINS, M. C., BELL, G., BRIGGS, D., DAVIES, M. C., FRIEDMAN, A., HART, C. A., ROBERTS, C. J. & RUTTEN, F. J. 2008. The application of ToF-SIMS to the analysis of herbicide formulation penetration into and through leaf cuticles. *Colloids and Surfaces B: Biointerfaces,* 67**,** 1-13.

PETRACEK, P., FADER, G., KNOCHE, M., BUKOVAC, M. 1998. Surfactant-enhanced penetration of benzyladenine through isolated tomato fruit cuticular membranes. *Journal of Agricultural and Food Chemistry,* 46**,** 2346-2352.

POPP, J., PETŐ, K. & NAGY, J. 2013. Pesticide productivity and food security. A review. *Agronomy for Sustainable Development,* 33**,** 243-255.

PRECIPITO, L. M. B., DARIO, G., OLIVEIRA, J. V. & OLIVEIRA, R. B. 2018. Evaporation and wettability of fungicide spray, with or without adjuvant, on leaves of vegetables. *Horticultura Brasileira,* 36**,** 320-324.

PRYCHID, C.J., RUDALL, P.J. 1999. Calcium oxalate crystals in monocotyledons: A review of their structure and systematics. *Annals of Botany*, 84, 725 - 739.

RAMSEY, R. J. L., STEPHENSON, G. R. & HALL, J. C. 2005. A review of the effects of humidity, humectants, and surfactant composition on the absorption and efficacy of highly water-soluble herbicides. *Pesticide Biochemistry and Physiology,* 82**,** 162-175.

SALAZAR-LÓPEZ, N., ALDANA, L., SILVEIRA-GRAMONT, M.-I. & AGUIAR, J.-L. 2016. Spirotetramat — An alternative for the control of parasitic sucking insects and its fate in the environment.

SCHÖNHERR, J. & BUKOVAC, M. J. 1972. Penetration of stomata by liquids. Dependence on surface tension, wettability, and stomatal morphology*, Journal of Plant Physiology.* 49**,** 813-819.

SCHÖNHERR, J. & RIEDERER, M. 1988. Desorption of chemicals from plant cuticles: Evidence for asymmetry. *Archives of Environmental Contamination and Toxicology,* 17**,** 13-19.

SCHÖNHERR, J. & RIEDERER, M. 1989. Foliar penetration and accumulation of organic chemicals in plant cuticles. *In:* WARE, G. W. (ed.) *Reviews of Environmental Contamination and Toxicology.* New York, NY: Springer New York.

SCHREIBER, L., SKRABS, M., HARTMANN, K., DIAMANTOPOULOS, P., SIMANOVA, E. & SANTRUCEK, J. 2001. Effect of humidity on cuticular water permeability of isolated cuticular membranes and leaf disks. *Planta,* 214**,** 274-282.

SCHROEDER, W. J. & SIEBURTH, P. J. 1997. Impact of surfactants on control of the root weevil *Diaprepes abbreviatus* larvae with *Steinernema riobravis. Journal of nematology,* 29**,** 216-219.

SCHUSTER, A.-C., BURGHARDT, M. & RIEDERER, M. 2017. The ecophysiology of leaf cuticular transpiration: are cuticular water permeabilities adapted to ecological conditions? *Journal of Experimental Botany,* 68**,** 5271-5279.

SHAPIRO-ILAN, D., GOUGE, D. H., PIGGOTT, S. J. & FIFE, J. P. 2006a. Application technology and environmental considerations for use of entomopathogenic nematodes in biological control. *Biological Control,* 38**,** 124-133.

SHAPIRO-ILAN, D. C., T.E.; BROWN, I.; GARDNER, W.A.; HUBBARD, R.K.; WOOD, B.W. 2006. Effect of soil moisture and a surfactant on entomopathogenic nematode suppression of the pecan weevil, *Curculio caryae*. *Journal of Nematology,* 38**,** 474-482.

SHISHKOFF, N. M., M. 2002. AQ10 Biofungicide combined with chemical fungicides or AddQ spray adjuvant for control of cucurbit powdery mildew in detached leaf culture. *Plant Disease*86**,** 915-918.
SHTEIN, I., SHELEF, Y., MAROM, Z., ZELINGER, E., SCHWARTZ, A., POPPER, Z. A., BAR-ON, B. & HARPAZ-SAAD, S. 2017. Stomatal cell wall composition: distinctive structural patterns associated with different phylogenetic groups. *Annals of Botany,* 119**,** 1021-1033.
SIMAY, E. L. 1988. Occurence of hyperparasitism of *Botrytis cinerea* Pers. by Gliocladium. *Acta Phytopathologica et Entomologica Hungarica,* 23**,** 133-135.
SPARKS, T. C. & NAUEN, R. 2015. IRAC: Mode of action classification and insecticide resistance management. *Pesticide Biochemistry and Physiology,* 121**,** 122-128.
SQUIRE, G.R., MANSFIELD T.A. 1974. The action of fusicoccin on stomatal guard cells and subsidiary cells, *New Phytologist*, 73, 433 - 440.
STAROV, V. & SEFIANE, K. 2009. On evaporation rate and interfacial temperature of volatile sessile drops. *Colloids and Surfaces A: Physicochemical and Engineering Aspects,* 333**,** 170-174.
STEURBAUT, W. 1993. Adjuvants for use with foliar fungicides. *Pesticide Science,* 38**,** 85-91.
TAYLOR, P. 2011. The wetting of leaf surfaces. *Current Opinion in Colloid & Interface Science,* 16**,** 326-334.
TOPPARI, J., CHR. LARSEN, J., CHRISTIANSEN, P., GIWERCMAN, A., GRANDJEAN, P., GUILLETTE, L., JEGOU, B., K. JENSEN, T., JOUANNET, P., KEIDING, N., LEFFERS, H., MCLACHLAN, J., MEYER, O., MÜLLER, J., RAJPERT-DE MEYTS, E., SCHEIKE, T., SHARPE, R., SUMPTER, J. & E. SKAKKEBAEK, N. 1996. Male reproductive health and environmental xenoestrogens. *Environmental Health Perspectives,* 104 Supplement 4**,** 741-803.
TRUSKETT, V. 2003. Influence of surfactants on an evaporating drop: Fluorescence images and particle deposition patterns. *Langmuir,* 19, 8271 - 8279.
UN. 2015. *World population projected to reach 9.7 billion by 2050.* [Online]. Available: http://www.un.org/en/development/desa/news/population/2015-report.html [Accessed 06/07/2019].
VALCORE, D. L. 2007. Spray optimization through application and liquid physical property variables–II. *The Environmentalist,* 28**,** 31-33.
VAN BRUGGEN, A., GAMLIEL, A. & FINCKH, M. 2015. Plant disease management in organic farming systems. *Pest Management Science,* 72.
VERMA, N., MACDONALD, L. & PUNJA, Z. K. 2006. Inoculum prevalence, host infection and biological control of *Colletotrichum acutatum*: causal agent of blueberry anthracnose in British Columbia. 55**,** 442-450.
VERNAY, C., RAMOS, L., DOUZALS, J.-P., GOYAL, R., CASTAING, J.-C. & LIGOURE, C. 2016. Drop impact experiment as a model experiment to investigate the role of oil-in-water emulsions in controlling the drop size distribution of an agricultural spray. *Atomization and Sprays,* 26**,** 827-851.
VOGG, G., FISCHER, S., LEIDE, J., EMMANUEL, E., JETTER, R., A LEVY, A. & RIEDERER, M. 2004. Tomato fruit cuticular waxes and their effects on transpiration barrier properties: Functional characterization of a mutant deficient in a very-long-chain fatty acid-ketoacyl-CoA synthase. *Journal of Experimental Botany,* 55**,** 1401-10.

WALSH, D. B., BOLDA, M. P., GOODHUE, R. E., DREVES, A. J., LEE, J., BRUCK, D. J., WALTON, V. M., O'NEAL, S. D. & ZALOM, F. G. 2011. *Drosophila suzukii (*Diptera: Drosophilidae): Invasive pest of ripening soft fruit expanding its geographic range and damage potential. *Journal of Integrated Pest Management,* 2**,** G1-G7.

WANG, F., HU, Z., ABARCA, C., FEFER, M., LIU, J., BROOK, M. A. & PELTON, R. 2018. Factors influencing agricultural spray deposit structures on hydrophobic surfaces. *Colloids and Surfaces A: Physicochemical and Engineering Aspects,* 553**,** 288-294.

WEBSTER, G. R., BISSET, N. B., CAHILL, D. M., JONES, P., KILLICK, A., HAWLEY, A. & BOYD, B. J. 2016. Kinetic resolution of the interactions between agrochemical products and adjuvant systems upon mixing. *Journal of Agriculture and Food Chemistry,* 64**,** 6139-47.

WEYERS, J.D.B., TRAVIS, A.J. 1981. Selection and preperation of leaf epidermis for experiments on stomatal physiology. *Journal of Experimental Botany*, 32, 129, 837 - 850.

WIER, M. and CALVERLEY, C. (2002) Market potential for organic foods in Europe. *British Food Journal*, 104, 1, 45-62.

WIKLUND, K. D., J. 1994. Cancer risks among female farmers in Sweden. *Cancer causes and control*, 5**,** 449-457.

WILLER, H. 2019. *A booming organic sector: more farmers, more land and a growing market. 57.8 million hectares of organic agricultural land – the organic market grows to almost 90 billion US Dollars* [Online]. FiBL. Available: https://www.fibl.org/en/service-en/news-archive/news/article/ein-boomender-biosektor-578-millionen-hektar-bioflaeche-biomarkt-waechst-auf-fast-90-milliarden-us.html [Accessed 01/07/2019].

WILLMER, C.M, MANSFIELD, T.A. A critical examination of the use of detached epidermis in studies of stomatal physiology, 1969, *New Phytologist*, 68, 363 - 375.

WOOD, S., KOZII, I., KOZIY, R., EPP, T. & SIMKO, E. 2018. Comparative chronic toxicity of three neonicotinoids on New Zealand packaged honey bees. *PLOS One,* 13**,** e0190517.

XU, L., ZHU, H., OZKAN, E., E. BAGLEY, W., DERKSEN, R. & R. KRAUSE, C. 2010a. Adjuvant effects on evaporation time and wetted area of droplets on waxy leaves. *Transactions of the ASABE,* 53.

XU, L., ZHU, H., OZKAN, H. E. & THISTLE, H. W. 2010b. Evaporation rate and development of wetted area of water droplets with and without surfactant at different locations on waxy leaf surfaces. *Biosystems Engineering,* 106**,** 58-67.

XU, W., LEELADHAR, R., KANG, Y. T. & CHOI, C. H. 2013. Evaporation kinetics of sessile water droplets on micropillared superhydrophobic surfaces. *Langmuir,* 29**,** 6032-41.

XU, X. & LUO, J. 2007. Marangoni flow in an evaporating water droplet. *Applied Physics Letters,* 91, 12.

YEATS, T. H. ROSE., J.K.C. 2013. The formation and function of plant cuticles. *Plant Physiology,* 163**,** 5-20.

YU, Y., ZHU, H., FRANTZ, J. M., REDING, M. E., CHAN, K. C. & OZKAN, H. E. 2009. Evaporation and coverage area of pesticide droplets on hairy and waxy leaves. *Biosystems Engineering,* 104**,** 324-334.

ZABKIEWICZ, J. 2002. Adjuvants and herbicidal efficacy—Present status and future prospects. *Weed Research,* 40**,** 139-149.

ZEISLER-DIEHL, V. M., Y.; SCHREIBER, L. 2018. Epicuticular wax on leaf cuticles does not establish the transpiration barrier, which is essentially formed by intracuticular wax. *Journal of Plant Physiology,* 227**,** 66-74.
ZHOU, Z. L., CAO, C., CAO, L. D., ZHENG, L., XU, J., LI, F. M. & HUANG, Q. L. 2017. Evaporation kinetics of surfactant solution droplets on rice (*Oryza sativa*) leaves. *PLOS One,* 12**,** e0176870.
ZIDACK, N. K., BACKMAN, P. A. & SHAW, J. J. 1992. Promotion of bacterial infection of leaves by an organosilicone surfactant: Implications for biological weed control. *Biological Control,* 2**,** 111-117.
ZOLLINGER, R. 2012. Spray adjuvants the rest of the story. *Proceedings of the Integrated Crop Management Conference,* 24th Annual Integrated Crop Management Conference. November, 2012.