

USING NANOTECHNOLOGY TO ENHANCE PLANT GROWTH

BY

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Abstract

Efficient and effective delivery of fertilisers, herbicides, pesticides and growth regulating compounds to plants is the subject of much ongoing research. The objective of this research was to develop nano-formulations for delivery of compounds to plants. Two formulations were developed: the first was solution-based focused on encapsulation of the active ingredient in a nanoemulsion. Nanoemulsions should be ideal for facilitating transfer of compounds to plant leaves as their size correlates well with the nanoscale surface features of leaves, achieving significantly greater total contact area between the oil droplets and the leaves. The second nano-formulation was solid-state based, focused on locating the active ingredient within the tubules of a nanotube clay.

For proof-of-concept two synthetic plant hormones, N-phenyl-N'-(2-chloro-4-pyridyl)urea (CPPU) or forchlorfenuron, a synthetic cytokinin, and 2,4-dichlorophenoxyacetic acid (2,4-D), a synthetic auxin, were chosen for encapsulation. CPPU is a phenylurea derivative that shows strikingly strong cytokinin-like activity in plants, including delaying senescence. It is highly water insoluble, but soluble in organic solvents. It is widely used in a variety of crops, particularly kiwifruit and table grapes. Delivery of CPPU safely, efficiently and at the appropriate dosage is a priority as overdosing or incorrect timing of application causes detrimental effects on fruit firmness and other quality attributes. Auxins are also a group of plant hormone. 2,4-D is a synthetic auxin which has been widely used at high concentrations as a herbicide, at medium concentrations for fruit thinning, and at low concentrations promotes root initiation, but at even lower concentrations promote root elongation. Consequently, careful control of dosage is required to obtain the desired effect.

The nanoemulsion system developed was water/polysorbate 80/glycerol/soybean oil. The active ingredient, CPPU, was incorporated into the nanoemulsion via the oil phase in a pre-concentrate which was then crash diluted to yield the final nanoemulsion. Nanoemulsions are created only when the concentrate is located in the bicontinuous or oil-in-water microemulsion regions of the phase diagram. The droplet size of the nanoemulsions was measured using dynamic light scattering with droplets ranging in size from 30 – 100 nm. The CPPU-loaded nanoemulsions were stable for more than three days.

To determine if the nanoemulsion was an effective delivery system, a leaf senescence bioassay was conducted to test the senescence-delaying effect of the CPPU-loaded nanoemulsions when applied to explants. The nanoemulsions were applied directly to the leaves of dwarf bean explants. Chlorophyll was extracted from the leaves and measured spectrophotometrically before and several days following treatment. The CPPU-loaded nanoemulsions enhanced the effectiveness of CPPU in delaying leaf senescence compared with the control experiments, including direct application of CPPU. A >10-fold reduction in CPPU concentration was achieved.

The second delivery method was a solid-state preparation, using halloysite clay nanotubes loaded with 2,4-D. A rooting bioassay using mung bean explants was used for proof of concept. Application of 2,4-D nanotubes to the cut end of a young stem, without roots, stimulated root formation compared to controls after 10 days and at a lower applied concentration. The retardation of root elongation, relative to controls after 13 days, potentially indicated continued slow release of the active ingredient from the nanotubes.

Results obtained from this research indicate that nano-formulations have the potential to deliver biologically active compounds to plants in the horticultural and agricultural sectors at effective concentrations lower than in current usage.

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List of Abbreviations

NAA	1-naphthaleneacetic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
BA	6-benzyladenine
ABA	Abscisic acid
ANOVA	Analysis of variance
Chl a	Chlorophyll a
Chl b	Chlorophyll b
cZ	cis-zeatin
EM	Coarse emulsion
cryo-TEM	Cryogenic transmission electron microscopy
DAPF	Days after petal fall
DNA	Deoxyribonucleic acid
DMF	Dimethylformamide
DLS	Dynamic light scattering
EPA	Environmental Protection Agency
Ph Eur	European Pharmacopoeia
FAO	Food and Agriculture Organisation of the United Nations
GA3	Gibberellic acid
HPLC	High-performance liquid chromatography
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
iP	isopentenyladenine
LbL	Layer by layer
LSD	Least significant difference
LC	Liquid crystal

MRLs	Maximum residue limits
ME	Microemulsion
NE	Nanoemulsion
CPPU	N-(2-chloro-4-pyridyl)-N'-phenylurea
DPU	N, N'-diphenylurea
PPU	N-phenyl-N'-(4-pyridyl) urea
N	Nitrogen
OECD-FAO	Organisation for Economic Co-operation and Development- Food and Agriculture Organisation of the United Nations
O/W	Oil-in-water
O/W/O	Oil-in-water-in-oil emulsions
PIT	Phase inversion temperature
P	Phosphorus
PGRs	Plant growth regulators
K	Potassium
RP-HPLC	Reverse phase-high performance liquid chromatography
SPE	Solid-phase extraction
tZ	trans-zeatin
TD	Thidiazuron
Φ	Volume fraction
W/O	Water-in-oil emulsions
W/O/W	Water-in-oil-in-water emulsions

CHAPTER 1

INTRODUCTION

1.1 RELEVANCE OF RESEARCH STUDIES

1.1.1 Importance of High Agricultural Productivity for Future Generations

Agriculture is the world's largest industry. More area on earth is used for agricultural activities than is covered by forest and woodlands[1]. Agriculture can be defined as the study and practice of cultivating land for the growing of crops and the rearing of farm animals[2]. Due to the increased demand for food production over the past five decades, world agriculture has shown remarkable improvement with countless new inventions in agricultural technology and practices[3] starting with the combined use of fertilisers, pesticides, herbicides and irrigation, followed by precision farming techniques, development of genetically modified crops and the latest is the application of nanotechnology in the agri-food sector[4].

1.1.1.1 An increasing world population requires increased food supply

Living in the 21st century at a time when the world's population is growing rapidly has given us the massive challenge of meeting, at a minimum, the basic needs of the world's inhabitants and ensuring sustainable development in all countries. In 2011 the world's population surpassed the 7 billion mark. It is now

estimated at 7.2 billion with an additional 82 million persons every year[5]. This number could reach between 8.3 billion and 10.9 billion by 2050[6].

An increase in food production means an increase in agriculture production. The large increase of the world population will unquestionably cause a very high demand for food towards 2050. The Food and Agriculture Organisation of the United Nations (FAO) report stated that agricultural production must increase by 70%[7] by 2050 in order to deliver enough food supply to this larger population and maintain the same dietary standard we have today.

In order to feed the growing world population, intensifying crop production seems the only remaining option[8]. Agricultural intensification has caused the world to change the ways in which it produces crops, from traditional farming systems largely based on the management of natural resources and ecosystem services, to those where the application of biochemistry and engineering are used to enhance production. From the high demand for food, as a result of increased global population, emerged the “Green Revolution” in agriculture which started in the 1960s. It was the result of increased agricultural production as governments in both developed and developing countries invested heavily in agricultural research[8]. Research into new techniques, such as the application of nanotechnology to agricultural systems, is required to increase agricultural productivity in the 21st century.

1.1.1.2 World agriculture and New Zealand agriculture

Agriculture is the world's oldest industry and one of the largest and most important industries in the world [9]. Looking back through history, agricultural activity was believed to have started some time around 11,000 BC when humans discovered that plants could be grown from seeds[9]. The agriculture sector can contribute to significant growth in economic development for some countries in the world, especially those of developing countries. According to the FAO statistical report for 2013, almost half of the world's population live in

rural areas and 2.5 billion of these rural people depend on agriculture as their source of income to sustain life with the largest portion being in Asia [10].

Nowadays, the world has shifted its attention to China, the fastest growing major economy in the world. In the latest OECD-FAO agriculture outlook, China has been their major focus as the world has witnessed rapid progress in China's agriculture industry over the past three to four decades, and China is expected to continue to influence the international agricultural markets [11].

New Zealand is well known for its primary industries that shape the economy, people and environment of the country. Other than being one of the main dairy and sheep meat exporters around the world[12], New Zealand has entered the world market competitively in another agricultural sector, horticulture. Half of the primary sectors stated by the government come from the horticultural industry, which shows how critically important horticultural crops are to New Zealand's future economy. The industry is valued at more than NZD5 billion, with exports at around 60% of total production to about 110 countries around the world[13]. Major exports are kiwifruit, apples, pears and wine.

This industry has a high reputation in the adoption of new technologies as tools to improve product quality and to give a good response to market demand. The research described in this thesis, is an attempt to introduce nanotechnology, using a synthetic plant hormone as the active ingredient, to improve plant growth. This is vital to increase productivity (both quality and quantity) of horticultural crops, and potentially could be applied to improve productivity of both agronomic crops and forestry. The investigation reported here is to provide proof-of-concept for the possibilities and benefits of the application of nanotechnology to the argi-economy.

1.2 PLANT HORMONES

Plant growth and development are the processes where a plant undergoes differentiation, builds shape and forms a diversity of cells, tissues and organs. These processes are achieved as a result of precise and effective communication between cells, tissues and organs within the plant. Normal development of plants is influenced by a number of internal and external factors. External factors include light, temperature, daylength and gravity, while internal factors are mostly chemical. Chemicals that control the regulation and coordination of plant growth are called hormones.

1.2.1 Plant Hormones

Plant hormones are a group of naturally occurring, organic substances which influence physiological processes at a very low concentration[14]. They are chemical messengers that influence the capability of the plants to respond to their environment. Plant hormones are significant in regulating growth and development of plants. They play an important role in controlling the rate of growth and differentiation during the process whereby plant cells and tissues become individual organ systems and develop into the mature plant[14][15].

The term 'Plant hormone' originated from the concept of hormones in mammals. Hormones in mammals are synthesised at one specific site and transported to the target site in the body[16]. The hormone stimulates specific physiological events at the target site[16]. Plant hormones, however, do not comply in this regard. The same plant hormone may be synthesized at different locations within a plant, in a wide range of tissues, or cells within tissues. They may be transported to other parts to interact with specific target tissues that cause physiological responses, but they may also act in the same tissues or cells where they are produced[16]. The same plant hormone may have multiple functions that could act at different locations and may even have an inhibitory effect for certain events in plants.

1.2.2 Natural Plant Hormones

Auxins, abscisic acid, cytokinins, ethylene, and gibberellins are the five classical classes of naturally occurring plant hormones[17][18]. Representative structures are shown in Figure 1.1. 75 years ago when Went and Thimann (1937) published the book *Phytohormones*, auxins, as the first-identified plant hormones[14], became synonymous with the term 'phytohormones'. Soon after, the other four classes joined the group of 'phytohormones'. This term, however, is infrequently used today. Over the last few years there has been active progress in the area of plant hormones with additional natural compounds being considered to be plant hormones. Examples of these include brassinosteroids, polyamines, jasmonates, oligosaccharins, sterols, phosphoinositosides, salicylic acid, nitric oxide, systemins[17] and the strigolactones. Most recently, brassinosteroids, jasmonates and strigolactones have been identified as authentic plant hormones and now rank with the five classical classes of major plant hormones[19].

Each class of plant hormone comprises compounds that are similar in both structure and their demonstrated effects in plant bioassays[16]. While mass spectrometry can determine the identity of a compound, a biological assay (bioassay) is the only means of determining the biological activity of a particular compound.

Indole-3-acetic acid (IAA; Figure 1.1) is the main auxin in most plants and induces cell expansion in tissue culture[15][16]. Together with cytokinin, auxin promotes undifferentiated growth, shoot formation or root formation in tissue culture depending on relative levels. Cytokinins and auxins will be discussed in more detail below as these two classes are the main focus in this research study.

The third class of plant hormones, gibberellins, is responsible for stem elongation by stimulating both cell division and cell elongation in xylem and phloem. They are synthesised in the young tissues of the shoots and developing seed. Over 135 compounds have been isolated in this class of plant

hormones. The most biologically active gibberellins include GA₁ (Figure 1.1) and GA₄[16].

Ethylene (Figure 1.1) is a simple gaseous hydrocarbon produced from an amino acid in many tissues in response to stress and acts as the fruit-ripening hormone. It diffuses from the site it is produced through the plant before being volatized into the air where it is also able to affect ripeness in surrounding plants. Auxin can increase ethylene production and ethylene itself can induce more ethylene production, causing over ripening of fruit and abscission¹ of leaves[15][16].

Another natural plant hormone is abscisic acid (ABA; Figure 1.1), a single compound that is synthesised from carotenoids in roots and mature leaves in response to water stress. It diffuses through vascular tissues and parenchyma. The main effects of ABA are in seed dormancy² and it is responsible for the regulation of stomatal closure. If leaves experience water stress, ABA amounts increase immediately thus triggering the stomata³ to close[15][16].

Brassinosteroids are a group of naturally occurring polyhydroxylated steroids. This group of steroid hormones play important roles in plants development including cell division and cell elongation in stems and roots, photomorphogenesis, reproductive development, leaf senescence and stress responses[20]. Brassinolide is the most widespread and active brassinosteroid in plants (Figure 1.1).

¹ The normal shedding from a plant of an organ (leaves or fruit) that is mature or aged.

² A state in which seeds are prevented from germinating even under optimal environmental conditions for germination.

³ Stomata is a plural of stoma, a tiny pore in a plant leaf surrounded by a pair of guard cells that regulate its opening and closure, and serves as the site for gas exchange.

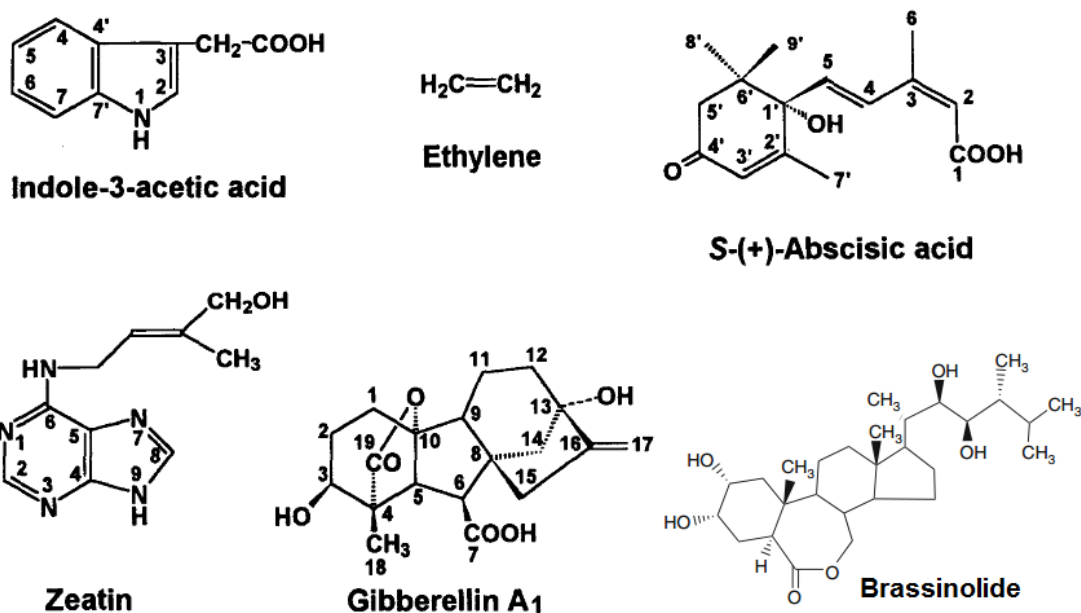


Figure 1.1: Structures of representatives of the six major plant hormones[18][21].

1.2.2.1 Cytokinins

The cytokinins, as one of the naturally occurring plant hormone groups, play an important role in the regulation of the plant cell cycle and other developmental processes in the plant. A cytokinin is defined as a compound that in the presence of auxin, causes cell division of plant cells in tissue culture. The first cytokinin identified was an adenine (aminopurine) derivative named kinetin (6-furfurylaminopurine; Figure 1.2) identified by F. Skoog, C. Miller and co-workers during the 1950s in their research for a compound that induced cell division[22][23]. Later, it was found that kinetin is an artefact and identified as a DNA degradation product of autoclaved herring sperm DNA[23][24][25]. The compound was named kinetin because of its promotion of cytokinesis (cell division) in tobacco callus tissue. A class name, cytokinins, was proposed and applied to zeatin and many other N6-substituted adenines as this group of compounds produced the same results as kinetin[26]. The first natural cytokinin was isolated from immature maize kernels and named zeatin (6-(4-hydroxy-3-methylbut-2-enylamino)purine; Figure 1.2) by Letham in 1963, almost ten years after the first cell division-inducing compound (kinetin) was obtained by Skoog.

Much more recently, in 2001, cytokinin biosynthesis in plants was confirmed by Japanese researchers[23][24][25].

Cytokinins can be found in all plant tissues. They are found in the root tips, the shoot apex and are abundant in immature seeds[23]. One of the most important effects of cytokinins in plants is stimulating cell division[15][23][24][25][26][27]. Using physiological concentrations, cytokinins have been shown to promote shoot apical meristem and shoot growth but to suppress root meristem and root growth. Cytokinins also induce cell expansion, especially in the leaves and cotyledons. Cytokinin can stimulate growth of lateral shoots but suppress lateral root initiation. The existence of cytokinins in tissues with actively dividing cells such as fruit, root tip, shoot apex and immature seeds indicates that cytokinins may naturally stimulate cell division in those areas[15][23].

Cytokinins retard senescence of leaves, both detached and on the intact plants[14][15]. Leaf senescence is a process where the leaf undergoes chemical changes as it ages[28]. During the process of leaf senescence, chlorophyll and photosynthetic proteins are degraded and the breakdown products exported from the leaf to either growing parts of the plant or into seeds or storage organs[28][29]. The yellow colour of the leaf is associated with the most rapid fall of protein content, chlorophyll loss and eventually death of the organ. Richmond and Lang (1957)[30] found that by keeping the petioles of excised leaves dipped into a solution of kinetin (Figure 1.2), both chlorophyll degradation and protein loss were retarded. Delay of senescence in detached leaves is used as a bioassay for cytokinins.

Figure 1.2 presents the structure of basic cytokinins. Naturally occurring cytokinins are adenine derivatives modified on the nitrogen atom at the 6 position of the six-member heterocycle, N⁶-position. Cytokinins can be classified as isoprenoid or aromatic, based on the N⁶-substituent[23]. The attached side chain also influences the biological activity of cytokinins as both classes are qualitatively similar in biological activities but may differ quantitatively in different processes. The most abundant class is the isoprenoid cytokinins, which exist as either isopentenyl (iP)-type cytokinins (isopentenyl

N⁶-side chain) or zeatin-type cytokinins that occur in either *cis*- or *trans*-configurations with a hydroxylated isopentenyl N⁶-side chain[23][24][27]. Other natural cytokinins are dihydrozeatin and 6-benzyladenine. Having the aromatic benzyl group at the N⁶ position, benzyladenine (6-BAP) increases stability and 6-BAP is often used in tissue culture[23].

Together with derivatives of adenine, cytokinin activity has also been detected in synthetic derivatives of phenylurea such as thidiazuron and diphenylurea[23][27]. These cytokinins are highly active but do not occur naturally. Their activity is similar to that of the most active natural cytokinins. Since cytokinins and their analogues can be produced by relatively simple chemical synthesis[24], many synthetic cytokinins with different target activity have been produced. One of them is CPPU, a synthetic compound that displays a significantly high cytokinin-like physiological activity[24]. Further discussion on CPPU follows, as it is the synthetic plant hormone that was used throughout this research study.

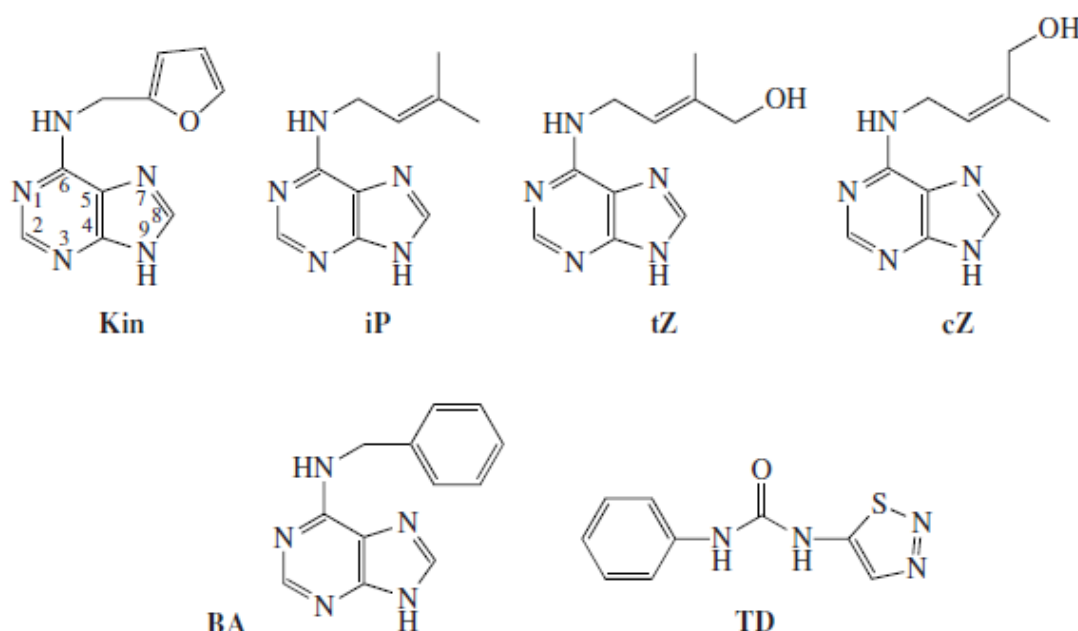


Figure 1.2: Chemical structures of typical cytokinins, some naturally occurring and synthetic cytokinins. The numbering of purine ring atoms is shown as for kinetin. (Kin–Kinetin; iP–isopentenyladenine; tZ–trans-zeatin; cZ–cis-zeatin; BA–6-benzyladenine; TD–thidiazuron)[[27].

Cytokinin Bioassay

In this study, a senescence bioassay was used to test the cytokinin activity of solutions. Cytokinin delays senescence, and the progress/delay of senescence can be measured by the amount of chlorophyll remaining following treatment of leaves with the test compounds.

Chlorophyll plays a vital part in photosynthesis and declines during senescence. Measurement of leaf chlorophyll content is a key technique in indicating the physiological status of a plant[31]. It can be extracted from leaves for quantitative analysis using a solution-based technique. Chlorophyll is green and is an example of a complex organic pigment. Chlorophyll molecules are bound to the thin rows of the membranes in the chloroplast[32]. Chlorophyll absorbs light energy and enables the synthesis of sugar. The energy contained within the molecules is called the chemical energy[32]. Therefore, the number of chlorophyll units (chlorophyll content) and the effectiveness of this system to convert light energy into chemical energy is directly related to how well the plant grows.

1.2.2.2 Auxins

Indole-3-acetic acid (IAA; Figure 1.1) is the main auxin in most plants. Auxin induces cell expansion in tissue culture. It also activates the differentiation of vascular tissue⁴ in the shoot apex⁵ and calluses⁶[15][16]. It is manufactured mainly in the shoot⁷ tips such as in leaf primordia⁸ and young leaves and travels from cell to cell through parenchyma⁹ to stimulate the cell enlargement, stem and root growth.

The role of auxin as an agent for inducing root growth has been summarized by Van der Lek (1941, cited in [33]) and Haissig and Davis (1994) [34]. It was

⁴ The vascular tissues include xylem, which conducts water and minerals from the roots upward and throughout the plant, and phloem, which transports dissolved foods in all directions within the plant.

⁵ Growing point at tip of shoot.

⁶ Undifferentiated cells that form on cut surfaces of dicotyledonous plants.

⁷ The stems and leaves of a plant.

⁸ The early, precursor cells of a leaf found on the surface of stems of higher plants.

⁹ A common ground tissue in plants, most abundant cell type and can be found in all plant parts.

reported earlier by Thimann and Went (1934) that IAA was found to be able to stimulate root formation on cuttings. This findings became a major breakthrough in the horticultural field for commercial plant propagation[35]. Later, the synthetic auxins, indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA), were synthesized chemically and found to be more effective in stimulating root formation on cuttings [36]. Rooting powders were first used by Grace (1937, cited in [33]) as a carrier for auxin. 2,4-D is another synthetic auxin that is capable of promoting root growth at a very low concentration. In this study, 2,4-D was used as the active material for the second formulation which was dry/powder nanoformulation. Halloysite nanoclay was used as the carrier for this synthetic auxin to be applied to the plant cuttings (Section 4.1.5). Root induction was the bioassay used to test for release of 2,4-D from the nanoclay.

After the discovery of IAA, Thimann (1936, cited in [33]) claimed that at the optimal concentration for root initiation, auxin blocked root elongation. The finding also applied to root primordia. This result triggered in depth studies on the requirement for auxin throughout root growth. De Klerk et al. (1999) built a model showing the successive phases in rooting of apple microcuttings where the group divided the phases into segments based on the different periods of root initiation and elongation growth. As shown in Figure 1.3, the first 24 hours is a period where the cells undergo dedifferentiation and become responsive to the rooting stimulus, auxin. 72 to 96 hours later is the start of cell division where root primordia are formed by the strong root induction action of auxin - the induction phase. However, after the induction period, auxin is no longer needed at such a high concentration and the concentration that once was an optimal concentration for root formation now is inhibitory for the outgrowth in and from the stem. They concluded that higher concentrations of auxin are needed to trigger root formation but much lower concentrations of auxin are needed at the root elongation phase. High concentrations of auxin will inhibit root elongation. This is an important point, since a supply of auxin has to be at the right concentration to firstly promote root initiation and then subsequently to promote root elongation.

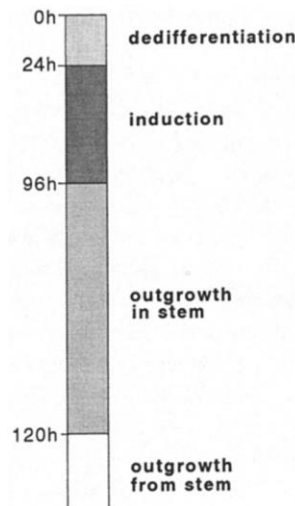


Figure 1.3: A scheme of successive phase in showing the plant hormones roles during the root growth[33].

1.2.3 Synthetic Plant Hormones

A synthetic plant hormone is a chemical compound that exhibits physiological and biochemical activities similar to those of natural plant hormones[14][17] and is classified into different classes of plant hormone according to similarities in structure and/or effects on the plant. Synthetic plant hormones are commonly referred to as plant growth regulators and sometimes as synthetic phytohormones or artificial plant hormones. The term plant growth regulator has been mainly used by the agrochemical industry to indicate synthetic plant growth regulators as opposed to the endogenous plant hormones.

According to Louis G. Nickell (1994) in his article Bioregulators for Crop Protection and Pest Control[37], the use of natural and synthetic plant hormones to modify crop plants has increased since the 1940s. They were used to change the rate and pattern of plant physiological activities such as vegetative growth, the aging process (senescence), fruit or plant maturity, reproductive development and postharvest preservation. Since then, much research has been done in this industry and people have begun to create and combine more than one type of synthetic plant hormone to control plant growth according to the market need.

1.2.3.1 A Synthetic Cytokinin – CPPU

A synthetic cytokinin is a compound that imitates natural cytokinin activity in plants, and carries with it an expectation that it may exceed the physiological activity of natural cytokinins[22]. There are two classes of chemical compounds that produce cytokinin-like activity: N⁶-substituted adenine derivatives and phenylureas. Nowadays, there are more than 200 types of natural and synthetic cytokinins that have been synthesized by researchers[38]. One of them is N-(2-chloro-4-pyridyl)-N'-phenylurea (CPPU), which has been shown to give the greatest cytokinin-like activity to plant cells[39].

CPPU, also known as forchlorfenuron, is a synthetic cytokinin-active diphenylurea which has physiological activity greater than naturally occurring adenine-substituted cytokinins such as *trans*-zeatin[40][41]. In 1978 Takahashi[39], together with a group of researchers in Japan, undertook a study on cytokinin activity by synthesising a series of derivatives which are analogues of kinetin[39][40]. Among them, N-phenyl-N'-(4-pyridyl) urea (PPU), a derivative of N, N'-diphenylurea (DPU) showed greater activity than kinetin or 6-benzyladenine. Both DPU and PPU had earlier been reported to possess growth-promoting properties, thus assigning cytokinin-like activity to these compounds. Shantz and Steward (1955) reported that DPU was able to stimulate cell division in mature carrot phloem cells but it was later shown to be a rather weakly active cytokinin, while PPU was first synthesized by Bruce and Zwar (1965) in their study on physiological action of 500 urea derivatives[42][43]. By inserting electron-withdrawing substituents into the pyridyl ring of PPU, Takahashi's group had created 35 phenylurea derivatives and tested their cytokinin-like activity using the tobacco callus bioassay[39][40]. Adding an electronegative Cl group at the α -position of the pyridyl ring of PPU resulted in a strikingly strong cytokinin-like activity. This compound stimulated a high frequency of cell division in tobacco callus culture, 10000 times higher than DPU and 10 times higher than kinetin[39][40]. CPPU is now used in the horticulture field around the world. Figure 1.4 shows the chemical structures of DPU, PPU, CPPU.

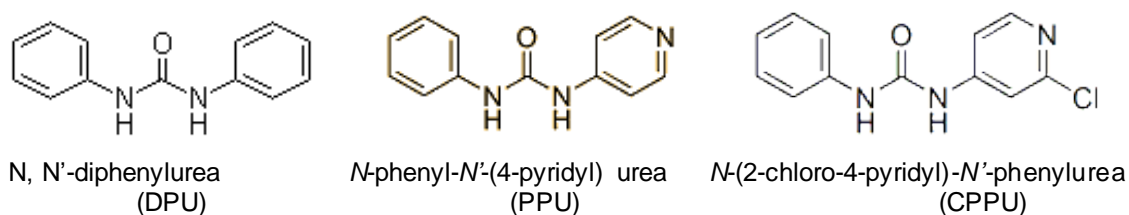


Figure 1.4: Structures of DPU, PPU and CPPU.

Uses and Importance of CPPU

CPPU has been extensively used for fruit crop production throughout the world over the past few decades. South Africa, Chile, Italy and Mexico are among countries that have had CPPU registered for use for table grapes since the mid-1990s. It has been registered in New Zealand since 1993 as a plant growth regulator for sizing kiwifruit and is being used by many kiwifruit farmers in New Zealand[44]. In April 2011, the Ministry of Agriculture and Forestry, New Zealand which incorporates the New Zealand Food Safety Authority, released a discussion document for public comment on their proposal to register forchlorfenuron to be used in apple orchards. The result is as yet unknown[45]. In 2004, the United States gained approval by the US Environmental Protection Agency (EPA) for the commercial use of CPPU as a plant growth regulator for grapes, raisins and kiwifruit[40]. Australia, also in 2005, joined the growing number of countries to register CPPU for use in increasing berry size in table grapes[46]. Recently, CPPU has been authorised as a plant growth regulator for kiwifruit by European Commission Directive[40]. CPPU has also been registered and widely used in grape, watermelon, cucumber, and kiwifruit in China[47] and is used by kiwifruit farmers in Japan to increase fruit size[48]. A considerable amount of research has been conducted by scientists in China and Japan to determine the effect of using CPPU in different types of fruits and vegetables.

CPPU stimulates high frequency cell division and cell expansion, mainly in fruits and vegetables[49][39][40][47]. It can induce callus formation, promote shoot regeneration, provoke seed germination, promote fruit expansion, and retard leaf senescence of plants[41]. The main reason that CPPU has become a primary choice in the horticulture field is its ability to effectively improve fruit set

and fruit enlargement in fruit crops such as grape, kiwifruit, gourd, watermelon, cucumber, avocado, apple and pear[40][50]. It also has been used in berry production to increase berry size and firmness, improve pack-out, reduce berry shatter¹⁰ and fruit abortion[50].

The application of CPPU for fruit development is very effective if it is applied at the correct developmental time with the right concentration. According to Kim et al. (2006)[49], when tested on a hardy kiwifruit, *Actinidia arguta* 'Mitsuko', the application of CPPU at 10 days after petal fall (DAPF) was most effective for increasing fruit growth, particularly in the longitudinal direction and the appropriate CPPU concentration was determined to be of 5-10 ppm (Figure 1.5). However, the storage quality and the cumulative effect of continuous CPPU treatment on fruit productivity needs to be further examined.

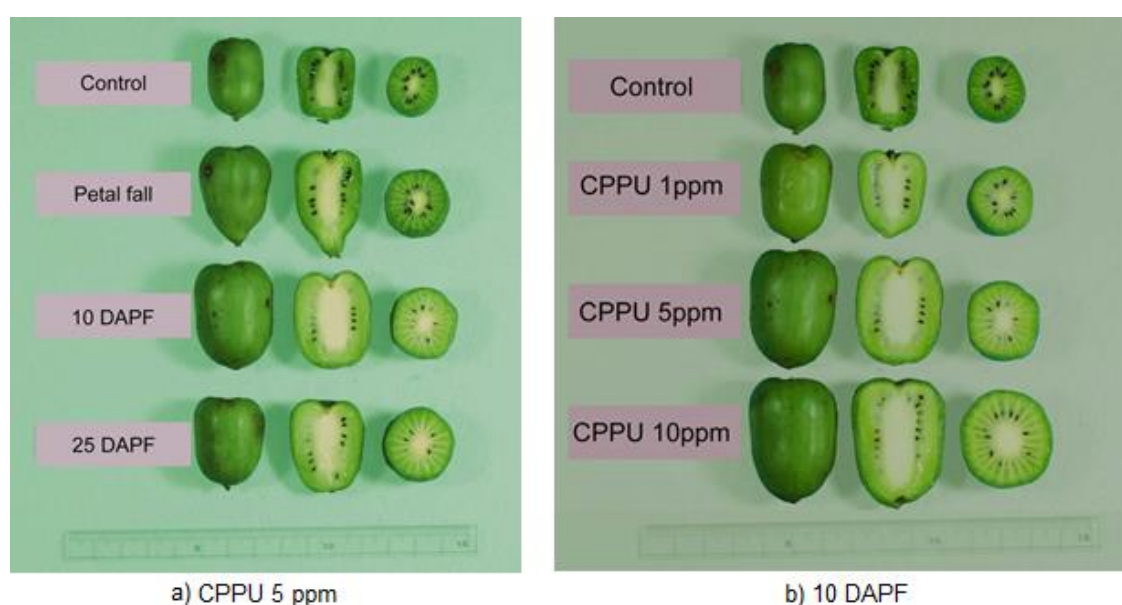


Figure 1.5: a) Fruit size in *A. arguta* 'Mitsuko' at different application times of CPPU and b) fruit size of *A. arguta* 'Mitsuko' at different CPPU concentrations (CPPU was applied 10 days after petal fall). *DAPF=days after petal fall[49].

Most of the papers published recently have shown that 5-10 ppm of CPPU is the suitable concentration to be applied to kiwifruit, grapes and berries after 10-14 DAPF[49][51][52][53][54]. Another interesting study was done by Feitosa (2002)[51], who looked at the effect of CPPU alone and the combination of

¹⁰ Loose berries that have detached from the stem

CPPU with another plant growth regulator (PGR), gibberellic acid (GA_3) on berry size increase. Using GA_3 is a conventional way of increasing fruit size as the hormone can also promote growth and cell elongation. During the fruit-set stage, when the berry size was about 8 mm in diameter, different concentrations of both PGRs were applied to the fruits. The result showed that application of 10 ppm of CPPU alone was the most effective as the fruit size and fruit set showed a significant difference compared to that in control tests and treatment with GA_3 (Figure 1.6). Treatment with both PGRs delayed the harvest for 8 days. The reason for the delay is yet to be elucidated.



Figure 1.6: A) Control test, B) 20 ppm of GA_3 and H) 10 ppm of CPPU[2].

Other than fruit size, CPPU can also advance fruit maturity as reported by Patterson et al. (1993)[48] and Iwahori et al. (1988)[55]. In terms of storage capability, Patterson et al. (1993)[48] found that the soluble solids concentration of fruit treated with CPPU was very high at harvest but after a few weeks of storage, the concentration became similar to that of untreated fruits. This means that CPPU does not affect the soluble solids concentration in ripe kiwifruit[48]. Fruit firmness was found to decrease in CPPU-treated fruit as it is softer than untreated fruit during harvest but it does not affect the rate of fruit softening during storage, but only if the concentration of CPPU used is below 20 ppm[48].

A recent study done by Rueda-Luna et al. (2015)[56], shown that watermelon fruit treated with 25 ppm CPPU had better production parameters and the highest concentration of glucose and sucrose. Xin et al. (2015)[57] used 15

ppm of CPPU with gibberellic acid and effectively improved grape fruit quality, but higher CPPU concentration (20 ppm) lowered fruit quality instead. An even lower concentration, 10 ppm used by Pramanick et al. (2015)[58] successfully increased fruit size as well as increasing total sugar content. Cruz-Castillo et al. (2014)[59] sprayed an even lower CPPU concentration than normally used, 4 ppm, on kiwifruit plants, a week before flowers were fully opened and functional. The harvested fruit was analysed and it was found that the fresh weight of the fruit was significantly increased by 12% and after three months of storage, no difference was detected between control fruit and CPPU-treated fruit in terms of dry matter content, fruit firmness and total soluble solids. This study has shown that lower dosages of CPPU could improve fruit size without affecting qualitative and nutritional characteristics of the fruit. Referring to this study by Cruz-Castillo et al. (2014), and other studies mentioned earlier, it was evident that lower CPPU concentrations could give acceptable results on targeted plants. If a proprietary formulation was developed that was active with even less CPPU, then residue levels would be further reduced and, therefore, impact less on the environment[59].

CPPU Delivery Methods

Methods or techniques for delivering additional chemicals to crops are very important in order for the chemical to be fully absorbed by the plant and for the plant to fully utilise the chemical being applied. The techniques used should not cause any negative impact or harm to the environment. The two most common methods of applying CPPU are dipping the fruit in CPPU solution or spraying the CPPU solution at the early post-bloom stage of fruit development[45][49]. These two techniques have been used in many countries around the world for more than three decades. However, the foliar spray technique, as shown in Figure 1.7, is the more favoured and it has been widely used among crop growers, mostly for delivering fertilisers and pesticides. The foliar spray technique (Figure 1.7) is the practice of applying liquid, generally an aqueous solution containing additional nutrients to plant leaves as a fine spray so that the plant can absorb the nutrients through its leaves[4]. It is unclear when this technique was first used, but after development of water soluble and liquid fertilisers, farmers began to apply fertilisers with sprayers.



Figure 1.7: Foliar spray technique[60].

Issues arising from CPPU Application

The application of additional synthetic plant growth regulators to plants has triggered concern regarding residues in fruit as well as in soil. The increase of CPPU usage in many fruits and vegetables has led to inquiries by the public with regard to the possibilities that this agrochemical may eventually reach the consumer[40] and may also cause problems during the export of the treated fruits[54]. Each country in the world has set their own limit for the amounts of CPPU residual for each different crop. Maximum Residue Limits (MRLs) allowed for CPPU are in the range between 0.01 mg/kg and 0.1 mg/kg depending on the crop and the country. For example, the maximum legal limit of CPPU for kiwifruit is 0.01 mg/kg in Australia, 0.04 mg/kg in USA, 0.05 mg/kg in EU and 0.1 mg/kg in Japan, while for apple it is 0.05 mg/kg within the European Commission, 0.1 mg/kg in Japan and is proposed to be 0.01 mg/kg in New Zealand[40][45][46][61].

Few papers have been published on the analysis of CPPU residues in agricultural products, even fewer in the soil or in water. The emphasis of these papers has been on the different methods and instruments used in order to detect the level of CPPU residue in each of these three environments, even in its lowest level. The lower the permitted residue level is, the more difficult it is to obtain a precise measure of the amount of CPPU residue, thus raising doubts

among consumers on the actual level of CPPU residues in agricultural products[47][52][62][63].

Sharma and Awasthi (2003)[62] studied the CPPU residue in grape, soil and water by applying CPPU to these three environments separately, using liquid chromatography with a UV detector as the analytical technique. By using both methods of CPPU application, dip and foliar spray, they found that CPPU residue was much higher in the grapes when the dip treatment was compared to the foliar application. The residue was below 0.01 mg/kg after 15 – 20 days of foliar treatment as compared to 65 days for the dip treatment for CPPU concentration, applied in the range of 2 – 4 ml/L for berry size from 4 mm to 6 mm. CPPU lasts more than 30 days in water and in the soil. This was shown to depend on the moisture content and soil type.

Similar results were found by Banerjee et al. (2008)[63] who also studied CPPU degradation in soils in India and its influence on soil enzyme activities. Their results suggest that the addition of CPPU only results in a small change in soil quality indicators, which does not present any serious threat to the soil's biological environment as long as CPPU is applied at the recommended dose.

In 2006, Hu and Li used solid-phase extraction (SPE) and high-performance liquid chromatography (HPLC) to study CPPU residue in watermelon[47]. The watermelons were dipped in 10 to 20 mg/L of CPPU at the female flower blooming stage and a fruit size of approximately 1 cm. After 10 days of treatment, the CPPU residue level was less than 0.01 mg/kg for all CPPU concentrations used and no CPPU residue was detected in watermelon with the harvest withholding period of 40 days.

Zhang et al. (2010)[52] used Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) to study CPPU residue in samples purchased in a market in China. This method was practicable for routine residue analysis of CPPU in a few fruit and vegetables in China. For kiwifruit and cucumber crops, CPPU residue was not detected, while for tomato the residue was 0.008 mg/kg and for grape, it was 0.034 mg/kg.

Other issues regarding the use of CPPU in crop production relate to the appropriate concentration to be used and the optimal time to apply the CPPU. Overuse and overdosing of CPPU could result in dramatic changes which lead to the disruptive effect of the product such as detrimental effects on fruit firmness[48]. In May 2011, the agricultural world was shocked by the news of the ‘exploding watermelon’ in Jiangsu Province, China[64]. It was believed that the misuse of CPPU caused the watermelon to burst. According to Liangju, the timing of application of the CPPU by the farmers was too late in the season; that it was too close to harvest time. The long hot weather followed by sudden heavy rainfall in the province may also have caused the fruit to be damaged[65]. Therefore, gaining further knowledge on the appropriate use of CPPU is vital and must be transferred to the farmers.

Based on the available data it can be concluded that the amount of CPPU residue in fruit is under the MRLs of most countries. This indicates that CPPU is safe to be used as long as it is being applied at the appropriate concentration and time before harvest. Further study needs to be done to determine the actual level of CPPU residue in more types of fruit and vegetables, also in soil and water and the effects CPPU can have on these different environments.

As mentioned previously, the main technique used to apply CPPU to plants is by foliar spraying. Even though it is described as a powerful method which can be used in plantation or personal gardening, it still has a few limitations. According to Nair et al. (2010)[4], the problems include low penetration rate of the chemical solution applied, run-off from hydrophobic surfaces, wash-off by rain, repeated application, and rapid drying of the spray solution which disables the penetration of the active ingredient. One of the factors that contributes to the effectiveness of foliar spraying is the chemical and physical characteristics of the spray solution, environmental factors (humidity, temperature and light) and leaf characteristics. Improving the formulation of the spray solution may overcome some of these problems.

Even with an optimised spray solution, there is still the possibility that the delivered chemical is being overly or incorrectly used, leading to

misunderstanding of the real effect on the plant. Therefore, in order to achieve high quality fruit and vegetables without consumer concern about the chemical residue and without introducing harm to the soil and water, a new formulation of CPPU solution that could be delivered to plants in the most efficient way is needed. In this research, an attempt has been made to produce a new formulation of spray solution consisting of nanometre droplets containing the active ingredient, CPPU, with optimum concentration. It was hypothesised that this method of CPPU delivery would allow better penetration of CPPU through the leaves because of the small droplet size thus avoiding repeated application to the plant which will cause extra cost and time to the farmers.

1.2.3.2 A synthetic Auxin – 2,4-D

2,4-D is a synthetic auxin that is widely used as a herbicide. It was first commercially used in the 1940s to control broadleaf plants while, at the same time, not affecting the grasses[66]. Because of its low production costs, 2,4-D remains one of the most commonly used herbicides in the world agricultural markets [67]. Figure 1.8 shows the structural formula of 2,4-D.

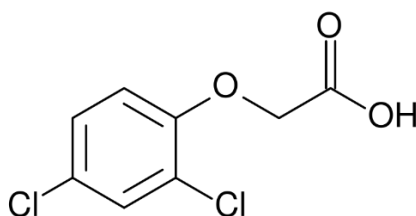


Figure 1.8: Molecular structure of 2,4-D[68].

2,4-D is favourably used in agriculture world because it displays relatively good selectivity. Selectivity means the herbicide affects only certain types of plants, while a broad spectrum herbicide affects a broad range of plants. 2,4-D controls a wide number of broadleaf weeds but it causes little or no harm to the grass when used at normal rates[67]. Many studies are recently conducted to improve the selectivity of other herbicides to a higher level. Since the main use of 2,4-D is to kill broadleaf weeds, the technique used has been foliar application; the 2,4-D solution is directly applied to plant leaves. According to Hallam

(1970)[69], among other phenoxyalkanoic acid herbicides under study, 2,4-D induced the most changes in morphology and internal structure of the chloroplast of the sample leaves, after just four hours of application. The study demonstrated that 2,4-D accumulated in and broke down the chloroplast, which then revealed the chloroplast as the initial site of herbicidal action in plant leaves, at the same time reflecting 2,4-D's phytotoxic properties.

Having the same concerns as CPPU application, the use of 2,4-D has also raised many public inquiries on the negative effects this herbicide has to the society and the environment, knowing that 2,4-D has been extensively used in agriculture fields, as well as domestic use. The adverse effects revolve around the environmental problems such as causing water pollution in ground, surface and drinking water, as well as its adsorption to soil[70]. A meta analysis by Goodman et al. (2015) [66] on studies done previously that looked to link 2,4-D with non-Hodgkin's lymphoma, gastric and prostate cancer found no associations overall between 2,4-D and any cancer end point.

Other than being used as a herbicide, this synthetic auxin is also used in laboratories for plant research, mostly to study the effect of 2,4-D on plant and root growth[69][71]. 2,4-D acts as an herbicide only if it is being employed at high physiological concentrations, but not at low concentrations. 2,4-D can be used for both promotion and inhibition of growth depending on the organs of the plant that the 2,4-D is being applied to and its concentrations (Fig 1.9). Different concentrations of 2,4-D may act either as promoting or inhibiting agents depending also on the stage of development of those different organs [72]. Figure 1.9 shows the different concentration of 2,4-D needed to promote or inhibit growth of three different plant organs: roots, buds and stems.

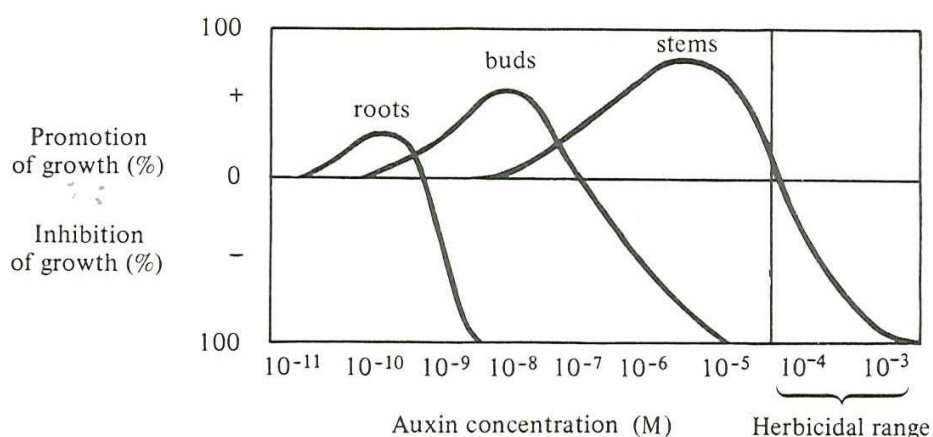


Figure 1.9: Differential sensitivity of plant organs to auxin. Reproduced from Kaufman et al. (1975)[72].

The graph in Figure 1.9 shows differences in auxin requirements between the three different organs. Suboptimal concentration for roots is the lowest among the other concentrations that affect the buds and stems. An optimal auxin concentration for roots growth is suboptimal concentration for buds growth, while supraoptimal (inhibitory) concentration for root growth is an almost optimal concentration for bud growth.

In this research, an attempt has been made to produce a solid state formulation that focused on root formation of plant cuttings. It was hypothesised that this method of 2,4-D delivery would allow a slow release of 2,4-D, thus giving the roots enough supply of this hormone for a longer period of time.

1.3 SOLUTION STATE FORMULATION – NANOEMULSIONS

Nanotechnology has rapidly spread, becoming increasingly prominent in human life with each passing year since it first emerged in the 1980s. However the central concepts defining the chemistry and physics of the nanoscale have been developed for many years prior to that. Nanotechnology can be broadly defined as the study, fabrication and application of systems by manipulating structures or objects with nanoscale dimensions between 1 nm and 100 nm[73]. This remarkable technology has been applied in almost all fields of science

including in colloid technology, with the development of nanoemulsions. Nanoemulsions are emulsions with droplet sizes in the nanometric scale, typically in the range of 20 nm to 200 nm[74][75].

1.3.1 Emulsions

An emulsion is a dispersion of liquid droplets of a certain size within a second immiscible liquid. It is therefore a two phase system of two immiscible liquids that are brought together by using a third component called an emulsifier(Figure 1.10). The presence of any two immiscible components in a system indicates the existence of an interface or boundary between the two phases[76]. An emulsion droplet interface at any point has the same interfacial tension. The most common example of an emulsion is a mixture of oil and water with one liquid being the dispersed phase and the other liquid the continuous phase of the system. Emulsions are thermodynamically unstable. They will eventually separate into two phases over time. However, they can be stable from a few seconds to several years as they are kinetically non-labile with stability conferred via repulsive entropic interactions.

Water is a polar molecule. Addition of a substance such as oil, an uncharged or non-polar molecule, to water, will cause the water molecules to associate with each other rather than interacting with the non-polar molecules. As a result, the oil molecules are expelled from the water and form a separate phase. Oil is therefore referred to as a hydrophobic substance[73]. For two such immiscible liquids to be homogenously dispersed to form a stable emulsion, another component is introduced to the system; an emulsifier or so-called surface active agent (surfactant). Surfactants are unique molecules. They contain both an “oil-loving” hydrocarbon chain and a “water-loving” hydrophilic headgroup which gives the surfactant molecule amphiphilic properties, such that it can be in either an aqueous or a hydrophobic environment[77].

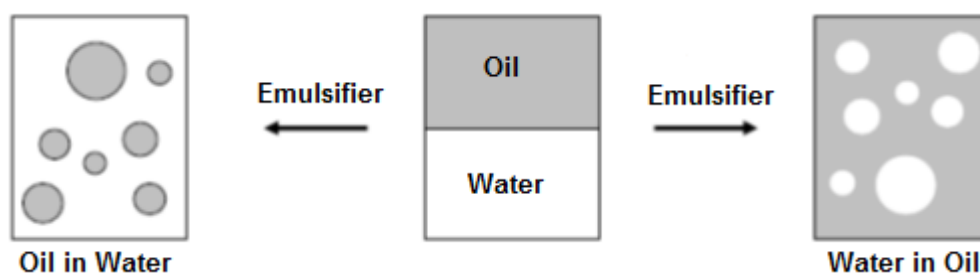


Figure 1.10: Formation of emulsion.

Emulsions are commonly used in many major chemical industries. In the food industry, emulsions are used to make mayonnaise, milk, margarine, butter and other fat-based foods. In the pharmaceutical area, they are used to improve solubility as well as delivery of hydrophobic and hydrophilic drugs, and also to increase stability and shelf-life of pharmaceutical products. In the agricultural industry, emulsions are widely used as delivery vehicles for insecticides and fungicides. Water insoluble chemicals are applied to crops using a combination of the spray method and emulsion technology which allows them to be effectively diluted and improves the efficiency and efficacy of the method[78].

1.3.1.1 *Emulsion Classifications*

Emulsions can be classified based on two criteria which are the size of the liquid droplets and the nature of the dispersed phase in the system. Different types of emulsions have different behaviour and are used in different applications. In Table 1.1 are given the classifications of emulsions based on the size of the liquid droplets.

The size classification of emulsions has been debated for decades. Different scientific papers have defined all three emulsions differently with the droplet sizes, particularly for microemulsions and nanoemulsions, overlapping. There are, however, significant measurable differences between microemulsions and nanoemulsions. Microemulsions are clear, transparent, isotropic mixtures of emulsion usually formed via addition of a co-surfactant. They are thermodynamically stable and the emulsion state is said to be indefinite[79]. In contrast, nanoemulsions appear to be transparent or translucent due to their

small droplet sizes, and phase separate over time. However, their lifetimes are enhanced compared to microemulsions with Brownian motion reducing sedimentation or creaming. Furthermore, nanoemulsions can be diluted with water without changing the droplet size distribution in common with microemulsions[79][80].

Table 1.1: Classification of emulsions

Droplet Size	Emulsions	Behaviour
Below ~20 nm	Microemulsion	Thermodynamically stable
~20 nm and above	Nanoemulsion	Thermodynamically unstable, kinetically non-labile
Above ~500 nm	Macroemulsion	Thermodynamically unstable, kinetically non-labile

Emulsions may also be classified based on the nature of the dispersed phase. There are three different emulsions: oil-in-water emulsions (O/W), water-in-oil emulsions (W/O) and multiple emulsions (of which there are two types water-in-oil-in-water emulsions (W/O/W) or oil-in-water-in-oil emulsions (O/W/O)). In O/W emulsions, oil is the dispersed phase or discontinuous phase and water is the continuous phase, while in W/O emulsion, water acts as the dispersed phase and oil is the continuous phase of the system. For W/O/W multiple emulsions, small water droplets are entrapped within larger oil droplets that in turn are dispersed in a continuous water phase[81]. Milk, cream and sauces are some examples of O/W emulsions whereas margarine and butter are examples of W/O emulsions. W/O/W emulsions were first established in 1976 by Matsumoto et al.[81]. Multiple emulsions are mainly applied in industries such as pharmaceuticals and cosmetics because of their special behaviour to prolong release of active ingredients trapped in the double emulsions[82].

To determine which liquid forms the dispersed phase and which will be the continuous one depends on the volume ratio of the liquids, the kind of emulsifying agent, and its concentration in strong connection with the temperature[83]. Several different tests can be done to identify the type of emulsion including the following: dye test, dilution test, electrical conductivity measurement, refractive index measurement and filter paper test. For example,

O/W emulsions will give a high conductivity value as water is the continuous phase and a low conductivity will be measured for W/O emulsions. Water soluble dyestuffs will spread and colour the solution if the emulsion is an O/W emulsion but not if it is a W/O emulsion. Filter paper used for this test consist of cobalt chloride (20%) paper prepared by soaking filter paper in cobalt chloride solution and then leaving to dry for a few hours. The paper is blue when dry and turns pink in water. The emulsion is confirmed to be O/W emulsion if the paper turns pink when soaked in the solution. For the dilution test, no phase separation will occur if extra water is added into milk, for example, as the dispersion medium is water but separation will immediately occur if oil is added thus confirming milk as an O/W emulsion.

1.3.1.2 Emulsion Formation and Stability

Preparation of an emulsion requires the formation of a very large amount of interfacial area between two immiscible phases resulting in an increase in the total energy of the system. For example, if 10 mL of an oil is emulsified in water yielding a droplet of diameter of 0.2 μm , the resulting O/W interfacial area will have been increased by a factor of $\sim 10^6$ [76]. The energy increase of an emulsion compared to the non-emulsified components is equal to ΔW . This energy is a measure of the thermodynamic instability of an emulsion. The energy required to generate one square centimetre of new interface of droplets is given by[76]:

$$\Delta W = \gamma \cdot \Delta A \quad \text{Equation 1}$$

where γ is the interfacial tension (N/m) between two liquid phases and ΔA is the change in interfacial area (the newly formed interface). ΔW is the energy of the interface and it corresponds to the reversible work done on the system to carry out the dispersion process. Since this amount of work remains in the system as potential energy, the system will be thermodynamically unstable and it will undergo rapid transformation to attain a minimum potential energy which leads to a decrease in the interfacial area via processes such as coalescence, creaming or sedimentation. If some material is added to the system that

reduces the value of γ , the magnitude of ΔW will also be reduced. However, the system remains in a thermodynamically unfavourable situation. The ultimate stability against coalescence processes is only achieved if γ approaches zero. Lowering the interfacial tension between phases is clearly an important factor in the formation and stabilization of emulsions. Ease of emulsion formation is determined by the amount of work needed to lower the interfacial tension. If less work is needed, the more readily the emulsion is formed[76][83][84]. Although thermodynamics is the major factor controlling the ultimate long-term stability of an emulsions, kinetics plays a dominant role in the emulsion's short-term stability and the most widely useful emulsions achieve their needed stability by kinetic movement or pathways[76].

Adsorption of surfactant on liquid/liquid interface

The presence of surfactant is to stabilize the emulsion system preventing the dispersed phase from coalescing into a macroscopic phase[85]. The adsorption of surfactant molecules at an oil-water interface will reduce the interfacial tension between the two phases and form a physical barrier[76][83].

The relationship between the adsorption of a molecule at the oil-water interface and the resulting interfacial tension is an important one and will be briefly discussed here. Gibbs derived a thermodynamic relationship between the variation of interfacial tension with concentration and the amount of surfactant adsorbed, Γ (moles per unit area), referred to as the surface excess concentration. Equation 2 and 3 are referred to as the Gibbs adsorption equation where c is the concentration of surfactant in a unit of mol dm^{-3} in dilute solutions. The equation shows that Γ can be determined from the experimental results of variation of γ with $\ln c$. The slope of γ versus $\ln c$ curve will give the surface excess of the dilute surfactant system[76][86].

$$\frac{d\gamma}{d \ln c} = -\Gamma RT \quad \text{Equation 2}$$

$$\Gamma = -\frac{1}{RT} \left(\frac{\partial \gamma}{\partial \ln c} \right) \quad \text{Equation 3}$$

The simple Gibbs equations above can give significant information on the relationship between emulsion droplet size and total surfactant concentration. The presence of surfactant can also control the type of emulsion that will form; O/W or W/O emulsion. With surfactant emulsions are easier to prepare and finer droplet sizes are produced.

1.3.2 Nanoemulsions

Nanoemulsions are emulsions with remarkably small and uniform droplet sizes in the nanometre range of 20-200 nm[74][75][87][88][89]. As for conventional emulsions, oil-in-water or water-in-oil nanoemulsions are possible. More complex variations also exist but usually result in large droplets. The size range of nanoemulsions is disputed in the literature with the lower limit being as high as 50 nm and the upper limit 500 nm[90][91][92]. Whatever the case may be, the exact size range is not the key issue since no qualitative differences are established by droplet size[87]. Since the size range of nanoemulsion droplets overlaps with that of microemulsion, there remains major misunderstanding in the literature with regard to the distinction and differences between nanoemulsions and microemulsions. Evidently, there are very clear fundamental differences between these two extremely small droplet size emulsions.

Thermodynamically stable particles are characterized by having a very low surface tension and this produces a very large surface area. As mentioned in 1.4.1.1, nanoemulsions are non-equilibrium, thermodynamically unstable materials which do not form spontaneously and that may undergo aggregation, flocculation, coalescence and eventual phase separation[87][93][80]. Some authors define nanoemulsions as metastable systems, but the distinction between a thermodynamically stable system and a metastable system is not always well defined[74][91][93].

Microemulsions, on the other hand, are equilibrium, thermodynamically stable systems that form spontaneously and have a broader structural variety than conventional emulsions. Their properties are independent of the order of addition of their components. Although microemulsions possess several advantages over the other type of emulsions, the relatively high surfactant concentration required for their formation[94] limits their extensive use in practical applications such as in the pharmaceutical industry. Therefore, attention has now been focusing on those emulsions with submicrometre droplet size that are between conventional emulsions and microemulsions that can be formulated over a wide range of constituent compositions, nanoemulsions[94].

Many nanoemulsions appear to be transparent or translucent with a bluish coloration (Figure 1.11). Their extremely small droplet size causes a large reduction in the effects of gravity acting on the droplets and as such, Brownian motion may be sufficient for overcoming gravity. Their high kinetic stability makes them resistant to sedimentation and creaming. The small droplets resist flocculation and coalescence since these droplets are non-deformable, hence surface fluctuations are energetically unfavourable resulting in a system that will remain dispersed with no phase separation over a period of time. Nanoemulsions provide extremely low interfacial tensions and large interfacial areas between the aqueous and oil phase[93][95].

Due to their remarkable characteristics, including high kinetic stability, low viscosity and optical transparency, nanoemulsions have become of interest in fundamental studies and in practical applications in many industrial fields. They are widely used in the pharmaceutical industry as a carrier for drug delivery[93], in agrochemicals for pesticide delivery[74], in cosmetics as personal care formulations[79], and in the chemical industry as polymerization reaction media[88].



Figure 1.11: Picture of a nanoemulsion (left) and a macroemulsion (right) with droplet diameters of 35 nm and 1 μm , respectively[89].

1.3.2.1 Preparation of Nanoemulsions

Nanoemulsions cannot be produced spontaneously. The most common approach in forming nanoemulsions is the high energy emulsification method, the so-called dispersion method[89][93][96]. More recently, a low energy emulsification method has been developed in which the phase behaviour and properties of the emulsifier are manipulated to produce extremely small droplets[74][89][93][94]. This new method was established based on the understanding that stability depends on the method of preparation and that the system goes through low interfacial tension states as intermediates during this low energy emulsification process[94].

High Energy Emulsification Methods

Nanoemulsion formation by the dispersion or high energy emulsification method is generally attained using high-shear stirrers, high pressure homogenizers, ultrasound generators and microfluidizers. Very high energies are needed for the conversion of large droplets into smaller ones, significantly higher than the difference in surface energy $\gamma\Delta A$ (refer 1.4.1.2). Hence the preparation of nanoemulsions by these high energy techniques in the presence of a surfactant or a surfactant mixture will produce nanometre-sized droplets[93][97].

It has been shown that an apparatus supplying the available energy in the shortest time and having the most homogenous flow produces the smallest size of droplets[89]. High pressure homogenizers fill these requirements and are the most common emulsifying instruments used to prepare nanoemulsions. This technique ensures better effectiveness and a more homogeneous droplet size distribution with the possibility of obtaining nanosized droplets even when using different types and amounts of surfactants, oils and water soluble materials[98]. As a high force dispersion device, ultrasonication is very efficient in reducing droplet size but is only applicable for preparation of small quantities whereas high pressure homogenizers or microfluidizers are favourable for the emulsification of larger quantities[97][99].

In Figure 1.12 is shown the general schematic presentation of high energy emulsification techniques widely used in industry and fundamental studies[93]. The aqueous and oily phases are first heated together and dispersed. The first emulsification process for the mixture is carried out using a high shear mixer resulting in a coarse emulsion. This raw emulsion then undergoes homogenization using a high pressure homogenizer with several passes through the homogeniser to obtain a homogenous dispersion of small droplet sizes, i.e. a nanoemulsion[93].

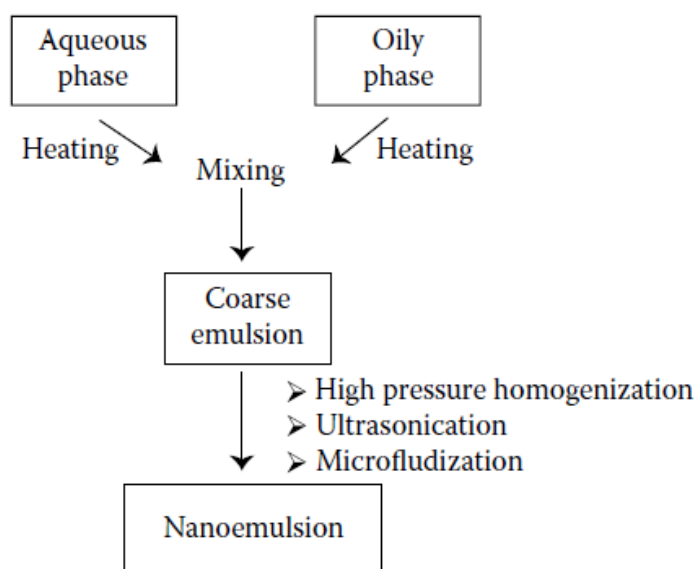


Figure 1.12: Schematic representation of high energy emulsification technique[93].

During the homogenization process, several parameters can be adjusted such as the homogenization pressure, the number of homogenization cycles and the homogenization temperature. These parameters are important for the physical stability of the final product[98][99]. The high energy inputs required in the formation of nanoemulsions are based on consideration of the Laplace pressure¹¹ (p) given by Equation 4. To break up a drop into smaller ones, the droplets must be strongly deformed and this deformation increases the Laplace pressure, p as shown when a spherical drop deforms into a prolate ellipsoid. For a spherical drop, there is only one radius of curvature R (Equation 5), whereas for a prolate ellipsoid there are two radii of curvature R_1 and R_2 , and more energy is needed to be delivered from the devices in order to provide sufficiently large deforming forces that are able to break the droplets into smaller droplets[100].

$$p = \gamma \left(\frac{1}{R_1} + \frac{1}{R_2} \right)$$

Equation 4

R_1 and R_2 are the principal radii of curvature of the drop. For a spherical drop, $R_1=R_2=R$ and,

$$p = \frac{2\gamma}{R}$$

Equation 5

Through the addition of surfactant, the interfacial tension will be reduced, hence reducing the p which consequently reduces the total energy input required. The smaller the droplets are to be formed, the more total energy and/or surfactant that is needed, which makes this preparation method unfavourable for industrial application where very small droplets are desired[80][100].

The low energy emulsification techniques that are currently being explored allow small droplet emulsions to be formed using knowledge of the underlying phase behaviour. This study will focus on these methods. They will be applied to obtain nanoemulsions for further use in agroindustry.

¹¹ Laplace pressure is the pressure difference between the inside and outside of a bubble or droplet.

Low Energy Emulsification Methods

By manipulating the physiochemical properties of a two phase system, for example the system's phase behaviour, solubility or stability against temperature, it is possible to induce the formation of nanoemulsions[101]. In the case where nanoemulsions are prepared using the low energy emulsification technique, the phase transition that occurs during the emulsification process is taken advantage of to produce small-sized droplets. The two most common low energy techniques are self-emulsification or spontaneous emulsification and the phase inversion temperature method (PIT)[74][80][93][94][102], where the system goes through a low interfacial tension state during the emulsification process[94].

Spontaneous emulsification

Spontaneous emulsification, widely used in applications of personal care and cosmetics as well as in pharmaceuticals, is a very simple method where an emulsion is produced by mixing two liquids at room temperature. One is a pure aqueous phase while the other is a mixture of oil, surfactant and a water-miscible solvent[102]. Bouchemal et al. (2004)[103] have demonstrated a spontaneous emulsification by three steps (Figure 1.13). The first step is preparation of a homogenous oily phase containing oil and lipophilic surfactant (e.g., Span 80, Span 85) in a water miscible solvent (e.g., acetone). The second step is injecting this organic phase into a homogenous aqueous phase containing water and hydrophilic surfactant (e.g., Tween 20, Tween 80) under magnetic stirring. The O/W emulsion is formed instantaneously by diffusion of the organic solvent in the external aqueous phase, leading to the formation of nanodroplets. The third step is removing the water-miscible solvents by evaporation under reduced pressure. Nanodroplets of oil now are dispersed in an aqueous solution of water and hydrophilic surfactant. This method has been used by a number of research groups[93]. According to Miller (1988)[104], when the solvent displaces itself from the oily to the aqueous phase, considerable turbulence at the water/oil interface is induced leading to the spontaneous emulsification process. If a very high solvent/oil ratio is used, solvent diffusion is very fast and as a result, the turbulence generated is increased causing nano-sized droplets to form by this spontaneous

emulsification process. The formulation can also include other components such as surfactants, monomers and polymers[102].

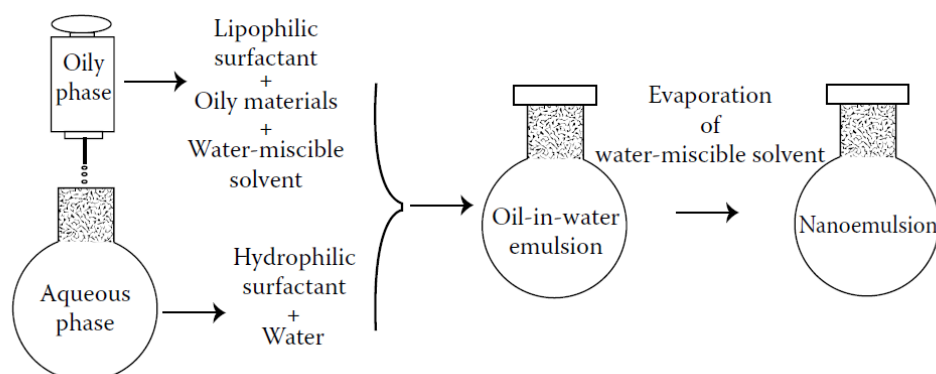


Figure 1.13: Schematic representation of the solvent evaporation technique, a formulation of O/W emulsions prepared using spontaneous emulsification technique[93].

Phase Inversion Temperature method

The Phase Inversion Temperature (PIT) method was introduced by Shinoda and Saito (1968)[101], and it is the most widely used method in industry[89]. This method exploits the changes in solubility of polyoxyethylene-type non-ionic surfactants as a function of temperature, and the resulting changes of the spontaneous curvature of the surfactant. Oil, water and non-ionic surfactants are mixed together at room temperature and stirred. The mixture is then slowly heated[102]. By increasing the temperature, this type of surfactant becomes lipophilic because of dehydration of the polyoxyethylene chain[105]. This method takes advantage of the changing behaviour of the spontaneous curvature of the surfactant monolayer. As shown in Figure 1.14, at low temperatures, the surfactant monolayer has a large positive spontaneous curvature that causes the emulsion to form oil-swollen micellar solution phase or O/W microemulsions which may coexist with an excess of oil phase. At high temperatures, the system is forced to undergo a transition from a O/W to a W/O microemulsion. The spontaneous curvature of the surfactant monolayer becomes negative and water-swollen reverse micelles coexist with excess water phase. At intermediate temperatures, the spontaneous curvature approaches zero with minimal surface tension and a bicontinuous microemulsion, containing equal amounts of water and oil phases, coexists with

both excess water and oil phase[105]. This phase is said to promote the formation of very finely dispersed oil droplets[105][80][102]. According to Solans (2005)[105], small sized droplets obtained at the PIT are unstable and coalesce very rapidly. Therefore, by rapidly cooling the emulsions prepared at a temperature near the PIT, very stable and small emulsion droplets can be produced[105][100].

Catastrophic Inversion (Figure 1.14) is one of the phase inversion phenomena in emulsions. It is induced by increasing the volume fraction of the disperse phase[80][100]. At constant temperature, a transition in the spontaneous radius of curvature will occur by changing the water volume fraction. Adding water stepwise into a mixture of oil and surfactant, with gentle stirring, will produce water droplets in a continuous oil phase. Increasing the water volume changes the spontaneous curvature of the surfactant from W/O emulsion to O/W emulsion at the inversion locus. Surfactants form flexible monolayers at the oil-water interface, resulting in a bicontinuous microemulsion at the inversion point. Also, the minimal interfacial tensions are achieved and facilitate the formation of small droplets at this point[93][80].

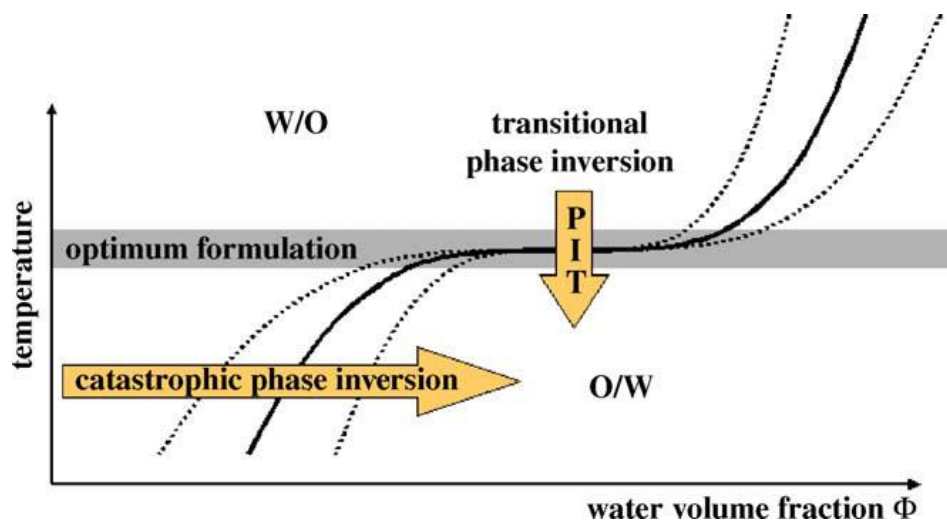


Figure 1.14: Schematic illustration of transitional phase inversion for the preparation of finely dispersed O/W emulsions. The solid black line marks the inversion locus, the dotted lines the hysteresis zone. Within the optimum formulation zone and at the inversion locus, the interfacial tension is minimal[80].

Izquierdo et al. (2002)[106] have successfully produced nanoemulsions by the PIT method using non-ionic surfactants and hexadecane as the oil phase of the system. The smallest sized droplets were obtained for compositions in which the emulsification started in a bicontinuous microemulsion phase. Forgiarini et al. (2001)[94] have studied the relationship between low energy emulsification methods and phase behaviour at constant temperature to form nanoemulsions. Three methods investigated by Izquierdo et al. (2002)[106] and Forgiarini et al. (2001)[94] were a stepwise addition of oil to a water-surfactant mixture, a stepwise addition of water into a surfactant-oil mixture and mixing all components in the final composition. Of these three methods, nanoemulsions were formed only when water was being added into an oil-surfactant mixture, not by the other two methods. In another study, Wang et al. (2007)[74] successfully produced nanoemulsions by a self-emulsification technique of low energy method. All appropriate components were mixed to generate a concentrate and a certain amount of the concentrate was injected into a very large volume of water, leading the emulsions to be crash diluted, under gentle stirring at constant temperature. Final nanoemulsion formation is achieved when the initial concentrate is a bicontinuous phase or an O/W microemulsion.

Figure 1.15 is an illustration of the mechanism involved in this low energy emulsification process[80]. In Method A, as the water phase is added into the oil phase, a W/O microemulsion is first formed. When the water volume fraction is increased, water droplets merge together and a bicontinuous or lamellar structure is formed. After the inversion point has passed, further increases in water content caused the structure to decompose into smaller oil droplets. Further dilution with water does not change the droplet size at this stage of droplet formation. In Method B, no transition through a bicontinuous or lamellar structure occurs as small droplets grow in size with increasing amount of oil and surfactant added into the system. Their size is a result of mechanical processes only.

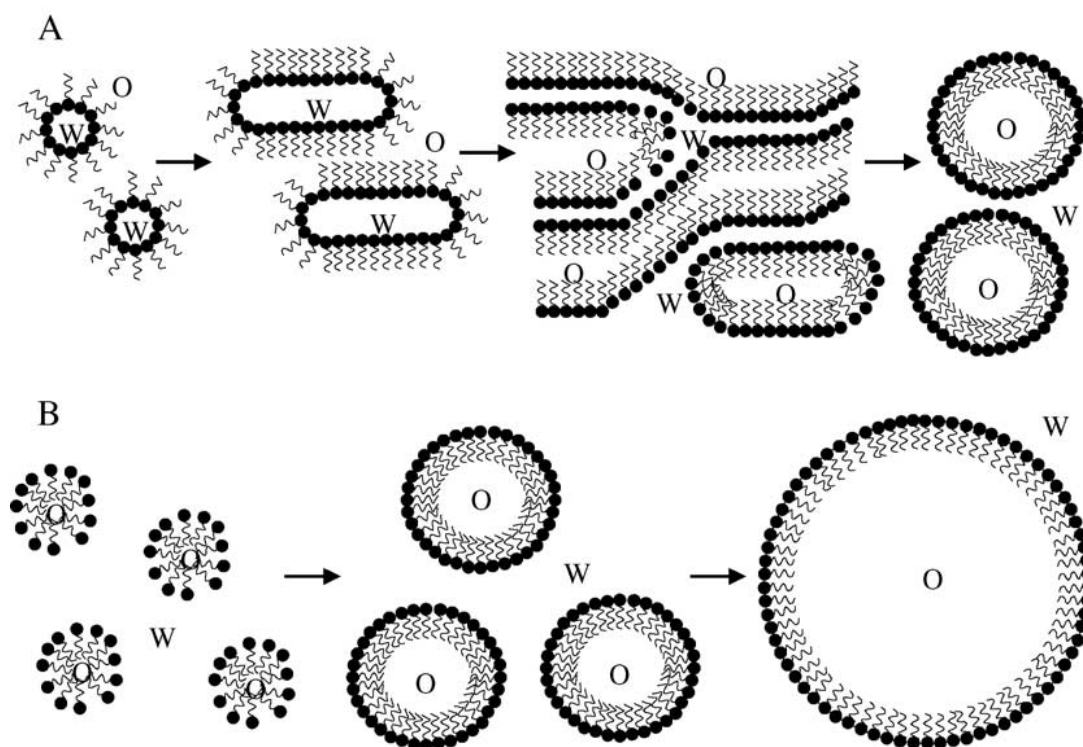


Figure 1.15: Scheme of the emulsification procedure (W=water phase; O=oil phase)[80].

1.4 SOLID STATE FORMULATION – HALLOYSITE CLAY NANOTUBES FOR CONTROLLED RELEASE OF ACTIVE MATERIALS

1.4.1 Controlled Release of Active Materials

The science of controlled release began to be explored in the 1950s when researchers attempted to develop such systems that could store active materials and then release them at controlled rates into other medium such as an aqueous system[107]. This technology has developed rapidly in the past few decades and there is now a variety of applications in several areas including agriculture, pharmaceutical, food science and personal care.

In the pharmaceutical area, the study of controlled release of drugs and other bioactive agents from polymeric devices has recently attracted interest from

chemists and chemical engineers around the world[108]. This research has shown a rapid improvement since it was first introduced. There are now systems ranging from coated tablets and gels to biodegradable microsphere and osmotic systems[108]. Controlling the rate, extent and time of a drug's delivery can optimize its performance in many ways. This technology can sustain drug delivery over days/weeks/months/years and targeted delivery (e.g., to a tumour, diseased blood vessel, etc.) on an 'on-time' or sustained basis[108]. The success of this approach depends on the ability to construct a biocompatible carrier that allows high loading of the drug molecule and that it reaches its destination without any leakage or premature drug release from the carrier[109] and that it reaches the destination at a specific time during treatment[110]. Controlled release is highly beneficial for drugs that are rapidly metabolized or eliminated from the body after administration[110].

Agriculture is another area that has made growing use of controlled release technology. The fertiliser industry is facing a challenge to improve the efficiency of its products. Whenever nutrients in the form of mineral fertilisers (nitrogen(N), phosphorus(P), potassium(K)) are applied to the soil to feed the plants, only a fraction of the delivered nutrients is taken up and used by the plants and crops[111]. Fertilisers are also lost through immobilisation, volatilisation and leaching, especially with nitrogen, to the environment. Controlled release fertilisers are fertilisers containing plant nutrients in a form which delays or gradually releases into the soil for plant uptake and use after application. The fertiliser is therefore available to the plant significantly longer than an ordinary nutrient fertiliser[111]. The term controlled release commonly refers to the coated or encapsulated products of controlled release fertilisers[111]. According to Shaviv (2000)[112], there are three types of coated fertilisers which are fertilisers coated with non-organic coatings (e.g., sulphur-coated urea), polymer coating of sulphur (e.g., modified of sulphur-coated urea by additional thin layer of an organic polymer) and fertilisers coated with organic polymers (e.g., resin-coated fertilisers and thermoplastic polymer-coated fertilisers). Figure 1.16 shows a simple example of controlled release fertiliser and its mechanism when applied to soil[113].

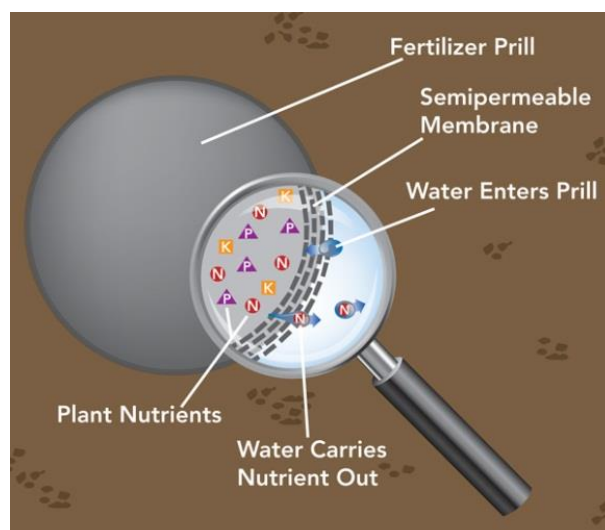


Figure 1.16: A picture of controlled release fertilizer applied to the soil[113].

Based on earlier research, controlled release technology has shown improved nutrient uptake and efficiency by the plant, reduced loss of nutrients, and reduced toxicity (caused by high ionic concentration of quick dissolution of conventional soluble fertiliser), thus minimizing negative environmental effects[112].

1.4.2 Halloysite Clay Nanotube

Clay minerals are naturally occurring nanomaterials whose primary particles range in size from Ångstroms to micrometres and are the most abundant naturally occurring nanomaterials known to humans[114] (Figure 1.17). Having excellent characteristic such as a very small size, expansive surface areas, anisotropic shape and reactive surfaces, these nanoparticles have been recognized in a wide range of disciplines and likely are the most utilized minerals[114]. Their application has been widely used in pharmaceuticals, as corrosion inhibitors, absorbents in wastewater treatment, and in paint and pottery[115][116][117].

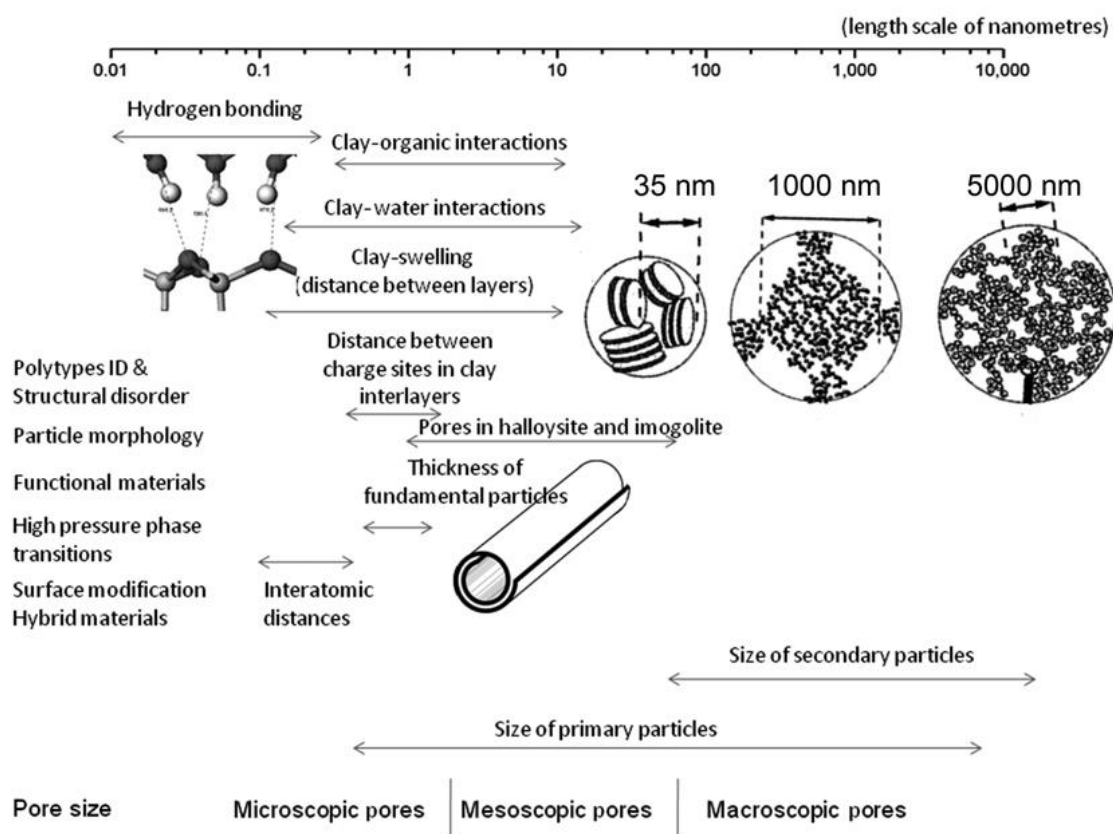


Figure 1.17: Length scales associated with the structure and surface chemistry of clay minerals[118].

Halloysite ($\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4 \cdot n\text{H}_2\text{O}$) is one of the clay minerals that is available in abundance in China and other locations around the world[117]. It is a two-layered aluminosilicate clay mineral, consisting of one alumina octahedron sheet and one silica tetrahedron sheet in a 1:1 stoichiometric ratio[115][117]. Halloysite consists of hollow nanotubules with 15 nm inner lumen diameter and 0.5-1 μm in length. They are formed by the rolling of two layered aluminosilicate kaolins¹² due to the strain caused by lattice mismatch between the adjacent sheets of silicon dioxide and aluminum oxide (Figure 1.18)[115]. The structure and chemical composition of halloysite is similar to kaolinite ($\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4$) but the unit layers in halloysite are separated by a monolayer of water molecules[119]. When $n=2$ the clay is in the form of hydrated “halloysite-10 Å” with one layer of water molecules between multilayers. The “10 Å” designation indicates the spacing in the multilayer walls as the kaolinite plates roll up into a multiwalled cylinder and the size is ~ 3 Å larger than kaolinite[119][120]. At $n=0$,

¹² Kaolin is a soft white clay mineral rich in kaolinite ($\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4$).

the structure is dehydrated “halloysite-7 Å” which may be obtained through an irreversible phase transition with loss of adsorbed water as the halloysite is heated to 90 – 150°C[120].

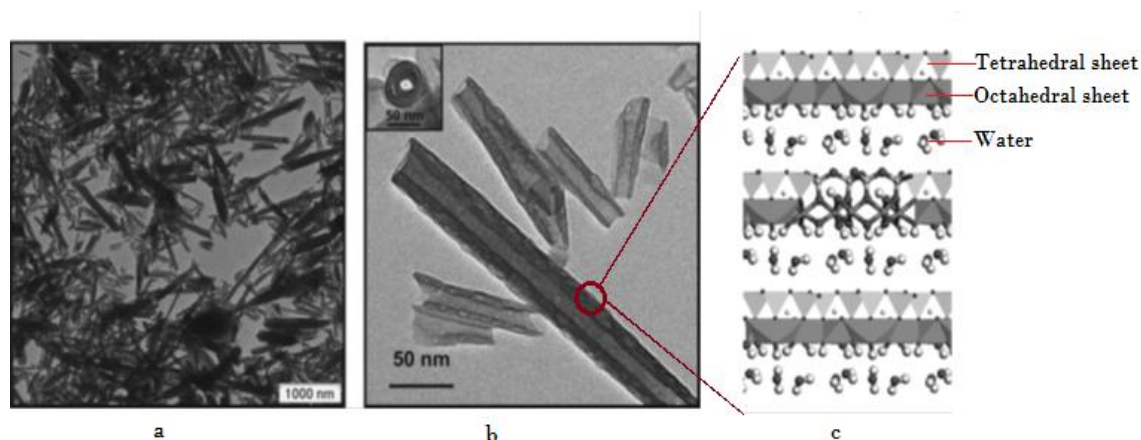


Figure 1.18: TEM images of halloysite nanotube (a) with cross section insert (b) and crystalline structure of halloysite nanotube (c)[115].

The most interesting halloysite nanotubes structure is the cylinder with small inner diameter which has attracted researchers interested in loading/release studies. For quicker release and higher loading efficiency, larger tubes may be used. Compared to other nano-sized material, halloysite nanotubes are readily obtainable and cheaper than other nanoparticles and suitable to be used as containers for the encapsulation of biologically active molecules such as biocides and drugs[117][120]. Their novel physical and chemical properties provide opportunities for advanced applications in electronics, catalysis, biological systems and functional materials[117].

1.4.2.1 Halloysite Clay Nanotubes for Controlled Release Active Materials

Halloysite clay nanotubes have shown a promising capability for macromolecule sustained release from its lumen due to its tubular structure, besides being a cheap and biocompatible nanotube[117]. Application of halloysite clay nanotubes as a nanocage for drug encapsulation and controlled release of biologically active molecules was first shown by Price et al. (2001)[121]. Its ability to be used in a drug delivery system was reported by Levis et al. (2003)[122]. Having silicon dioxide at external surfaces and

aluminium oxide at the internal surfaces, results in a negatively charged outer surface and a positively charged inner lumen surface. Multilayer films generated by layer-by-layer (LbL) nanoassembly can be produced due to the negative charge on the outer surface, while the positively charged inner surface is used to fill the lumen with negative macromolecules of active agents such as drugs used in the pharmaceutical industry[117].

Veerabadran et al. (2007)[123] have described the ability of halloysite nanotubes for use in the entrapment, storage and subsequent release of a few poorly soluble drugs. The insoluble drugs were loaded into a water-alcohol solvent to increase the drugs loading and the results showed an extension of 6-10 hours of release from 15 nm lumen diameter tubular halloysite for all tested drugs. Veerabdran et al. (2009)[115] have developed the controlled release of drugs by improving the system using the LbL nanoassembly technique (Figure 1.19). The study has elaborated an LbL assembly of polyelectrolyte multilayer shells on clay nanotubes, acting as the nanotemplate, decreasing the rate of drug release by four times: from 7 h release for bare clay tubes to 30 h for polyelectrolyte coated tubes. Biocompatible chitosan and gelatin multilayers were used for the encapsulation of halloysite nanotubes after the drug has been loaded into the lumen. LbL assembly in this study allows a control over permeability. Modification of the surface properties of similar bulk materials is possible and it can be used as a water-based process which is inexpensive and effective[115].

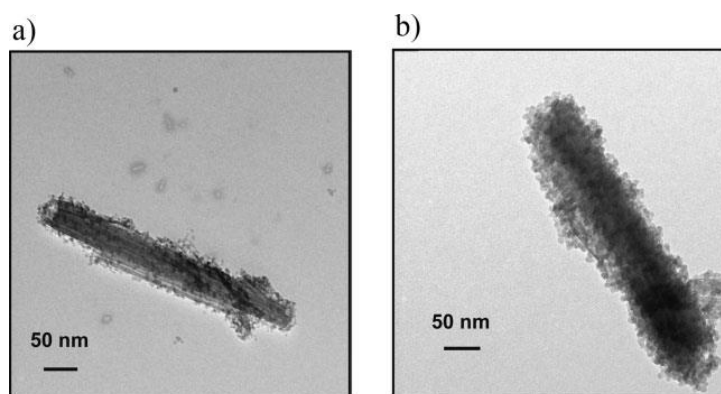


Figure 1.19: Transmission electron microscopy image of (PEI/PAA) halloysite clay nanotube shell assembly with 7 nm silica outermost (a, one silica layer; b, two silica layers, PEI=poly(ethyleneimine), PAA= poly(acrylic acid))[115].

The use of halloysite clay nanotubes as a carrier in controlled release of active molecules is still new and many improvements can be made to this technique. Work done by Price et al. (2001)[121], Levis et al. (2003)[122], Veerabadran et al. (2007)[123] and Lvov et al. (2008)[120] have opened a wide road to advance this technology into broader applications. To date, there are no studies on using halloysite nanotube for synthetic plant hormone delivery. In this research study, halloysite clay nanotubes were used for controlled release of 2, 4 - D as the active agent. The resulting solid powders of halloysite clay nanotubes were applied to the plants based on the same techniques used in the application of controlled release fertilisers.

1.5 RESEARCH OBJECTIVES

The main objective for this research was to successfully produce two new formulations (one a solid-state delivery and the other solution-based) for delivering synthetic plant hormones, (2,4-D and CPPU, respectively in the two formulations) with greater efficacy than standard methods to target plants, thus enhancing plant growth. It was hypothesised that the use of halloysite clay nanotubes as the nanocarriers of 2,4-D delivery would allow a slow release of 2,4-D, thus giving the roots a supply of this hormone for a longer period of time. For the other formulation, it was hypothesised that the nanoemulsion formulation used as a method of CPPU delivery would allow better penetration of CPPU through the leaves because of the small droplet size thus avoiding repeated application to the plant. The first step was development of the two new nanoformulations, and then bioassays were used to investigate the formulation's effectiveness in allowing the active ingredients to more efficiently reach the target plant's organs under study.

The two formulations were developed with chemical safety for the plants, humans and the environment as a primary consideration. Results obtained from this research represent a proof-of-concept for what is possible through the application of nanotechnology for use in the agricultural sector. Nanotechnology

plays an important role in improving the conventional methods used in the delivery of agrochemicals to the target plants. When nanomaterials are used as nanocarriers for these active ingredients, they should be able to deliver the active ingredients at an effective concentration with high solubility, stability and effectiveness to improve plant growth. This technology should be able to act on the plant more effectively than the normal agrochemicals, hence a single application only should be needed, reducing farming costs and additionally the adverse effects that the active ingredients could bring to the environment can be avoided.

What follows in the next chapters are the experimental sections. In Chapter 2 is described the full formulation of the nanoemulsion used as the carrier system for delivery of CPPU. The first of the two bioassay investigations is reported in Chapter 3. Dwarf beans were the plant used in this bioassay, whereby delivery of CPPU was via a nanoemulsion formulation. Both laboratory-scale and large-scale treatment tests were undertaken and the treatment was applied directly to the plant leaves. Delivery of 2,4-D to the roots of mung beans, using the halloysite clay solid-state treatment, is the focus of Chapter 4. A laboratory-scale bioassay investigation was undertaken. In the final chapter the major outcomes from this work are reported along with avenues for future investigations.

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CHAPTER 2

NANOEMULSION SYSTEM

The main focus of this research programme is to explore how nanoscale delivery systems can be used to effectively deliver growth hormones to plants. One of the major considerations is that the chemical being supplied is fully utilised by the plant and that the chemical itself interacts with the plant's cells and tissues in the most effective way. This ensures that cell division and expansion is induced thus giving the best possible outcomes with respect to plant growth. Furthermore it is desirable to use the smallest quantity of the delivered chemical agent to induce the required cell division. Therefore, understanding the behaviour of the plant and the chemical, here CPPU, in their mutual interaction is crucial for determining the best method of delivering the CPPU to the plant.

As discussed in Chapter 1, one of the methods for delivery of chemical substances, such as growth substances, nutrients and pesticides is the spraying technique. Here the diluted chemical substances are sprayed onto the target plant at an appropriate time. It has been widely used in farming activities. However, this technique can be improved, since in particular, many of the critical chemicals that need to be delivered have very low water solubility, including CPPU.

In the solution phase delivery system investigated here, the CPPU is encapsulated within a nanoemulsion. As discussed in Chapter 1, the method used to produce the nanoemulsion in this research is a low energy

emulsification technique. This method has been used to generate drug delivery systems for delivering drugs to humans in the pharmaceutical field with excellent results[1]. For example, Ragelle et al. (2012) have developed a stable nanoemulsion of fisetin (a chemical substance found in several fruits and vegetables) that improves the fisetin's bioavailability and antitumour activity in mice[2]. Previous literature does not report the testing of the method to deliver synthetic plant hormones, as the active ingredients, to plants..

2.1 NANOEMULSION SYSTEM

In this work, a new self-nanoemulsifying system, adapted from Wang et al. (2007)[3] was used to produce nanoemulsions containing CPPU as the active ingredient located within the oil droplet. The nanoemulsion system used in this study was water/polysorbate 80/soybean oil/glycerol/CPPU. This system, that consists of soybean oil as the lipid phase and polysorbate 80 plus glycerol as the surfactant phase, was selected for use in this study because it has been widely used in the fabrication of nanoemulsions, especially in the pharmaceutical industry and is safe to be used for humans and in the environment [4][5]. The soybean oil and CPPU combined constitute the oil phase of the system. The CPPU is solubilised in the soybean oil prior to formation of the nanoemulsion.

The phase diagram, of the pseudo-ternary¹³ polysorbate 80+glycerol/soybean oil/ water system constructed by Wang (2009)[6] is presented in Figure 2.1. It should be noted, that the ratio of surfactant and co-surfactant in this pseudo-ternary phase diagram is fixed at 1:1[6]. This system was chosen for investigation as the base delivery vehicle for CPPU to plants, specifically with respect to determining the relationship between successful nanoemulsion formation and the type of thermodynamically stable phases that are formed upon self-assembly, as summarised within the phase diagram, because the phase behaviour had been well studied previously and the characteristics of the

¹³ 'Pseudo-ternary' phase diagram is used here because the surfactant phase is a mixture of the surfactant and co-surfactant here polysorbate 80 and glycerol.

individual ingredients have been well studied with respect to humans and the environment. Upon changing the amount of lipid phase (surfactant plus co-surfactant, from here on just called surfactant), and/or oil phase, different types of final states can be formed. In the phase diagram shown in Figure 2.1, three different regions are identified: coarse emulsion (EM, a kinetically stable state), liquid crystal (LC, a thermodynamically stable state) and microemulsion (ME, a thermodynamically stable state).

The green lines shown in Figure 2.1 indicate how to read the diagram. For example, to determine the composition at point **X**, which lies inside the EM region of the phase diagram, a series of three lines are drawn through the point **X** with each line parallel to a side of the triangle. Point **X** therefore has a composition of A% of water, B% of oil and C% of surfactant + co-surfactant, which then represent 100% of the final mixture.

This system's phase diagram is dominated by the EM region. In this region, Wang (2009) [6] determined the average droplet diameter to be ~1000 nm. This region dominates when relatively low amounts of the surfactant phase is present but it extends to higher surfactant concentrations as the amount of oil is decreased. In contrast, the LC region exists at very high surfactant concentrations. For a fixed oil concentration, at low oil content, the microemulsion region is formed upon increasing water content as surfactant content decreases. The ME region extends all the way up to quite high surfactant concentration (~70 wt %). Here most of the droplets are under 100 nm in diameter.

Based on using the low energy method described in the literature, nanoemulsions are expected to be able to be generated for systems which form bicontinuous and/or O/W microemulsions as stable mixtures within the phase diagram [3][7][8][9]. Bicontinuous microemulsions do not have individual aggregates or droplets dispersed throughout the continuous phase but rather they have a sponge-like structure consisting of intertwined channels of oil and water in the mixture[10] (see Figure 2.5). From the work of Wang (2009) [6] it is known that O/W microemulsions exist within the ME region, hence it was

anticipated that nanoemulsions would be able to be formed when the low energy method was applied to this base system. Furthermore, the presence of such a large microemulsion region provides potentially greater flexibility with regard to determining the optimal dosage composition of each chemical to be used in the formation of the nanoemulsions once CPPU is added into the formulation process and then applied to plants.

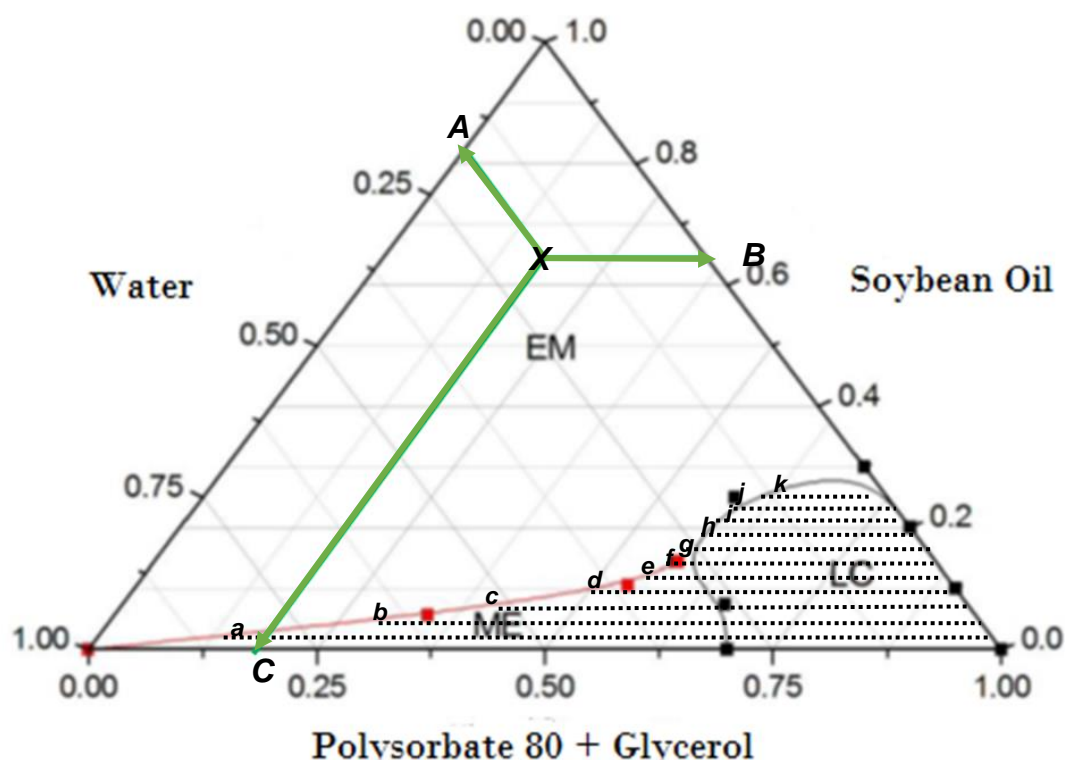


Figure 2.1: Phase diagram of the polysorbate 80+glycerol/soybean oil/water system (EM – Coarse Emulsion, ME – Microemulsion, LC – Liquid Crystal) taken from reference [6]. The dotted lines show (a-k) represent the domains explored here. The X and A, B, C points are present as guides to reading the diagram with respect to composition, as described in the text.

The focus of this chapter is the exploration of the ternary phase diagram of polysorbate 80+glycerol/soybean oil/water system with regard to identifying the region within the phase diagram whereby nanoemulsions are formed following crash dilution of concentrates that lie specifically within the ME and LC regions of the phase diagram. Droplet size, sample stability and visual appearance were all used to identify when nanoemulsions are produced. Determining the full nanoemulsion region is the critical first step to producing CPPU-loaded

nanoemulsions and finalising the full formulation procedure. After the nanoemulsion formulation process is optimised, CPPU is then embedded into the system, prior to application of the final formulation to the plant leaves in the biological studies described in Chapter 3.

2.2 MATERIALS AND METHODS

The surfactant used was liquid polysorbate 80 (Figure 2.2), purchased from Fluka Analytical and manufactured in Switzerland. The product is a clear viscous liquid of dark yellow colour. The sample complies with the European Pharmacopoeia¹⁴ (Ph Eur) quality standards. The molecular formula is $C_{64}H_{124}O_{26}$ with a molar mass of 1310 g/mol.

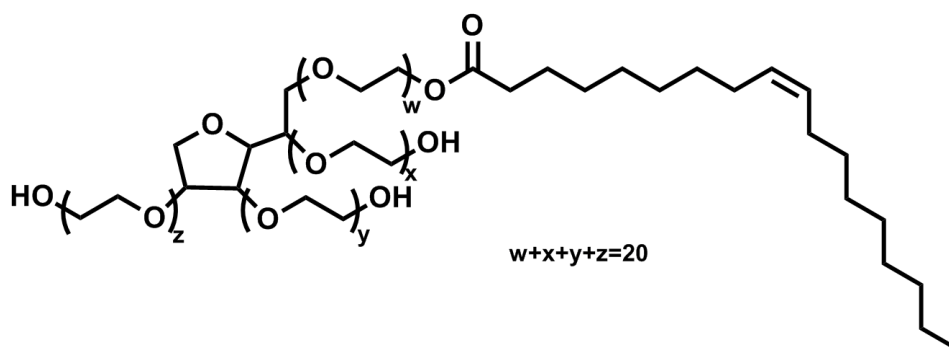


Figure 2.2: Chemical structure of Polysorbate 80. $w+x+y+z$ refers to the total number of oxyethylene subunits on each surfactant molecule which does not exceed 20.

Polysorbate 80, also known as polyoxyethylene-sorbitan-20-monooleate or Tween 80 is an amphipathic, non-ionic surfactant composed of fatty acid esters of polyoxyethylene sorbitan. It is widely used in the manufacture of foods and drugs and in the biotechnology and pharmaceutical industries[11]. Polysorbate 80 is derived from polyethoxylated sorbitan and oleic acid. The hydrophobic site is the hydrocarbon chain and the hydrophilic site is provided by the ethylene oxide subunits[11].

¹⁴ The European Pharmacopoeia is a reference work for quality control of medicine for pharmaceutical use, a quality standards that need to be applied before market their products.

The soybean oil used as the oil phase of the emulsion system was purchased from Sigma Aldrich Ltd. This material is very commonly used in the food and pharmaceutical industries and is considered safe for use in/on plants and animals [6]. The oil comes in the form of a clear liquid and is light yellow in colour. Soybean oil is a vegetable oil extracted from the seeds of the soybean. It consists primarily of glycerides of the following fatty acids: linoleic, oleic, palmitic and stearic. The major component is linoleic acid, a polyunsaturated fatty acids which make up more than 50% of the total fatty acid composition in soybean oil[12]. Compared with other oils, e.g. coconut oil and peanut oil, soybean oil has a very low melting point, -3.7°C [13] which makes it easy to work with. According to Carla et al. (2009), the use of soybean oils as the hydrophobic component in nanoemulsion formulation is in accordance with the principle of green chemistry as it is obtained from renewable sources and is environmentally friendly[14].

Glycerol, 99% purity with a molecular weight of 92.09 g mol^{-1} (Figure 2.3), was used as the co-surfactant in the initial formulation. It was kindly donated by Pure Science Ltd from New Zealand. Glycerol is a colourless, odourless and highly viscous liquid commonly used in the pharmaceutical industry [6]. Because it is non-toxic glycerol also has a wide range of applications in the food and cosmetic industries beyond its use in the pharmaceutical industry[15]. Generally, the addition of low molecular weight co-surfactants decreases the concentration of the more expensive surfactants used in the system. For example, in the study conducted by Luo et al. (2004)[16], they showed that the presence of 1-pentanol is helpful in forming stable and transparent aminosilicone oil microemulsions. Co-surfactants can influence the formation of microemulsions through both interfacial and bulk effects. The amphiphilic nature, short hydrophobic chain and terminal hydroxyl group of alcohol co-surfactants can make them interact strongly with the surfactant monolayers at the oil/water interface, affecting the packing of the interface and thus influencing the curvature of the interface and the interfacial energy[16].

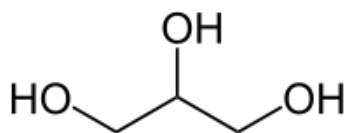


Figure 2.3: Chemical structure of glycerol.

CPPU (see Figure 1.6) was purchased from Jiangsu Fengyuan Bioengineering Co., Ltd., Jiangsu, China. It is a white crystalline powder, 99% purity of standard analytical grade. CPPU is essentially insoluble in water (39 mg/l at 21°C) but soluble in organic solvents such as methanol, ethanol, acetone and soybean oil. It is stable against heat, light, acid and alkali.

2.2.1 Preparation of Nanoemulsions

Nanoemulsions were prepared using the recently reported low energy emulsification method[3][7][17][18]. To produce the final emulsion with nanosized droplets, all the appropriate components used to construct the emulsion system are simultaneously mixed together in the specific ratio required to generate a concentrated sample of the appropriate internal structure (oil-in-water microemulsion or bicontinuous microemulsion). In this work the ratios were based on the pseudo-ternary phase diagram shown in Figure 2.1, determined by Wang (2009)[6].

In the making of the concentrates, the surfactant and co-surfactant were mixed to the ratio of 1:1 on a weight basis. Polysorbate 80 was mixed with glycerol and this mixture of surfactant and co-surfactant, here on referred to as the 'surfactant phase', was then used as a single solution in the nanoemulsion formulation. The oil was added into the surfactant phase (on a weight basis) and all three materials were gently stirred at room temperature until they were well combined. Deionized water, as the last material used to produce the concentrates, was slowly added dropwise under constant stirring, ensuring that the water was well mixed with the other materials. Adding water to the system immediately changed the physical appearance of the concentrates. It should be noted that the amount of water added into the concentrates is based on the

ternary-phase diagram (Figure 2.1). The appearance of the concentrates changes for concentrates in the ME and LC regions containing a small amount of oil, the initial few droplets of water added into the surfactant phase caused the mixture to become slightly turbid. Further addition of water resulted in a clear and transparent solution (Figure 2.4 (a)). As the oil composition of the concentrates increased, adding the water into the surfactant phase resulted in a cloudy mixture (Figure 2.4 (b)), unlike the situation described above.

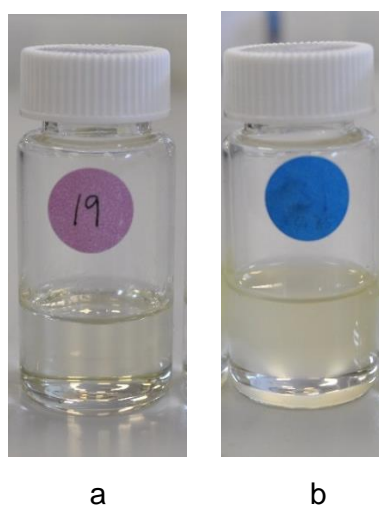


Figure 2.4: Examples of different physical appearance of the initial concentrates. Each of the concentrates is produced from different points in the ternary phase diagram, comprised of different amount of surfactant phase, oil and water, a) 2.5 wt % of oil/70 wt % of surfactant phase/27.5 wt % of water, b) 7.5 wt % of oil/65 wt % of surfactant phase/27.5 wt % of water.

A sample of the initial concentrate was then injected into a very large volume of water under gentle stirring to achieve the final system, which was either a diluted microemulsion or a nanoemulsion. Wang et al. (2007)[3] termed this method the 'crash dilution' method. The injected concentrate yields emulsions (micro or nano) with a final concentrate volume fraction of $\Phi = 0.03$ (1.5 mL of concentrate in 50 mL of water). When the initial concentrated systems were within the bicontinuous or O/W regions, that lay somewhere within the ME region of the phase diagram (Figure 2.1), nanoemulsion formation is ensured as a consequence of the 'crash dilution' step (Figure 2.5). In this study, this is the desired outcome; formulation of nanoemulsions. It should be noted that based on the work of Wang (2009)[6] it was known that somewhere within the

ME region either or both bicontinuous or oil-in-water microemulsions exist depending on the exact composition of the initial concentrate samples[6].

The formation of nanoemulsions was explored throughout the ME and LC regions of the phase diagram following the dotted lines shown in Figure 2.1. This allowed the so-called crash-diluted phase diagram to be mapped. From the work of Wang et al. (2007)[3] it was expected that concentrated samples with different compositions would generate nanoemulsions and diluted microemulsions with different droplet sizes. Exploration of the crash-diluted phase diagram was undertaken by preparing all concentrated samples a minimum of three times and undertaking repeated crash dilutions in order to ensure the reliability and consistency of the data obtained.

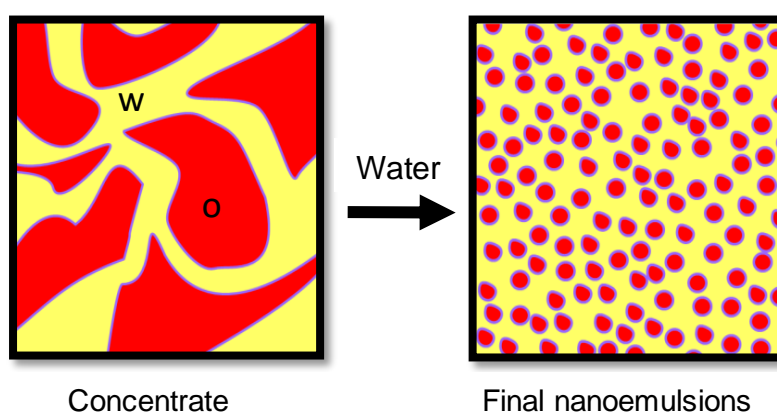


Figure 2.5: Nanoemulsions are created only when the concentrate mixture is located in the bicontinuous microemulsion or O/W microemulsion regions.

The droplet sizes of the concentrated systems and the final crash-diluted solutions were measured using dynamic light scattering, described in Section 2.2.3.

2.2.2 CPPU-Loaded Nanoemulsion Formulation

Once the appropriate concentrations of each of the surfactant, oil and water phases had been determined, that ensured nanoemulsion formation, CPPU was dissolved in the soybean oil phase, prior to this oil phase being added to the surfactant phase. To this mixture was subsequently added water producing

the CPPU active-loaded concentrate. Nanoemulsion formation then proceeded as described in Section 2.2.1.

Concentrations of 5-10 ppm CPPU are commonly used in the literature[19][20][21] as being appropriate to produce good cell division and plant growth. In this study the focus is based on exploring whether using nanoemulsions to deliver the CPPU enhances its efficacy and hence CPPU concentrations ranging from 0.003 ppm to as high as 30 ppm were examined. Note that the stated concentration is that relative to the total final crash-diluted solution. Due to the very low solubility of CPPU in water, but correspondingly high solubility in soybean oil, CPPU is expected to be wholly contained within the oil droplet phase. Oil droplets are coated with surfactant and co-surfactant, lowering the oil/water interfacial energy, and dispersed in the water phase, as shown in Figure 2.6.

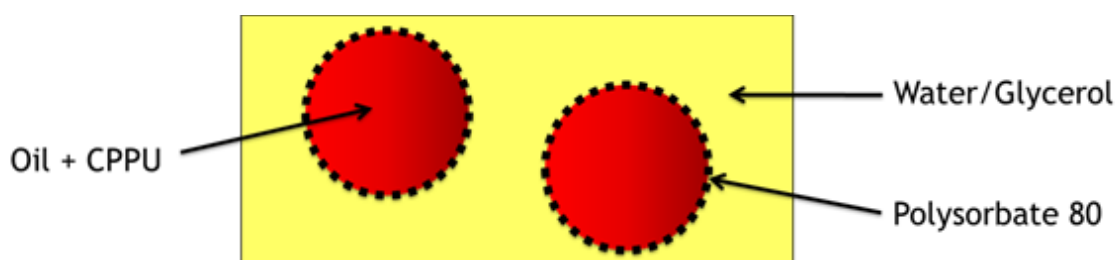


Figure 2.6: Schematic representation of the nanoemulsion system of water/polysorbate 80/ glycerol/soybean oil with added CPPU. It is highly likely that the droplet surfaces are also covered in glycerol.

2.2.3 Droplet Size Measurement

The light geometrical structure of small particles and their state of motion can be probed using light scattering[22]. In this research study, dynamic light scattering (DLS) was used to determine the droplet size distribution of the nanoemulsions and microemulsions produced after crash diluting the concentrate.

When light strikes matter, the electric field of the light induces an oscillating polarization of electrons in the molecules of the sample. In return, a secondary

source of light is promoted and scattered[23]. DLS is one of the most powerful and widely used techniques to measure the sizes of molecules and particles in the nanometre range and is commonly used to study droplet size distributions in emulsions and nanoemulsions[17][24].

DLS, also known as photon correlation spectroscopy or quasi-elastic light scattering, is a non-invasive, well-established technique to characterize the size of colloidal dispersions by using the illumination of a suspension of particles or molecules undergoing Brownian motion with a laser beam[25]. In DLS the hydrodynamic diameter of the droplets is measured. The hydrodynamic diameter of a droplet includes the droplet 'core', any surface structure, and the concentration and types of ions in the medium associated with the droplet. This means that the diameter includes any other molecules or solvent molecules that move with the droplets. The hydrodynamic diameter is the sphere defined by the droplet rotating in all directions and it is a measure of how easy it is to move the droplets through the solvent. It has the same translational diffusion coefficient as the droplet being measured. All droplet sizes quoted here are the droplet diameters.

To measure the droplet size using the Zetasizer NanoZS, a disposable cuvette was filled with the nanoemulsion and placed into the optical path of a laser. When a laser beam (typically red light at 633 nm) is passed through the sample, the light will be scattered upon interacting with the particles that are moving by Brownian motion. This scattered light is then captured by one of two detectors present in the instrument; one is the forwardscatter detector at 13° and the other is the backscatter detector at 173° . By using the backscatter detector, the laser does not have to travel through the entire sample, thus it will measure the droplets close to the cuvette wall as the light passes through a shorter path length in the sample. Thereby reducing the effect of multiple scattering, where light from one droplet is itself scattered by other droplets. Because of the many advantages of backscatter detection, this technique is most suitable to be used to measure very small droplets or low concentration samples such as those investigated here.

DLS, used in this research, works by monitoring the intensity of light scattered by droplets as a function of time. Since all droplets move under Brownian motion, induced by the bombardment of solvent molecules that are moving due to their thermal energy, there will be interference (constructive or destructive) that causes a change in the intensity of the light. This leads to time-dependent fluctuations in the intensity of the scattered light. By measuring the time scale of the light intensity fluctuations, DLS provides information on the average size, size distribution, and polydispersity of droplets in solution. When the droplets are illuminated with a laser beam, the intensity of the scattered light fluctuates at a rate depending on the size of the droplets; smaller droplets move a greater distance and move more rapidly than bigger droplets. The faster the droplets diffuse, the faster the intensity will change. Time-dependent fluctuations of the intensity of the scattered light are measured by an autocorrelator which determines the correlation function of the signal. The fluctuations are directly related to the droplet's rate of diffusion through the solvent which can then be analysed to determine the hydrodynamic diameter of the droplets. The correlation of the signal decays exponentially. This signal changes slowly if the droplets are large and the correlation continues for a long time. Smaller droplets move more rapidly and as a result the correlation decreases more quickly.

2.2.4 Stability of Nanoemulsions

The stability of the so-formed nanoemulsions was determined by measuring the droplet size as a function of time using DLS. The stability of the fabricated emulsions varied between a few minutes to more than a few months because of the Brownian motion of the droplets and the repulsive barriers between the droplets during droplet-droplet encounters. Even when macroscopic phase separation does not occur after a certain time, droplet size can still be an increasing function of time and hence the system can be considered to become less stable over time[3]. This occurs via two main destabilization mechanisms; coalescence and Ostwald ripening[3][17][26][27]. Ostwald ripening or molecular diffusion occurs due to emulsion polydispersity and the difference in stability between small and large droplets due to the Laplace pressure increasing or

decreasing droplet size[26]. Coalescence is a process where two or more droplets merge during contact to form a single droplet with higher droplet size, causing a surface fluctuation and eventually phase separation of the solution.

2.3 RESULTS AND DISCUSSION

2.3.1 CPPU Incorporation and Solubility in the Lipid Phase

Previous studies have been focused on producing nanoemulsions; mostly exploring drug delivery systems, targeted for human use. Many different oils have been used to incorporate different drugs[28]. Which oils are used in testing the effect and/or delivery of the drugs are generally those that solubilise the drug to the highest extent and themselves do not negatively influence either the function and/or efficacy of the drug or the receptor of the drug. According to Müller et al. (2000), the main point in evaluating the suitability of the drug's, or active material's, carrier system is its loading capacity. A sufficient loading capacity can be obtained by having a sufficiently high solubility of the active material in the oil[24].

By using a simple test, where CPPU was mixed with a variety of different oils under gentle stirring at room temperature, it was found that CPPU powder was most readily dissolved in soybean oil after 24 hours observation. Hence only soybean oil was developed further and reported in this chapter.

The ternary phase behaviour of the soybean oil with polysorbate 80/water has been explored as described above and below. In order to find the maximum solubility of CPPU in soybean oil, CPPU was added little by little into the soybean oil under gentle stirring until the mixture was fully concentrated and further addition of CPPU resulted in no further change to the solution, with an excess of crystalline CPPU observed. 1.2 mg was identified as the maximum loading of CPPU in 1 mL of soybean oil after gentle stirring for 24 hours. This correlates to a concentration of 1200 ppm of CPPU. Hence the range of CPPU

concentration used here (0.003 – 30 ppm) corresponds to the dilute range for CPPU solubilisation in soybean oil.

2.3.2 Phase Diagram Region of Interest

Table 2.1 provides the summary of the concentrate samples explored and subjected to crash dilution and the resulting final emulsion samples produced along each of the dotted lines, a to k, depicted in Figure 2.1.

Consider the first samples formed in the polysorbate 80+glycerol/soybean oil/water system that all lie within the low oil content range (2.5 – 5 w/w% soybean), in particular those samples which correspond to line a (see Figure 2.7 for images of the final samples). The droplet diameter of the final diluted emulsion samples were always less than 20 nm for all surfactant concentrations used (90 – 20 w/w%). All the resulting crash-diluted samples were colourless and transparent. Based on these two pieces of data (solution transparency and droplet size) all of the diluted samples are therefore dilute microemulsions, no nanoemulsions were formed.

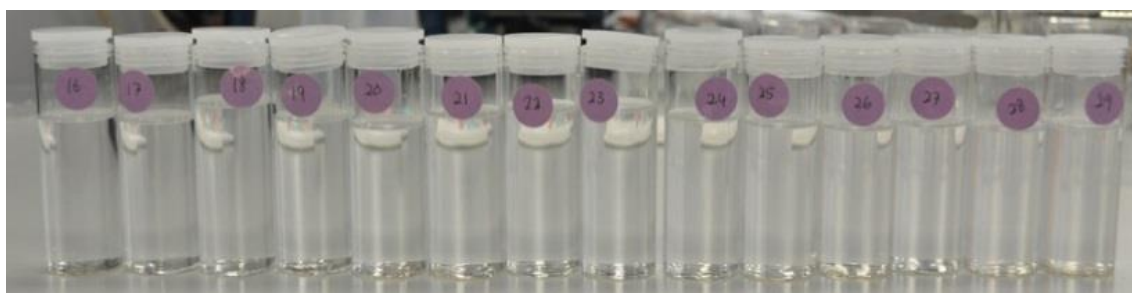


Figure 2.7: Images of solutions having a final concentrate volume fraction of $\Phi = 0.03$, formed from concentrated samples along the **a** line of the polysorbate 80+glycerol/soybean oil/water phase diagram shown in Figure 2.1.

The remaining ten dilution lines (b-k, depicted in Figure 2.1) were explored with oil concentration increments of 2.5 wt% to a maximum of 27.5 wt% oil. As the oil content of the concentrates increased, the range of surfactant content decreased (see Figure 2.1). Samples explored along line b were comparable to line a. For line c (7.5 wt% oil, shown in Figure 2.8) a different behaviour was observed upon crash dilution, two regions could be distinguished. The droplet

diameters were larger at the lower surfactant concentration end of the range and the visible appearance of the samples was changed. Samples were now bluish and translucent. These are characteristics of nanoemulsion formation, rather than microemulsions. Microemulsions were still formed at the high surfactant concentration end of the range of oil concentrations explored. From these results it appears that successful nanoemulsion formulation requires more than 5 wt% oil and less than 75 wt% surfactant in the original concentrate.

This trend continued up to an oil concentration of 15 wt%, line f in Figure 2.1. Increasing the oil content further resulted in a third behaviour trend on increasing surfactant concentration (Figure 2.9). Here again two regions are evident, consider for example the droplet sizes along line j which are larger at the lower surfactant concentration end of the range than at the higher end. There is also an obvious difference in the sample appearance where the samples are now cloudy to milky. The droplet size is more than 200 nm. This region, again based on droplet size and visual appearance of the sample, corresponds to macroemulsion formation. These samples are more unstable than the nanoemulsions which are formed and are subject to phase separation in a shorter period of time than those with smaller droplet size. Line j also shows that when a higher amount of oil (17.5 – 27.5 wt%) is used it is still possible to produce nanoemulsions as long as a higher concentration of surfactant is also used (70 – 80 wt%). But the nanoemulsion region is now very small and close to the border of the macroemulsion region.

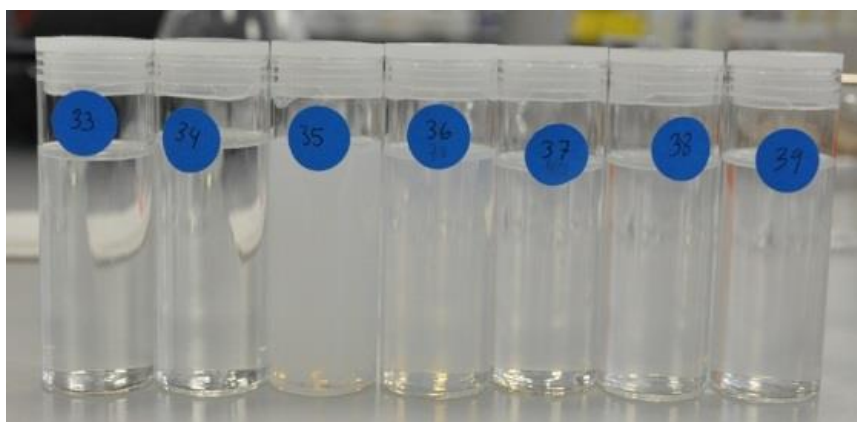


Figure 2.8: Images of solutions having a final concentrate volume fraction of $\Phi = 0.03$, formed from concentrated samples along the **c** line of the polysorbate 80+glycerol/soybean oil/water phase diagram shown in Figure 2.1.

Table 2.1: Summary of the characteristic of the final crash diluted emulsions of the polysorbate 80+glycerol/soybean oil/water system

Line as depicted in Figure 2.1	Concentrations (w/w%)				Characteristics of the final crash-diluted emulsions (concentrate to water volume fraction $\Phi = 0.03$)		
	Oil	Surfactant and Co-surfactant (1:1)	Water	No of samples along line	Droplet size diameter (nm)	Physical appearance	Type of emulsions
a	2.5	95 - 20	2.5 - 77.5	16	10 - 17	Colourless and transparent	Micro-emulsion
b	5	90 - 30	5 - 65	13	11 - 22	Colourless and transparent	Micro-emulsion
c	7.5	90 - 70	2.5 - 22.5	5	11 - 13	Colourless and transparent	Micro-emulsion
		65 - 45	27.5 - 47.5	5	29 - 49	Bluish and translucent	Nano-emulsion
d	10	85 - 75	5 - 15	3	13 - 18	Colourless and transparent	Micro-emulsion
		70 - 55	20 - 35	4	45 - 79	Bluish and translucent	Nano-emulsion
e	12.5	85 - 75	2.5 - 12.5	3	16	Colourless and transparent	Micro-emulsion
		70 - 60	17.5 - 27.5	3	70 - 90	Bluish and translucent	Nano-emulsion
f	15	80 - 75	5 - 10	2	18 - 20	Colourless and transparent	Micro-emulsion
		70 - 60	15 - 25	3	98 - 103	Bluish and translucent	Nano-emulsion
g	17.5	80 - 75	2.5 - 7.5	2	22 - 26	Colourless and transparent	Nano-emulsion
		70 - 60	12.5 - 22.5	3	127 - 162	Cloudy and milky	Macro-emulsion
h	20	75 - 70	5 - 10	2	23 - 29	Colourless and transparent	Nano-emulsion
		65 - 60	15 - 20	2	197 - 206	Cloudy and milky	Macro-emulsion
i	22.5	75 - 70	2.5 - 7.5	2	30 - 35	Bluish and translucent	Nano-emulsion
		65 - 60	12.5 - 17.5	2	193 - 209	Cloudy and milky	Macro-emulsion
j	25	70	5	1	43	Bluish and translucent	Nano-emulsion
		65 - 60	10 - 15	2	204 - 226	Cloudy and milky	Macro-emulsion
k	27.5	70	2.5	1	50	Bluish and translucent	Nano-emulsion
		65	7.5	1	212	Cloudy and milky	Macroemulsion



Figure 2.9: Images of solutions having a final concentrate volume fraction of $\Phi = 0.03$, formed from concentrated samples along the **j** line of the polysorbate 80+glycerol/soybean oil/water phase diagram shown in Figure 2.1.

From these data the crash-diluted phase diagram of the polysorbate 80+glycerol/soybean oil/water system at room temperature can be depicted, as shown in Figure 2.10. The equilibrium phase diagram boundaries and phases are superimposed for ready comparison with Figure 2.1. A wide range of different droplet sizes are realised following crash dilution from the original ME and LC regions, ranging from 10 nm to 270 nm. Based on droplet size alone, the red region is the region that most likely corresponds to the nanoemulsion region as the droplet size is between 30 nm to 100 nm. Furthermore, all samples within this region are bluish or turbid, together these results are indicative of nanoemulsion formation.

The ME and LC regions were fully explored. Four separate regions in the crash-diluted phase diagram can be differentiated based on the droplet size of the final diluted emulsions. These regions span across the original ME and LC regions. The biggest region is that shown in blue in Figure 2.10. Within this region microemulsions comprised of very small droplets ranging from 10 nm to 20 nm are formed. All samples are colourless and transparent. This region forms at very low oil content for all surfactant contents, upon increasing oil content, higher surfactant phase content is also needed.

Within the region coloured purple, droplet size varied from 21 nm to 45 nm and all samples are colourless and transparent. This larger droplet microemulsion

region was found at a higher oil content, the small and stable oil droplets being maintained because of the very high surfactant phase content.

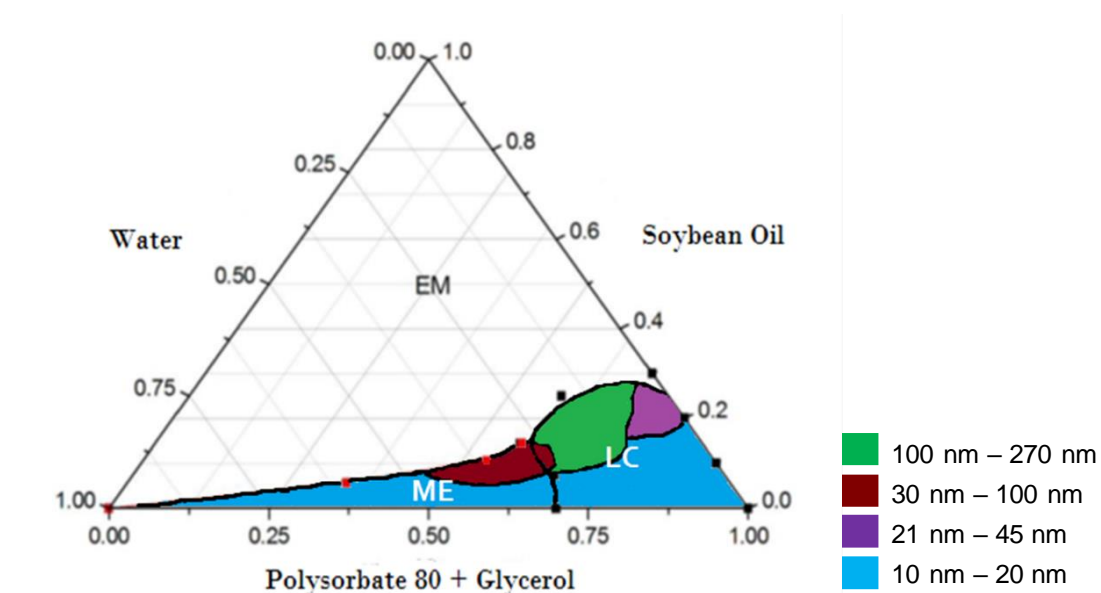


Figure 2.10: Crash-diluted phase diagram of the polysorbate 80+glycerol/soybean oil/water system. The different colours represent the different droplet sizes generated following crash dilution of the concentrated samples. The ME and LC regions of the underlying phase diagram are depicted for ease of comparison.

The third region coloured green has the largest droplet sizes of 100 nm to 270 nm and samples are very turbid. This region was observed at high oil content but lower content of surfactant phase.

The last region is that coloured red with droplet size of 30 nm to 100 nm. Samples were bluish to turbid and here nanoemulsions formed. This is the most important region from the perspective of this investigation.

2.3.2.1 The Nanoemulsions: The Red Region

Nanoemulsions result upon crash dilution of concentrates with compositions within the red region shown in Figure 2.10. This indicates that the original concentrate region consists of bicontinuous or O/W microemulsions. Based on the original work by Wang et al. (2007)[3], nanoemulsions are only formed using the crash dilution method if the original concentrated solution is a

bicontinuous or O/W microemulsion[8]. The final nanoemulsions produced are translucent with a very unique bluish colouration due to the small droplet size as shown in Figure 2.11 (a). This slight blue colour is evident when the droplets are smaller than the wavelength of the light passing through the nanoemulsion solution[29]. Solution **a** in Figure 2.11 represents a typical nanoemulsion produced from within the red area shown in Figure 2.10. In contrast the solution shown in Figure 2.11 **b** is typical for those formed within the green region where the droplets are significantly larger.

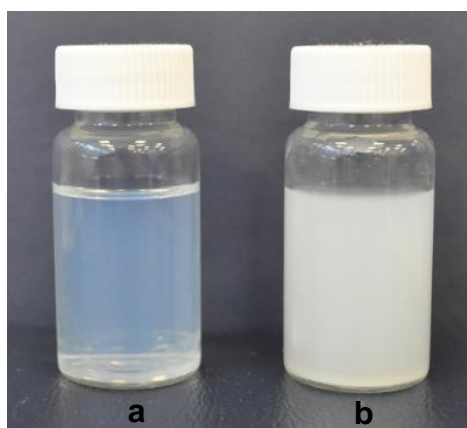


Figure 2.11: Sample a is a nanoemulsion with a measured droplet size of 54 nm and b is a macroemulsion with droplet size 212 nm.

Once the nanoemulsion region had been identified, an investigation was performed to 1) see if the droplet size systematically changed as a function of composition of the concentrate and 2) determine the variability in the droplet size obtained upon crash dilution of concentrates with identical composition.

As is expected with kinetically stabilised samples, there was variability in the characteristics of the final nanoemulsions prepared. To exemplify this, the samples that resulted upon crash dilution of three separate concentrates containing the same amount of all components (water, soybean oil, surfactant and co-surfactant) are shown in Figure 2.12. The concentrate was taken from the red region of Figure 2.10. Nanoemulsions result from all three concentrates but there is a noticeable difference between the three solutions. This difference in colour results from the difference in droplet size present in the samples.

Droplets are 45 nm and 57 nm respectively in samples **b** and **c**, while in sample **a** droplets are 94 nm (Figure 2.12). The nanoemulsion region is thermodynamically unstable which leads to different droplet sizes being produced even though the initial concentrate was taken from the same point within the red region. This is expected behaviour for such systems. It should be noted that for a single concentrate, the nanoemulsions obtained always had approximately the same size droplets.

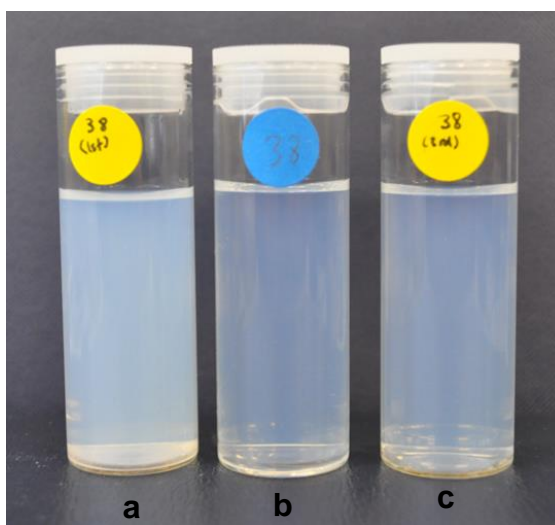


Figure 2.12: Nanoemulsion samples formed from three 'identical' concentrate samples (three samples having the same composition). Each nanoemulsion has a different final droplet diameter; a) 94 nm, b) 45 nm and c) 57 nm.

The droplet sizes obtained throughout the red region were considered with respect to composition to determine if it varied systematically with for example oil or surfactant concentration. No trends could be determined.

According to Wang et al. (2007), when the initial concentrated emulsions are located within the bicontinuous or O/W microemulsions regions, the oil phase is completely dispersed (or solubilised) throughout the water phase due to sufficient surfactant being present. When these concentrated emulsions are diluted in excess water, the surfactant concentration is suddenly decreased, which also unavoidably decreases the oil solubilisation. The system is then supersaturated with oil which may lead to homogenous nucleation of oil, thus producing small monodisperse droplets [30].

2.3.3 Effect of Oil Content on Droplet Size

The droplet sizes of the final emulsions as a function of oil content at different surfactant phase concentration are shown in the Figure 2.13. The general trend for all surfactant concentrations is an increase in droplet size as oil content increases. At the same time, the data show that smaller droplets form at higher surfactant content, even when the oil content is increased, demonstrating that the addition of surfactant allows for stabilisation of smaller droplets. As it is widely known, the role of surfactant or emulsifier during emulsification is to absorb at the oil/water surface and lower the surface tension, which then causes the deformation of oil droplets [31]. This leads to a decrease in droplet size, which has been verified here using the DLS measurement. The surfactant stabilizes the emulsion system preventing the dispersed phase from coalescing into a macroscopic phase[9]. When smaller droplets are formed for a given oil content there is a higher number of droplets and a larger surface area. This higher surface area can be stabilised only at higher concentrations of surfactant.

Based on the collective data reported above an appropriate nanoemulsion region had been identified. The next step required was to investigate loading nanoemulsions within this region with CPPU.

2.3.4 CPPU Incorporation into the Nanoemulsion Formulation

The main purpose of any agrochemical formulation is to make handling and application of the active ingredients as straightforward as possible for the user, more effective delivery and advantage to the plants and be non-polluting to the environment. The use of emulsions for agriculture purposes (pesticide formulation) has been studied for several decades [32]. As the technology has evolved, nanoemulsions have been shown to be an ideal option for optimizing delivery of water-insoluble active agents in agrochemicals[3]. The size of the CPPU-loaded oil droplets of the nanoemulsions should permit them to deposit uniformly on the plant leaves[3]. The low surface tension of the system and the low interfacial tension of the O/W droplets would then enhance wetting,

spreading and penetration of the nano-sized droplets into the plant leaves and therefore aid CPPU delivery.

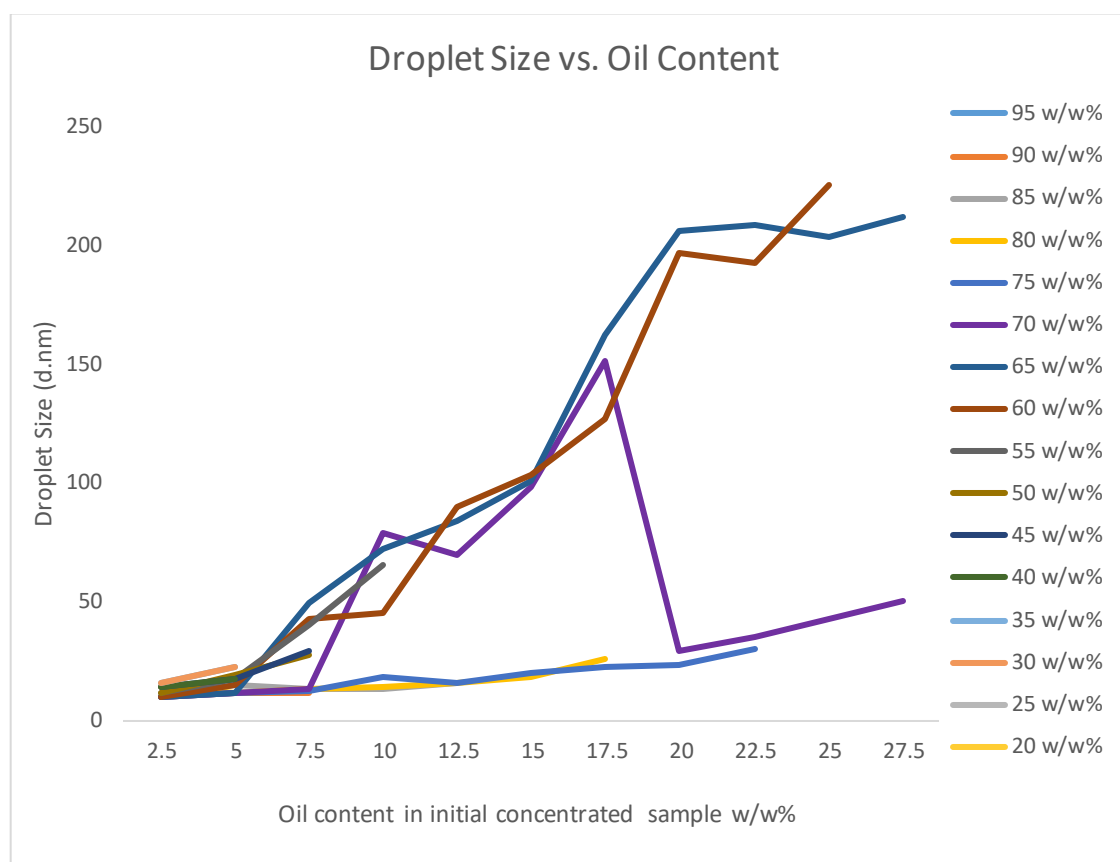


Figure 2.13: Droplet size of crash-diluted emulsions vs. the oil content present in the initial concentrates.

The nanoemulsion region of the water/polysorbate 80/soybean oil/glycerol system depicted as the red region in Figure 2.10 is therefore expected to be the optimum formulation zone. Hence this region was the region of focus for incorporating the active material, CPPU, into the original concentrate in order to fabricate CPPU-loaded nanoemulsions. As shown above any concentrate from within this region will produce nanoemulsions following crash dilution. The concentrate with 10 wt% of soybean oil, 60 wt% of surfactant and co-surfactant and 30 wt% of water (sitting along line *d* in the red region) was chosen to exemplify CPPU-loaded nanoemulsion preparation. However the entire region was explored in this thesis work. CPPU was dissolved in the soybean oil prior to formation of the concentrate. The droplet size was evaluated in the same way as discussed above.

This CPPU-loaded nanoformulation was used to prepare all CPPU-loaded nanoemulsions which were then used in the biological studies discussed in Chapter 3. In particular, with the focus being to understand and explore their capacity to enhance plant growth using senescence delay as the indicator.

Figure 2.14 shows a schematic representation of the dilution process to produce CPPU-loaded nanoemulsions. After the crash-dilution process (yielding samples with concentrate to water volume fraction, $\Phi = 0.03$), nano size droplets were achieved. Further dilution was then undertaken to obtain the appropriate final concentration of CPPU in the final CPPU-loaded nanoemulsions before it could be used in direct application to plants in the biological studies. It is noted that the bluish colour evident in the initially formed CPPU-loaded nanoemulsions is not always visible when more water is added into the system. However the droplet size is not altered upon this second dilution [8]. This loss of blue colour occurs because the distance between each droplet is increased which changes the interaction with the incident light causing loss of the bluish coloration.

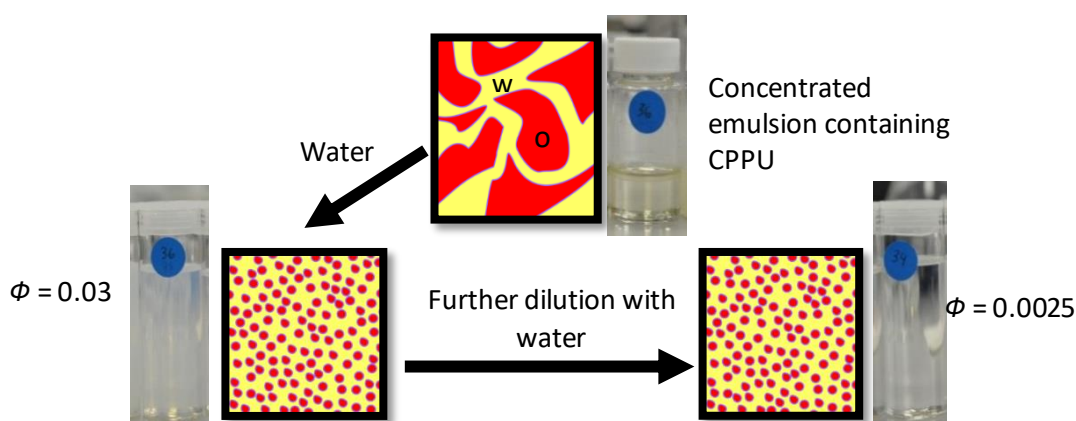


Figure 2.14: Dilution process of CPPU-loaded nanoemulsions. Concentrated emulsions containing CPPU were subjected to the crash-dilution process (volume fraction of concentrate to water, $\Phi = 0.03$) and then further dilution (volume fraction of second dilution $\Phi = 0.0025$) is done to achieve much lower CPPU and oil concentrations for plant application.

Visibly, throughout the entire process, no difference was observed when CPPU was predissolved in the soybean oil as compared with when no CPPU is present.

2.3.5 Stability of Nanoemulsions and CPPU-loaded Nanoemulsions

Stability is always an issue in emulsion studies. Nanoemulsions that contain very small droplets maintain their stability against phase separation due to the Brownian motion and relatively high diffusion rates and movement of the droplets bombarding into each other and other molecules (surfactant etc.) and high repulsive barriers between the droplets. From visual observation, the final nanoemulsions produced in this study, after the crash-dilution process, are very stable. There was no visible phase separation for samples stored for over a year.

Droplet sizes of the prepared nanoemulsions were measured over a period of 3 days (appropriate time scale for application of the solution to the plants (the concentrate was stable for months, as would be required in a commercial product with the user undertaking the final dilution just prior to use) and the results are shown in Figure 2.15. Two different concentrates from the red region are shown as examples. All samples showed no variation in droplet size over a three day period. The size distributions were almost identical with and without CPPU (3 ppm). Little change in droplet size was observed irrespective of how much CPPU was present in the range of CPPU concentrations used for the biological investigations. Further dilution of these samples is needed to be able to use the nanoemulsion in the biological studies. As mentioned previously, further dilution does not alter the droplet sizes. The diluted CPPU-loaded nanoemulsions also behave in the same way, remaining unchanged over the period of three days (data not shown, essentially the same as those shown in Figure 2.15). This remarkable behaviour of nanoemulsions produced by this system gives a good opportunity for the formulation to be used in many applications that need it to be kept for a longer time.

Nanoemulsions have received considerable attention in the past few years for their possible applications, spanning from within the food to pharmaceutical industries[33][34]. The potential of using nanopesticides for crop protection[35] has also been considered in the agricultural industry. Research on nanopesticides has been rapidly developed over the last two years, evident by

the increase in the number of articles published on the topic[35]. Most of the intentions for this research appear to be to increase the water solubility of active ingredients (mostly essential oils) and reduce the oil droplet size, thus producing a better delivery to the target organisms/plants. For example, Anjali et al. (2012)[36] have successfully produced a nanosized neem oil emulsion and because of its small droplet size, it was found to be an effective larvicidal agent.

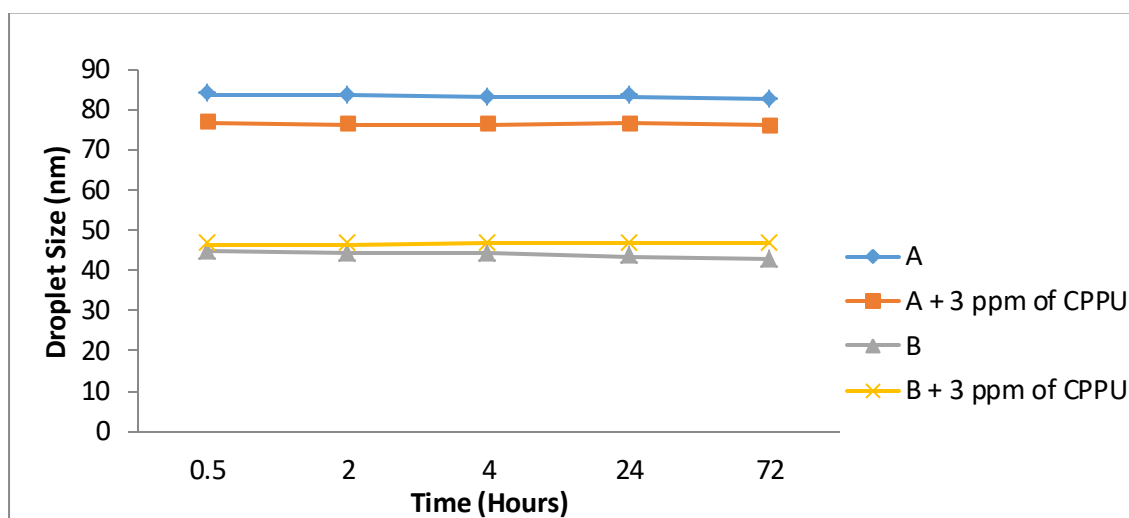


Figure 2.15: Droplet sizes obtained from post crash- dilution nanoemulsions (concentrate to water ratio: $\Phi = 0.03$) for two different droplet sized nanoemulsions, A and B, with and without CPPU incorporation. Sample A comprised of 7.5 wt % of oil/65 wt % of surfactant phase/27.5 wt % of water, while sample B is 10 wt % of oil/60 wt % of surfactant phase/30 wt % of water.

This research study on CPPU-loaded nanoemulsion, is also looking for the same response as that found in the pesticide and drug delivery studies: to produce small sized and stable nanoemulsions for better delivery to the target organisms. What makes this research different is that the active ingredient used was synthetic plant hormones, and the target organism is plants. To the best of our knowledge, this is the first study using synthetic plant hormone as the active ingredient, embedded into the core of an oil nanodroplet system. The process used to produce the nanoemulsions was a low energy emulsification process which is a widely studied technique to obtain a minimum size nanoemulsion [37]. It has been shown here that the technique is capable of producing stable nanosized droplets, with or without CPPU. This technique, which falls under the

study of phase inversion emulsification continues to receive great attention due to its ability to control the size of the dispersed phase and the stability of the final products.

2.4 SUMMARY

From the data presented, nanoemulsions, with droplet sizes in the range of 30 – 100 nm, were successfully produced using the polysorbate 80+glycerol/soybean oil/water system and applying the crash-dilution process. The method used in building the nano-solution formulation was a low energy emulsification process, no high energy equipment was required at any stage in the process, making it fit for practical applications. A key factor for the formation of nanoemulsions is that the initial concentrate must be a bicontinuous or O/W microemulsion. Such a region was found to exist within the polysorbate 80+glycerol/soybean oil/water phase diagram. Once the nanoemulsion was produced, further dilution was found to not change the droplet size and characteristics of the nanoemulsion. This indicates that this system and method of formulation provides good flexibility for use in various agriculture applications that need different concentrations of the formulation. Furthermore, different sized droplets could be formed which allows for fine tuning, in some cases, or robustness and ease of use in others.

CPPU was successfully incorporated into the nanoemulsion system, without substantially changing the droplet size or its stability, thus making it possible to proceed to the next focus of the experimentation: biological studies. Here, the potential of the CPPU-loaded nanoemulsions as a good carrier to deliver CPPU effectively to the sample plant will be investigated. The high stability of the nanoemulsion alone and the CPPU-loaded formulation produced later, allow the nano-formulation to be kept for a long time. These characteristics make the new CPPU-loaded nanoemulsion a promising methodology from both environmental and economical points of view.

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CHAPTER 3

BIOLOGICAL ACTIVITY OF CPPU-LOADED NANOEMULSIONS

As discussed in Chapter 1, water-based formulations have been shown to be an efficient method for delivery of plant growth regulating compounds to crops and have been widely used for decades. In this study, nanoemulsions, which are water-based formulations, were developed as a delivery medium for use in a plant-contact method in order to preferentially enhance delivery of CPPU, as a proof-of-concept active agent, and thereby increase its effectiveness in promoting plant growth. An additional benefit of this delivery medium would be if it resulted in reduced amounts of CPPU needing to be used to produce the same results in the field.

To test the efficacy of the CPPU-loaded nanoemulsion formulations, a senescence bioassay was used to determine if an active delivery system had been formulated. Measurement of leaf chlorophyll content is a key technique in indicating the physiological status of a plant[1]. Chlorophyll plays a vital part in photosynthesis and declines during senescence. Loss of chlorophyll from aging leaves can be prevented by treating leaves with cytokinins. Treating the leaves with CPPU, a synthetic cytokinin-like plant hormone, should produce the same results as natural cytokinins. Using the experimental protocol described by Bidwell (1975)[2], the effect of CPPU and CPPU-loaded nanoemulsions on leaf senescence was investigated by treating the primary leaves of young seedlings with nanoemulsions loaded with and without this synthetic plant hormone.

The nanoemulsions containing CPPU were directly applied as a solution to the leaves of plants. The change in the leaves after application of the CPPU-loaded nanoemulsions was examined using the delay of leaf senescence, as measured by the amount of chlorophyll in the treated leaves relative to control leaves, as the bioassay.

3.1 MATERIALS AND METHODS

3.1.1 Growing the Model Plant – Dwarf Beans

The plants used in all CPPU investigations were *Phaseolus vulgaris* L. from the Fabaceae family, commonly known as garden bean. There are various types of beans classified as *P. vulgaris* which come in different seed sizes and colours such as red kidney beans, black beans and green beans. Dwarf beans of *P. vulgaris* cv. Long John produced by Yates Australia, were purchased from a local gardening shop. *P. vulgaris* cv. Long John was selected because of the shape and size of the leaves which are bigger compared with the other types of beans, thus giving more surface area for the treatment procedures with the various formulations investigated here.

Initially, the seeds were planted and grown in vermiculite¹⁵ for two or more weeks. The germination period was between 7-10 days. The beans then required a few extra days to grow to the height and size required to conduct the experiments. Extra water was supplied to the seeds on day one. After 2-3 days, the beans were re-watered. Daily watering was not required and in fact could lead to the seeds going mouldy. However, too infrequent watering led to death. In the initial small scale experiments, all plants were grown in the laboratory with the optimum germination temperature being between 18-25°C. Plants were ready to be used when the primary leaves had extended and the first trifoliate¹⁶

¹⁵ Vermiculite is a growing media for plant propagation. It is a naturally occurring mineral that has been heated and expanded, which makes it able to hold a high capacity of water and has great aeration properties.

¹⁶ Plants with three leaflets.

had appeared. At this stage of growth, the seedlings were cut at soil level and the cotyledons¹⁷ removed from the seedlings. The explants were then immediately placed in jars containing ~100 mL of water. Figure 3.1 shows the different stages of growth of the beans.

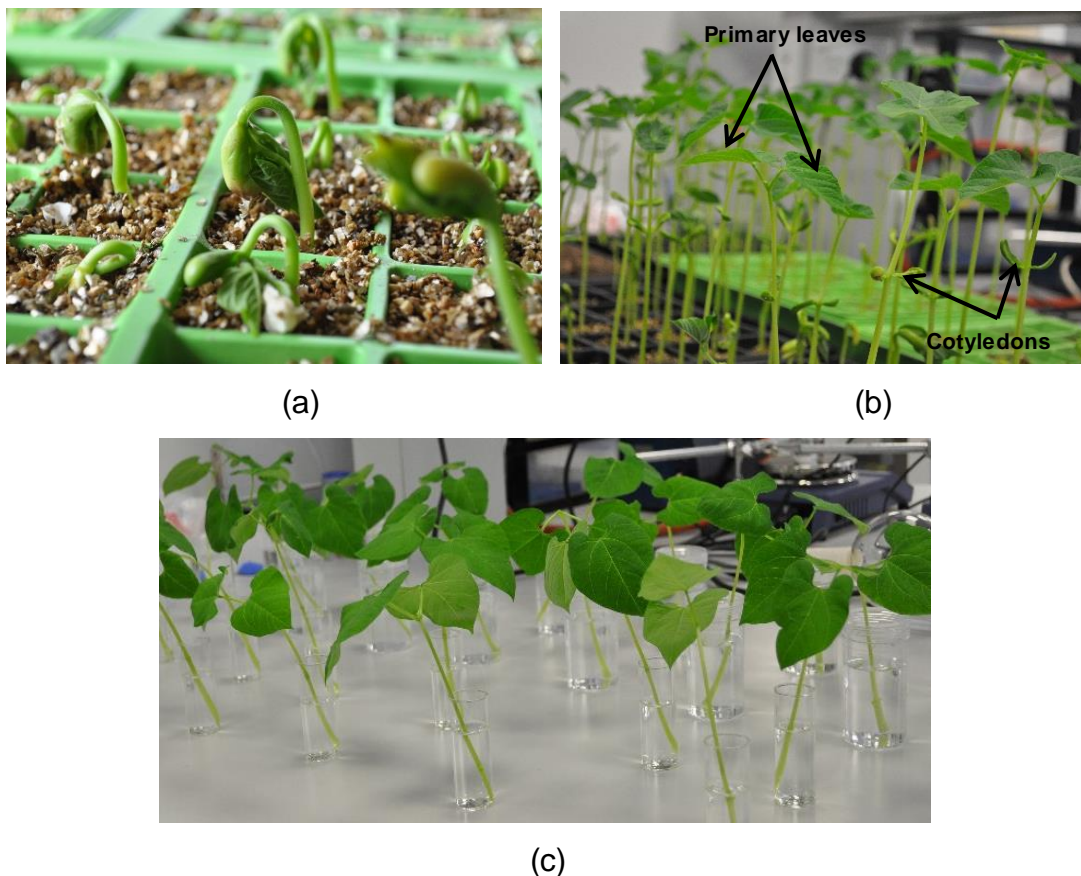


Figure 3.1: Dwarf bean seedlings with (a) the young seedlings starting to emerge from the vermiculite after ~7 days, (b) the primary leaves fully expanded after ~2 weeks, showing also the cotyledons and (c) the seedlings after being cut and placed in a water.

Once the plants had been cut and placed in water (Figure 3.1 (c)) they were ready for the first stage of the experimental treatment. This required painting one of the two primary leaves with one of the various treatment solutions, with the paired primary leaf remaining untreated. Before discussing in further detail the experimental procedures, challenges in obtaining good plants to be used in the experiments are discussed below. Obtaining good plants was critical in

¹⁷ Part of the embryo within the seed of a plant that provides energy and nutrients for the developing seedling.

order to obtain statistically significant data. This was one of the most time consuming and difficult elements of the research.

There are many things that need to be taken into consideration when growing plants for biological studies. Of particular importance for the bioassay was having sufficient numbers of pairs of primary leaves that were fully expanded, wrinkle free and equivalent in size and shape (Figure 3.2). This minimised the variability between replicates when performing the various treatments and analyses and was therefore a critical factor in obtaining reproducible results and statistical confidence in the data obtained for different treatments.



Figure 3.2: Bean explants prepared for the bioassay with matching, fully expanded primary leaves. The photo is close to full size. These are examples of good plants that could be used in the treatment trials.

Getting the ‘perfect’ shape for the primary leaves was quite challenging. In many of the growth trials, problems were encountered at the very first stage: not all of the seeds that were sown germinated or grew properly post germination, a problem that might have been due to low seed viability and seedling vigour, but might also have been due to the low nutrient source from the vermiculite, and/or the glasshouse temperature. Fertiliser was not used because the plants needed to be restricted in nutrients so that the CPPU-treated leaves would act as ‘sinks’, withdrawing nutrients from the paired leaf,

causing it to senesce, while senescence of the CPPU-treated leaf was delayed. This might have taken much longer if the plants had been grown in a high nutrient environment. The large cotyledon is also a store of nutrients for the developing plant, and thus was always removed from the plants.

The considerable variation in germination rates and growth which occurred can clearly be seen in Figure 3.3, where some seeds have not germinated, some seedlings appear unhealthy, some have already lost one of the primary leaves and others have no leaves at all.



Figure 3.3: Variable germination of *Phaseolus vulgaris* cv. Long John seeds. Seeds of the dwarf beans were grown in pots all planted at the same time and treated in an identical manner.

As an example of the issues faced, and in preparation for a large bioassay, plants were grown in the glasshouse at the University of Canterbury, Christchurch, New Zealand during September and October. Approximately, 1200 seeds were planted in unfertilised potting mix. Fewer than half of the seeds produced young seedlings (Figure 3.4). Due to poor seed viability and seedling vigour, only ~10% of the seeds produced plants suitable to be used for any further treatments. Plant development was also delayed, which was likely to have been due to low night temperatures.



a)



b)

Figure 3.4: *Phaseolus vulgaris* cv. Long John plants growing in the greenhouse at the University of Canterbury.

Once germinated, the next biggest problem faced in producing good plants was where one of the primary leaves, and sometimes both, became crinkled and shrunk after approximately two weeks of growth, thus yielding an uneven shape and size of the two primary leaves, as shown in Figure 3.5. These plants could not be used in the treatment experiments and were discarded. With some considerable experimentation, primarily related to the quantity and timing of the water supplied, the occurrence of this problem was reduced but could not be completely eliminated. The reason why the leaves grew in this way was never fully resolved.



(a)



(b)

Figure 3.5: (a) Example of crinkled leaves and (b) uneven primary leaf size.

The next major problem occurred once the plants had been cut, removed from the medium and then placed in the water, at which point plants suddenly

withered (Figure 3.6). For some of these plants, after being left in water for 24 hours, the stem became firm again and the plants revived. However, the remainder continued to wither and finally died. Again, there was no straightforward explanation or easy-fix for this response of some of the plants.

Taking into consideration all of these issues and after much trial and error, it was routinely possible to get at least a 50% yield of 'perfect' plants from each planting for use in the bioassay. Batches of seeds were continuously sown to ensure that there were sufficient numbers of plant samples for all the experiments with sufficient replication and also to repeat the experiments, thereby allowing statistically sound experimental data to be obtained.



Figure 3.6: Examples of plants that suddenly withered after being cut, removed from the growth medium and placed in water.

3.1.2 Experimental Design: Bioassay

3.1.2.1 *Leaf senescence*

Following the experimental protocol described by Bidwell (1975)[2], after growing the bean seedlings, cutting them at soil level and then placing them in a jar containing water, the next step was to remove the first trifoliate leaf and the cotyledons, so that only the two primary leaves remained. The adaxial surface of one of the primary leaves was then painted with various treatment solutions. A small cotton bud was first soaked with the treatment solutions for a

few seconds or until it fully absorbed the solution. The wet cotton bud was then gently rubbed over the entire adaxial surface of the leaf. The painting process was undertaken with care to prevent the leaf cuticles¹⁸ from being damaged during the process as the cuticle helps keep the leaf from drying out and protects it from bacteria, insects and fungi[3]. The paired primary leaf was left untreated. The bean explants were then kept under very low light intensity (generally in a darkened room or cupboard) to speed up the senescence process and painted again with the appropriate treatment solutions every 3-4 days for two weeks or until the two primary leaves showed a significant colour difference by eye. Figure 3.7 shows the different stages of the experimental procedure.

Any developing trifoliolate and lateral roots were immediately removed in order to inhibit the production of cytokinins, as those parts of the plants produce natural cytokinins. Therefore these must be removed to ensure that the senescence of the primary leaves was only due to the treatment being applied in the study. The plants were observed every 3-4 days for any visual sign of developing senescence of the leaves.

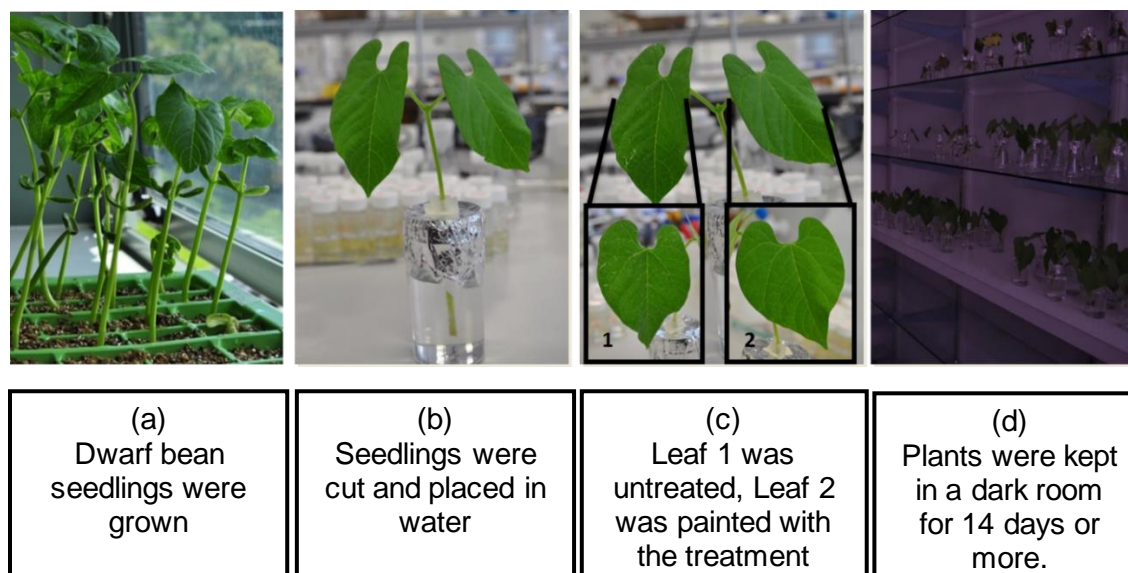


Figure 3.7: Bioassay procedure. The pictures show the sequence of the experimental procedure from (a) to (d). Total time from planting to sampling the leaves for analysis was 4-6 weeks.

¹⁸The waxy layer on the outer surface of the leaf epidermis.

The range of experiments conducted included a series of control tests whereby two components used in the formulation of the nanoemulsions (polysorbate 80 and glycerol) were individually applied to the leaves. These components were diluted to the same concentration as that used in the final nanoemulsions. Those control tests allowed investigation of the response of the plants towards two of the chemical components used in the formulation of the nanoemulsions.

Based on the investigation reported in Chapter 2, the nanoemulsion region was identified within the water/polysorbate 80/glycerol/soybean oil phase diagram. Initial investigations revealed that obtaining an exact size of the soybean oil droplets was not possible, as expected for kinetically stabilised nanoemulsions and that furthermore there was no easily determined trend in droplet size as a function of oil or surfactant concentration. Moreover, there seemed to be, within experimental uncertainty no difference in plant response for different sized droplets within the nanoemulsion region. Hence a single concentrate sample was used for all experiments reported here. The concentrate containing 10 wt% of soybean oil, 60 wt% of polysorbate 80 + glycerol, in a weight ratio of 1:1 and 30 wt% of water was chosen from the nanoemulsion region of the ternary phase diagram was used as being representative of the nanoemulsion region. Nanoemulsion and CPPU-loaded nanoemulsion preparation used in both laboratory-scale and large-scale bioassays was as described in Chapter 2.

3.1.2.2 *Chlorophyll assay*

After the bean leaves were treated with a particular solution for a set period of time, samples were collected for chlorophyll measurement by cutting out four leaf disks of equal leaf area using a hole punch [4], 5 mm diameter, from both the treated and untreated leaves, as shown in Figure 3.8. The leaf disks were weighed, then placed into micro-centrifuge tubes. Dimethylformamide (DMF), equivalent to 20 μ L per mg tissue, was immediately added to the tubes. The tubes were then wrapped in tin foil and incubated at 4°C overnight or longer (until all the chlorophyll was extracted from the leaf disks) followed by centrifugation to produce a pellet of the ground leaf tissue. It should be noted

that the leaf disk samples can also be frozen under liquid nitrogen and stored in a -80°C freezer for some time before being immersed with DMF. However, extracting the samples immediately after being cut from the leaves is preferred as they are still fresh.

The concentration of total chlorophyll was determined using a NanoDrop™ spectrophotometer[5]. The absorbance of the diluted samples was measured at a fixed wavelength, 664 nm for Chl a, and 647 nm for Chl b, using 2 µL samples [5]. Total chlorophyll was calculated using the following equations established by Arnon (1949)[6] and presented as mg/g.

$$\text{Chl a} = (11.65A_{664}) - (2.69A_{647}) \quad \text{Equation 1}$$

$$\text{Chl b} = (20.81A_{647}) - (4.53A_{664}) \quad \text{Equation 2}$$

$$\text{Total Chl (mg/g)} = \text{Chl a} + \text{Chl b} \quad \text{Equation 3}$$

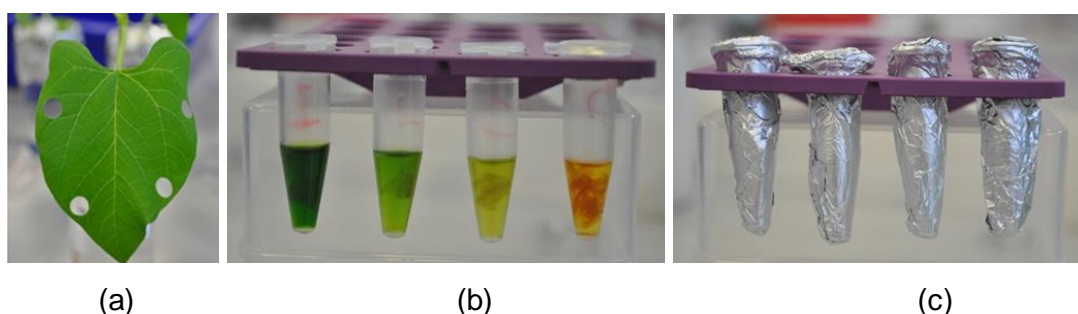


Figure 3.8: Sampling method for the chlorophyll bioassay. (a) Sample material was collected by removing four leaf disks with a hole punch, these were then weighed; (b) the sample disks were immersed in DMF, volume based on total leaf mass; and (c) samples were wrapped in tin foil and incubated at 4°C overnight.

3.1.3 Laboratory-Scale Plant Tests

Preliminary lab-scale studies were performed for all treatment solutions. These small scale tests were run in order to determine all the appropriate control conditions and to determine the optimum CPPU concentration for the CPPU-loaded nanoemulsions that produced the most effective results in delaying the senescence process. The results from these experiments were used to inform which treatments would be investigated in the larger scale plant experiments.

All plants were grown directly in the laboratory for the laboratory-scale studies. A maximum of three explants was used for each treatment.

3.1.3.1 Test 1: Reference test - Individual chemical components of the nanoemulsion

Glycerol and polysorbate 80, used to construct the nanoemulsions, were applied to the explants to determine if they were toxic to the plants. Each of the chemicals was diluted to the concentration expected to be used to produce nanoemulsions, which was ~0.075 w/w%, noting that the glycerol:polysorbate 80 ratio was always maintained at 1:1. The difference in colour of the leaves after being treated with each of these two chemicals over a two-week period was determined by visual examination.

3.1.3.2 Test 2: CPPU-loaded nanoemulsions vs. CPPU and nanoemulsion alone

As discussed in Chapter 1, based on the literature, 5 to 10 ppm of CPPU is the concentration most frequently applied to kiwifruit, grapes and berries. To assess the appropriateness of the leaf senescence bioassay, 3 ppm of CPPU solution was initially tested. A CPPU solution was made by dissolving the CPPU powder in ethanol to make a stock solution of CPPU. An aliquot of the stock solution was taken and diluted with distilled water to 3 ppm. This was the reference concentration for all other experiments. Polysorbate 80 was then added at 0.1% (v/v) concentration to yield the working solution. The second test solution used was nanoemulsion only and the third treatment solution was the CPPU-loaded nanoemulsion with a final CPPU concentration of 3 ppm. These three tests are summarised in Figure 3.9.

Four leaf disks for chlorophyll analysis were collected from each of the pairs of primary leaves, on the first day of the experiment (Day 0), counted as Time 1 (T1), which was prior to the 'painted leaf' receiving the treatment. The chlorophyll bioassay was immediately run on these leaf samples to yield the

base chlorophyll amount in the fresh leaves prior to application of any treatment solutions. The different treatment solutions were then applied to the 'painted leaf'. On Day 14 (T2), four sample disks were again collected from the leaves and the chlorophyll analysis run. The difference in the amount of total chlorophyll between Day 0 and Day 14 allowed the effect of the treatments or plant senescence to be determined.

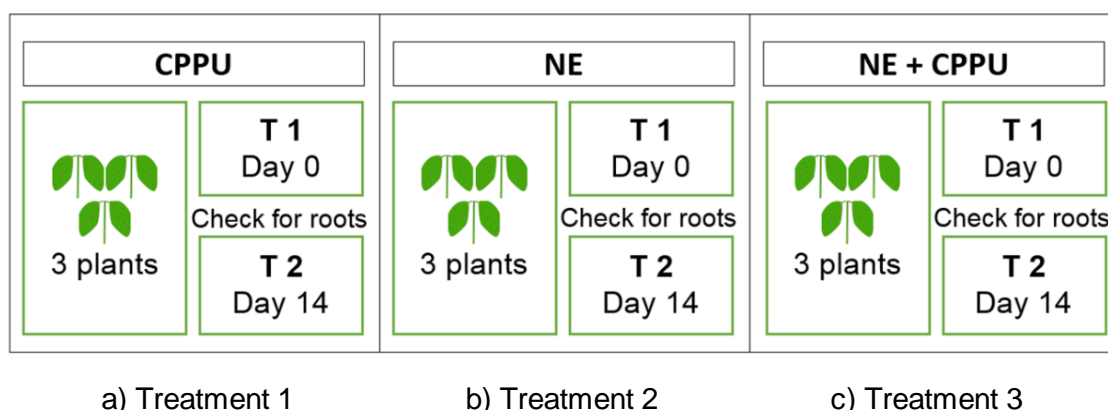
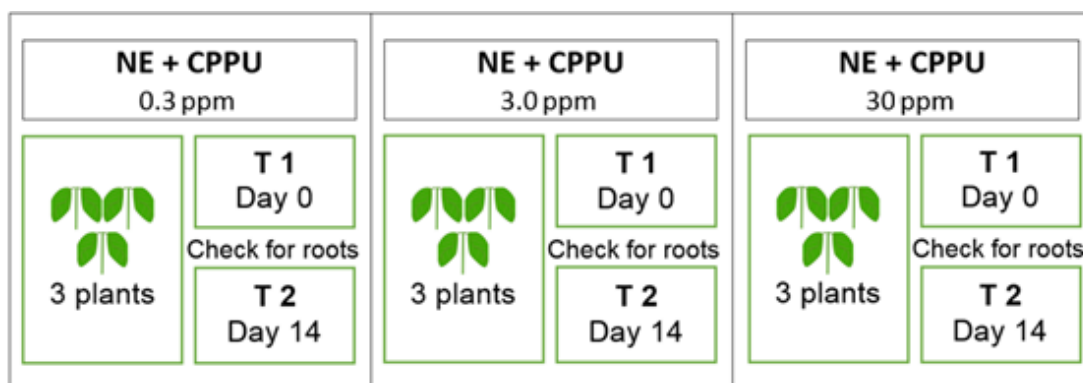


Figure 3.9: Bioassay design for the laboratory scale tests. Treatments 1 and 2 were CPPU and nanoemulsion (NE) alone; Treatment 3 used the CPPU-loaded nanoemulsion. In each case the treatment solutions were applied to one of the primary leaves on each of three plants at the equivalent of 3 ppm CPPU.

3.1.3.3 Test 3: CPPU-loaded nanoemulsions with varying CPPU concentrations

The next series of small-scale laboratory experiments were based on studying the effect of different CPPU concentrations in the nanoemulsion formulations on the final chlorophyll amount between the two primary leaves. The same procedures as outlined in 3.1.3.2 were repeated with two additional CPPU concentrations; 0.3 ppm and 30.0 ppm (Figure 3.10).



a) Concentration 1

b) Concentration 2

c) Concentration 3

Figure 3.10: Bioassay design to test the nanoemulsions (NE) loaded with three different concentrations of CPPU.

3.1.4 Large-Scale Plant Experiments

Bioassays were also undertaken using more replications per treatment for those CPPU concentrations that showed statistical significance in the laboratory-scale experiments. The experiments were designed to assess the potential of the CPPU-loaded nanoemulsion formulations in maintaining their effects on the explants, and to enhance confidence in these findings due to the increased replication.

3.1.4.1 Test 1: CPPU-loaded nanoemulsions vs. CPPU and nanoemulsion alone

Plant production on a large scale was undertaken in the Treehouse greenhouse facilities at the Botanical Gardens in Wellington (Figure 3.11) during January until April. The greenhouse was able to maintain its temperature at 21-24°C throughout the entire experimentation time. This constant temperature, with the help of consistent external warm weather and good quality seeds meant that almost 50% of the seeds planted in this greenhouse were routinely harvested and produced good quality explants. This significantly shortened the total experimental time to obtained usable plants and therefore reliable data. The growth medium used was unfertilised potting mix consisting basically of bark, pumice and coir fibre. Note the stark difference between these plants (Figure

3.11): number germinated and quality of primary leaves compared with those shown in Figures 3.3 and 3.4.



Figure 3.11: Dwarf bean plants grown in the greenhouse at the Botanical Gardens in Wellington.

A change was introduced to these large-scale plant trials based on the number of times the treatment was applied. Here, the treatment solution was applied only once, on T1 (Day 0). The two main reasons for this change were 1) to replicate treatment on farm and 2) the time required to apply the treatment to so many plants would have been significant. The method of applying the treatment solutions to the explants remained the same as for the laboratory-scale trials (Section 3.1.3): the primary leaves were manually painted using a cotton bud that was dipped in the treatment solutions until the solution was evenly distributed on the surface of the leaf. The explants were then moved to the dark room and observed for 14 days. The chlorophyll analysis was performed as described previously (Section 3.1.2.2).

Figure 3.12 shows the experimental design for the large-scale experimentation. Here 12 explants were used for each treatment with water, CPPU solution (3 ppm), nanoemulsion solution and CPPU-loaded nanoemulsion (3 ppm).

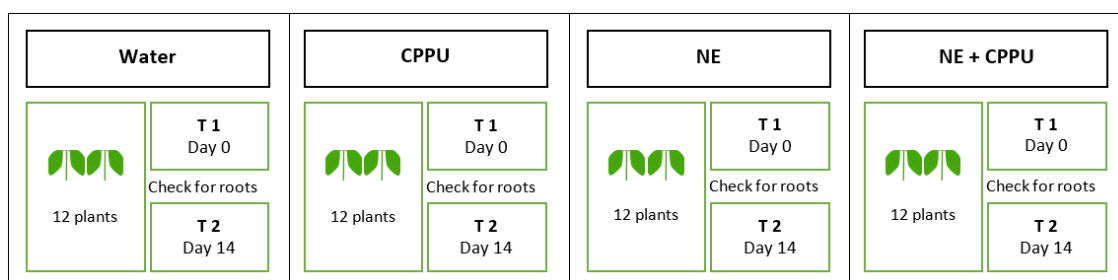















Figure 3.12: Bioassay design for the large-scale tests. Treatments were water, CPPU, nanoemulsion (NE) alone and CPPU-loaded nanoemulsion. In each case the treatment solutions were applied to one of the primary leaves on each of 12 plants at the equivalent of 3 ppm CPPU.

3.1.4.2 Test 2: CPPU-loaded nanoemulsions with varying CPPU concentrations

One way of investigating the effectiveness of CPPU-loaded nanoemulsions is to determine if less CPPU could be used within the nanoemulsion with the same effect as from a greater concentration when applied in normal solution. Here, concentrations lower than the standard 3 ppm of CPPU were applied to the sample plants. Explants treated with just water were used as the control (Figure 3.13). The CPPU concentrations used for both the CPPU-loaded nanoemulsions and the CPPU solution, were 1 ppm, 0.3 ppm, 0.1 ppm, 0.03 ppm, 0.001 ppm, 0.003 ppm (Figure 3.13). All plants were examined for any developing lateral roots between times T1 and T2. Any roots were removed as described previously. The number of samples used was 12 plants for each treatment described in Figure 3.13.

Water											
 12 plants		T1 Day 0									
		Check for roots									
		T2 Day 14									
CPPU 1.0 ppm		CPPU 0.3 ppm		CPPU 0.1 ppm		CPPU 0.03ppm		CPPU 0.01 ppm		CPPU 0.003 ppm	
	T1 Day 0		T1 Day 0		T1 Day 0		T1 Day 0		T1 Day 0		T1 Day 0
	Check for roots		Check for roots		Check for roots		Check for roots		Check for roots		Check for roots
	T2 Day 14		T2 Day 14		T2 Day 14		T2 Day 14		T2 Day 14		T2 Day 14
NE + CPPU 1.0 ppm		NE + CPPU 0.3 ppm		NE + CPPU 0.1 ppm		NE + CPPU 0.03ppm		NE + CPPU 0.01 ppm		NE + CPPU 0.003 ppm	
	T1 Day 0		T1 Day 0		T1 Day 0		T1 Day 0		T1 Day 0		T1 Day 0
	Check for roots		Check for roots		Check for roots		Check for roots		Check for roots		Check for roots
	T2 Day 14		T2 Day 14		T2 Day 14		T2 Day 14		T2 Day 14		T2 Day 14

treated leaves, with a level of significance of 0.05. The two means are significantly different if the calculated p value is <0.05 ; i.e. the differences are statistically significantly different to the 95% limit. A Three-way mixed ANOVA was run to understand the effect of different treatments, concentrations and two primary leaves on the percentage of total chlorophyll loss. At each concentration, A Two-way mixed ANOVA was also used to understand if there is an interaction between different treatments and the (two) primary leaves on the percentage of total chlorophyll loss.

3.2 RESULTS AND DISCUSSION

The biological assay used to assess the nanoemulsions was based on leaf senescence and chlorophyll content. During leaf senescence, many of the components in the leaf are degraded and recycled to other parts of the plant. The leaf changes colour as the chlorophyll is degraded [7][8]. By treating the primary leaves of young seedlings with CPPU, it was possible to delay chlorophyll loss and prolong the life of the leaves. Moreover, when senescence is delayed in the primary leaf treated with cytokinin, that leaf can become metabolically active and act as a strong sink attracting nutrients and hastening senescence of the paired leaf, as shown by Mothes and Engelbrecht (1963)[9]. According to Mothes and Engelbrecht (1961, 1963)[10], if kinetin, a type of cytokinin, is sprayed on a limited area of an excised mature leaf, numerous substances such as amino acids and other metabolites in the untreated parts will migrate to this 'kinetin-locus'. This directed migration ('active transport') leads to an accumulation of metabolites, which results in increased metabolic activity at the sprayed site, including promoting the synthesis of nucleic acids and proteins. This activity causes the kinetin-treated tissue to behave physiologically like a young tissue[10]. This response was used as a bioassay to explore if a CPPU-loaded nanoemulsion was, firstly, releasing the CPPU and, secondly, was more effective than a traditional water-based CPPU application.

3.2.1 Laboratory Scale Tests

3.2.1.1 *Test 1: Reference Test - Individual chemical components of the nanoemulsion*

All the chemical components used in the nanoemulsion formulation are common substances used in many pharmaceutical studies of drug delivery systems[11]. Each is safe for use in the human body and regarded as environmentally benign[11]. Despite this, it was crucial to specifically investigate the response of the plant towards the chemical components (particularly the glycerol and polysorbate 80), in order to be able to understand and analyse the data from the nanoemulsion formulations, and to determine that the components were not toxic to the plant. Plant response to soybean oil was not tested as the oil itself is immiscible in water and coating only oil on the surface of the leaves may block the stomata which would then reduce the transpiration rate[12] and may result in data error. A study performed by Baudoin et al. (2006)[13] showed that application of 1.5% of oil (by volume in water) to the leaves of grape cultivars could decrease photosynthesis in the leaves. However, the cultivars differed in their response to oil application, potentially due to differences in the amount of oil retained by the leaves. However, in this study, the final concentration of the soybean oil in the applied nanoemulsion is very low ~0.025 w/w% and was considered unlikely to be an issue.

The leaf senescence bioassay offers a simple test for direct observation/measurement of the colour transition in both primary leaves over a two-week period. Figure 3.14 shows pictures of leaves that were treated with the individual components of the nanoemulsions taken on Day 0, 8 and 15. Each of glycerol and polysorbate 80 alone and a mixture of both components were diluted to the same amount as would be used in the diluted nanoemulsions (0.075 w/w%), prior to their application.

The bioassay was based on the findings of Mothes and Engelbrecht (1963)[9] and the experimental design outlined by Bidwell (1975) [2]. In the initial

experiments the development of senescence was defined by visual examination. The colour difference was assessed by noting the colour of the leaves as being dark-green (1), pale-green (2), yellow-green (3), or yellow (4). The visual results indicate that glycerol alone slowed leaf senescence whereas polysorbate 80 increased the rate of senescence. However, a mixture of both chemicals had no visual effect on senescence. As shown in Figure 3.14, by Day 15, the colour of the primary leaf painted with polysorbate 80 had undergone a dramatic change from being dark-green (1) to yellow (4) over most of the leaf. The same result can be seen for its paired leaf. However, senescence was more advanced on the painted leaf. Following treatment with glycerol, the painted leaf maintained its greenness and did not show any obvious colour loss by Day 15. Its paired leaf showed some changes as the colour had gone from dark-green (1) at Day 0 to slowly becoming yellow-green (3) at Day 15. However, and more importantly, was that when the mixture of both these chemicals was applied, there was no visual difference in the leaf colour from Day 0 to Day 15. This then meant that these components could be used to develop the nanoemulsions as described in Chapter 2.



















	Day 0	Day 8	Day 15
Glycerol (0.075 w/w%)			
Untreated leaf			
Treated leaf			
Polysorbate (0.075 w/w%)			
Untreated leaf			
Treated leaf			
Glycerol (0.075 w/w%) + Polysorbate 80 (0.075 w/w%)			
Untreated leaf			
Treated leaf			

Figure 3.14: Primary leaves treated and untreated with each of the individual components of the nanoemulsion and a mixture of the two observed over a period of 15 days.

3.2.1.2 Test 2: CPPU Nanoemulsions vs. CPPU and nanoemulsion alone

The main objective for this research was to successfully produce a formulation for delivering synthetic plant hormone that is more efficient than applying CPPU alone in reducing plant senescence. As evidenced from the results presented above, the combined components of the nanoemulsions were non-damaging to the plant leaves. The next laboratory-based experiment involved testing CPPU in the bioassay, along with the CPPU-loaded nanoemulsion and the nanoemulsion system alone. It was necessary to establish that CPPU on its own delayed senescence in the bioassay since its use in this bioassay has not previously been reported in the literature. The extent to which senescence is delayed can be quantitatively indicated by the amount of total chlorophyll left in the leaf 14 days after treatment.

It should be noted that visual inspection and classification as described and discussed above were completed for all growth trials undertaken but will not be reported further here, the quantitative chlorophyll data rather than qualitative visual data will be presented.

The three different treatments, as summarised in Figure 3.9, and the difference in the amount of total chlorophyll in each of the primary leaves for all explants was determined. It should be noted that due to insufficient explants a true water control was not included in this experiment.

Many different outcomes from the different treatments are possible, three of these possible outcomes are illustrated in Figure 3.15. Picture (a) shows an obvious difference in the degree of senescence exhibited by the untreated and treated leaves which means that the possibility of there being a statistically significant difference in the amount of chlorophyll between the paired leaves is very high. While for (b), any difference in senescence between the leaves is less obviously visible, and there may or may not be a statistically significant difference in the amount of chlorophyll in the two leaves; and for (c), both of the leaves appear to have senesced to the same degree.

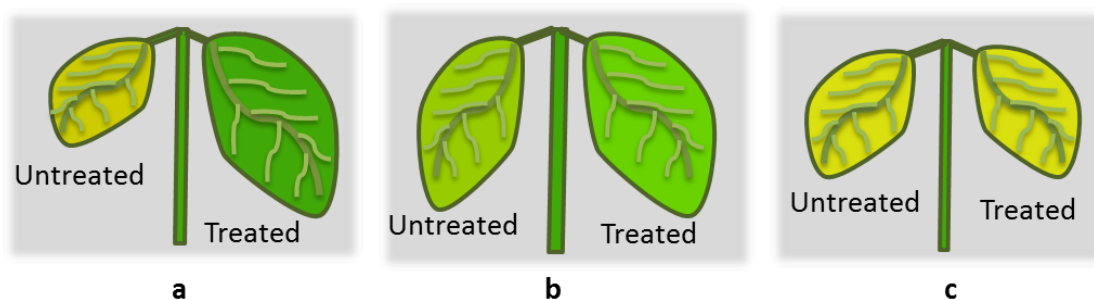


Figure 3.15: Three possible outcomes of the bioassay. The three outcomes show different senescence responses of the treated and untreated leaves.

Figure 3.16 shows one of the plant samples from the laboratory-scale plant trials. Visually, there is an obvious difference between the paired primary leaves, which directly represents the level of senescence that occurred in the leaves. Leaf 2 was painted with CPPU-loaded nanoemulsion and Leaf 1 is the unpainted leaf.

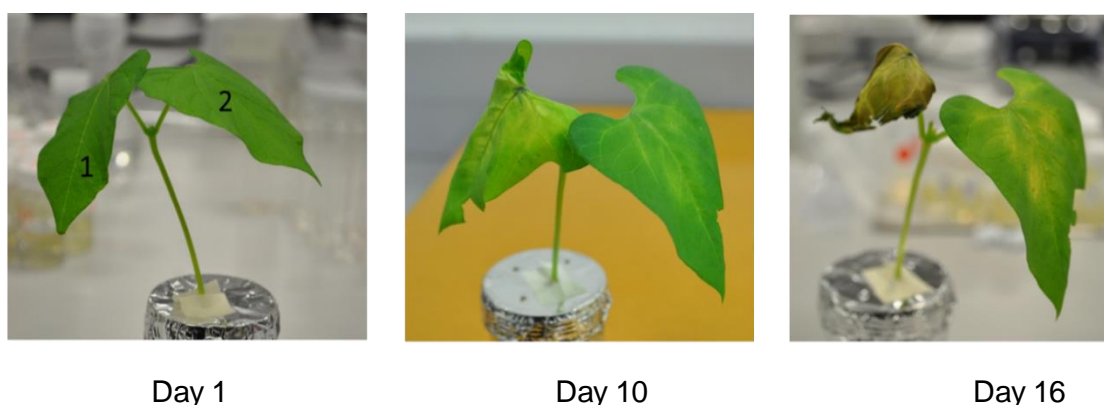


Figure 3.16: Pictures of an explant after being treated with CPPU-loaded nanoemulsion for 16 days. (1: untreated leaf, always on the left; 2: treated leaf).

Figure 3.17 shows the corresponding quantitative chlorophyll assay results. The results are presented as the mean percentage of total chlorophyll loss after 14 days of observation with $n = 3$ plants. The percentage difference between the total chlorophyll loss for the untreated leaf compared with the leaf painted with CPPU was 16%, compared with a 3.0% difference for the pair treated with nanoemulsion alone. However, this difference was not statistically significant. There was a 37% difference between the two primary leaves in the CPPU-loaded nanoemulsion treatments, the greatest difference compared to the other

two treatments. Statistical analysis showed that there is a statistically significant difference ($p < 0.05$) in the total chlorophyll loss (%) between the paired leaves treated with CPPU-loaded nanoemulsion. It should be noted that the small number of plants means that the statistical analysis provides, at this stage, an indicator only, informing the large-scale experiment described below.

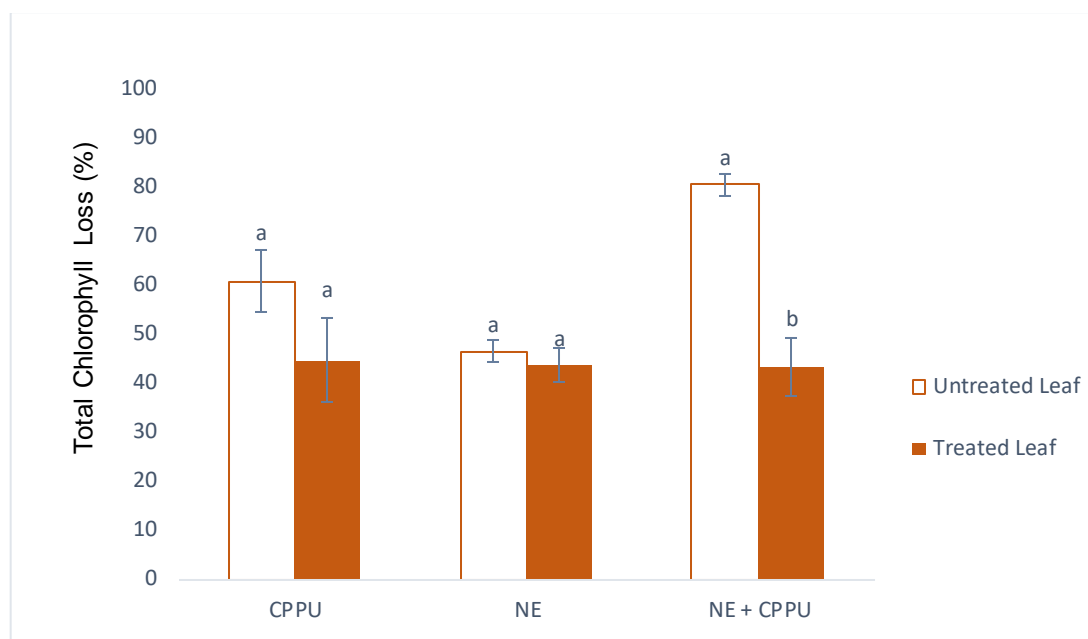


Figure 3.17: Percentage of total chlorophyll loss after 14 days for untreated and treated leaves with CPPU solution (3 ppm), nanoemulsion (NE), and CPPU-loaded nanoemulsion (3 ppm of CPPU), $n = 3$ plants. Bars are mean percentage of total chlorophyll loss \pm SD. Letters that differ within a treatment indicate significant difference between the treated and untreated leaves ($p < 0.05$).

These data show that the CPPU-loaded nanoemulsion caused senescence of the paired leaf, indicating that the nanosize droplets containing CPPU are being effectively absorbed by the painted leaf allowing more effective delivery of CPPU as compared to the standard water-based delivery system. The substantially reduced amount of chlorophyll remaining in the untreated leaf indicates a metabolically very active painted leaf, after being treated with the new nano-formulation.

For treatments with nanoemulsion solution only, the difference in the total chlorophyll loss between the two primary leaves was very small, 3.0%. Ideally the nanoemulsion solution should not have any effect since its role should be

just as a carrier for the active ingredients to be delivered to the plants. The result obtained was therefore as expected, as both of the leaves had essentially the same percentage of chlorophyll loss after two weeks, $46.5 \pm 2.2\%$ and $43.7 \pm 3.6\%$ which was not a statistically significant difference.

3.2.1.3 Test 3: Nanoemulsions loaded with different CPPU concentrations

The data presented above showed that 3 ppm of CPPU in the nanoemulsion delayed leaf senescence to a greater extent than CPPU alone. The data presented below is now based on exploring lower and higher concentrations of CPPU: 0.3 ppm and 30 ppm CPPU-loaded nanoemulsions. Studying these two additional concentrations was undertaken to provide a better indication of the optimum CPPU concentration to be applied to the type of bean under study and also the range of concentrations that have an effect. There is a possibility that the dwarf bean under study will show a senescence delay when less than 3 ppm CPPU is used or will give a much larger response when more than 3 ppm of CPPU is used. However, the key rationale behind this test was that a positive response with a lower concentration of CPPU would be a significant advantage to this new CPPU-loaded nanoemulsion formulation as it would provide evidence that this new formula was effective in delaying leaf senescence with a lower CPPU concentration than the standard water-based delivery solution.

Figure 3.18 shows the effects on plant samples that were treated with 0.3 ppm, 3 ppm and 30 ppm of CPPU nanoemulsions. The leaf that was painted with 0.3 ppm CPPU showed a very marked difference in terms of colour and size compared to its untreated leaf after 14 days. The same extreme difference could also be seen when 30 ppm of CPPU was used, here the colour and size were entirely changed after 14 days. All three cases show that the treated leaf is still fresh and green after 14 days. These positive results provided the opportunity in the experimentation process to explore a much lower concentration of CPPU when working with these bean plants and a very large range of possible delivery concentrations using the nanoemulsion delivery system. In the case of environmental consideration, the lower the amount of

CPPU that could be used to delay leaf senescence, the better it would be for the environment. The application of many agrochemicals to plants has triggered concern by the public, including with regard to the possibilities that agrochemicals may eventually reach the consumer if they are overly used or used at high concentrations by farmers[14]. Maximum Residue Limits (MRLs) allowed for CPPU are in the range of between 0.01 mg/kg and 0.1 mg/kg depending on the crop and the country as discussed in Chapter 1.

A chlorophyll analysis was run to determine the percentage of total chlorophyll loss after two weeks observation. The results are shown as Figure 3.19. As expected, treatment with 30 ppm of CPPU gave the biggest difference in terms of total chlorophyll loss between its treated and untreated leaves (64%), while 3 ppm and 0.3 ppm gave moderate differences, 34% and 31%, respectively. All three treatments gave statistically significant differences for the percentage of chlorophyll loss between untreated and treated leaves with $p < 0.05$. Note that in this second trial the difference for 3 ppm CPPU + nanoemulsion was 34%. In the previous section the reported difference was 37%, showing good reproducibility between separate plant trials.

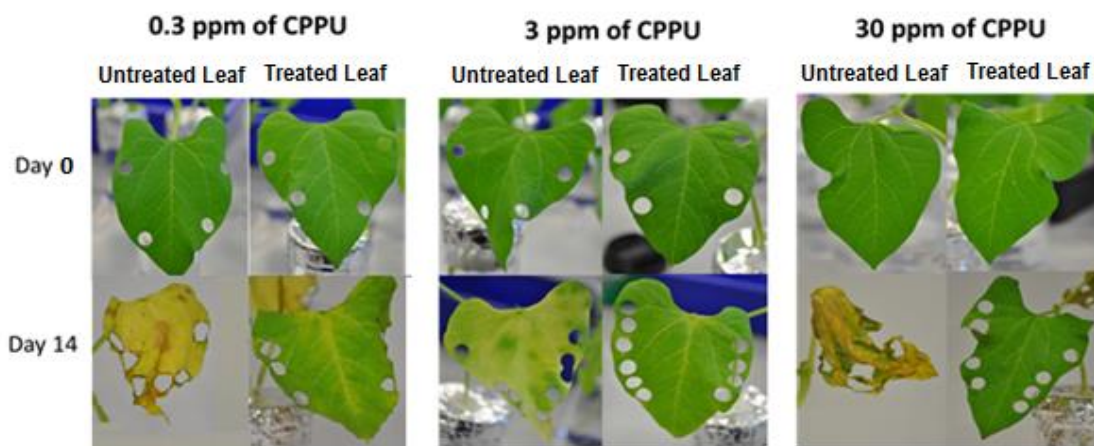


Figure 3.18: Photos of leaves being treated with different concentrations of CPPU-loaded nanoemulsions at Day 0 and Day 14.

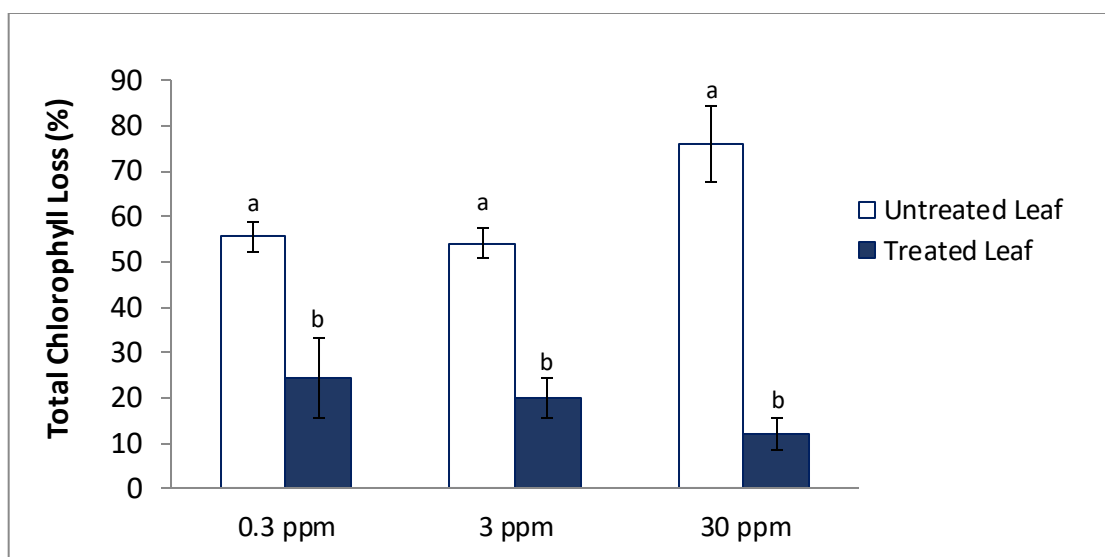


Figure 3.19: Percentage of total chlorophyll loss after 14 days observation for leaves treated with different concentrations of CPPU-loaded nanoemulsions. $n = 3$ plants. Bars are mean percentage of total chlorophyll loss \pm SD. Letters that differ within a treatment indicate significant difference between the treated and untreated leaves ($p < 0.05$).

What is also clearly evident in these results, particularly when comparing the 30 ppm CPPU-loaded nanoemulsion result with the 0.3 ppm CPPU-nanoemulsion result, is the occurrence of source transitioning as has been described above. The senescence process of the leaf paired with that treated with the 30 ppm CPPU-loaded nanoemulsion is significantly more advanced (having lost $76.0 \pm 8.8\%$ of its chlorophyll) when compared with the corresponding untreated leaf for the 0.3 ppm CPPU-loaded nanoemulsion-treated explant ($55.6 \pm 3.4\%$ loss of its chlorophyll). The higher CPPU concentration causes increased metabolic activity, leading to not only the retention of amino acids and other metabolites in the treated leaves, but also the active transport of metabolites out of the untreated leaves to the treated leaves[9].

3.2.2 Large-Scale Plant Experiments

Having obtained statistically significant data for $n = 3$ plants and reproducibility across different small-scale tests, large-scale tests were performed. The large-scale plant experiments were designed so that the solution could be applied to a larger number of explants to enhance the power of the statistical analysis. As

mentioned in the previous sections, growing larger numbers of plants was a significant challenge and a number of issues arose during this process. The number of treatments applied in the large-scale test to the plants was also increased, which therefore required an even larger number of usable explants. Achieving this was a significant challenge.

3.2.2.1 Test 1: CPPU nanoemulsions vs. CPPU solutions and nanoemulsion alone

Tests of CPPU-loaded nanoemulsions, CPPU water-based solutions and nanoemulsions only were carried out using plants grown at the Botanical Gardens in Wellington. A water-only treatment was used as a comparator. Explants treated with distilled water were not expected to show significant differences between the two primary leaves, as water should not affect the rate of senescence or increase the metabolism of the treated leaf. A difference in the total chlorophyll loss between the control and painted leaves was also not expected for treatments with nanoemulsion alone, since the laboratory-scale experiments showed that the combined nanoemulsion ingredients did not promote senescence (Figure 3.17).

In this experiment, the CPPU solution used was 3 ppm and the CPPU-loaded formulation was also 3 ppm of CPPU. 12 plants were used for each treatment. The results showed the expected differences based on the laboratory-scale test. It can be seen that treatment with water and nanoemulsion alone gave no difference between the untreated and treated leaves within experimental uncertainty. For treatments with CPPU solution and CPPU-loaded nanoemulsions when compared with their respective untreated leaves, there is a significant difference in the percentage of chlorophyll loss between the untreated and treated leaves (Figure 3.20).

The total chlorophyll loss between the treated and untreated leaves for treatment with CPPU-loaded nanoemulsion (36%) appeared greater than the treatment with CPPU solution (15%). This means that the explants treated with

the CPPU-loaded nanoemulsion retained a greater amount of chlorophyll in the treated leaf. As described before, kinetin-treated tissues behaved physiologically like young tissue, accumulating amino acids and arresting others[10]. This finding is in agreement with a study by Masclaux et al. (2000) [15] that young leaves behaved as sink leaves as they accumulate free amino acids and efficiently use carbohydrates for building cellular structures and for producing energy. This experiment has shown that it is likely that the CPPU-loaded nanoemulsion has reached the internal tissues of the leaf and triggered the active transport of metabolites to occur, thus delaying the senescence process of the treated leaves.

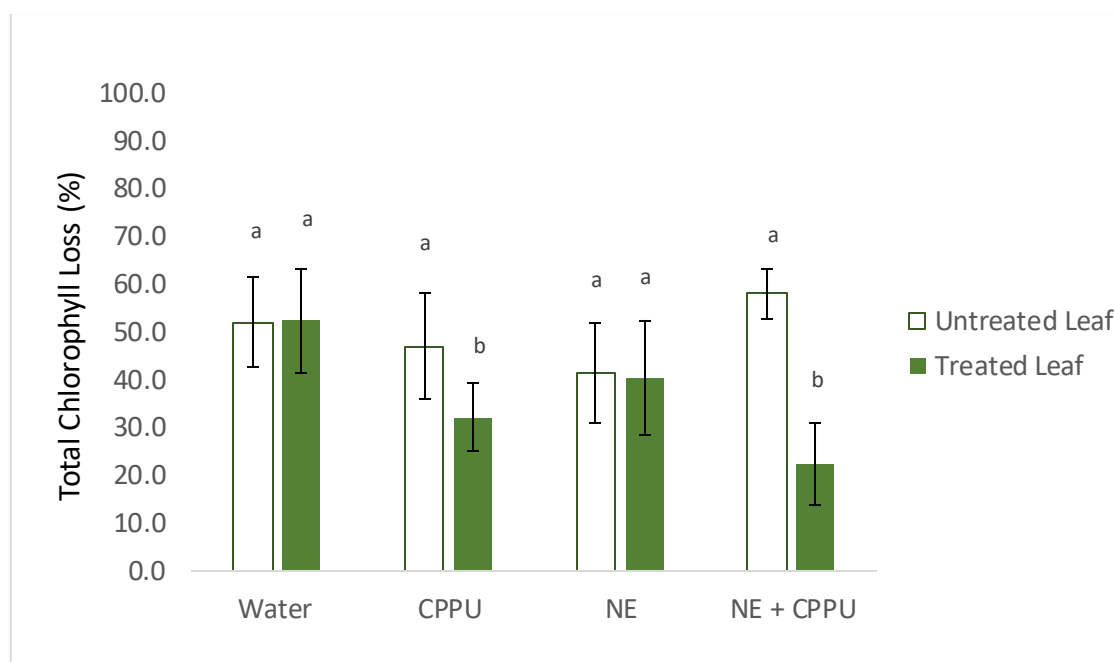


Figure 3.20: Percentage of total chlorophyll loss after 14 days following treatment with water, CPPU solution (3 ppm), nanoemulsion solution and CPPU-loaded nanoemulsion (3 ppm). N = 12 plants. Bars are mean percentage of total chlorophyll loss \pm SD. Letters that differ within a treatment indicate significant difference between the treated and untreated leaves ($p < 0.05$).

3.2.2.2 Test 2: Nanoemulsions loaded with different concentrations of CPPU

The second test performed on a large scale was done to compare the total chlorophyll loss between treated and untreated leaves for nanoemulsions loaded with different CPPU concentrations. From the earlier results discussed in the laboratory-scale tests, it was found that 0.3 ppm of CPPU gave a

significant result with regard to delaying the leaf senescence process (Figure 3.19). For this large-scale test, the nanoemulsions were loaded with a series of much lower CPPU concentrations: 0.1 ppm, 0.03 ppm, 0.01 ppm and 0.003 ppm. The results are shown in Figure 3.21. From the statistical analysis, it was found that 0.1 ppm CPPU gave a statistically significant difference ($p < 0.05$) in the total chlorophyll difference between untreated and painted leaves following treatment with CPPU-loaded nanoemulsions. The lower concentrations gave statistically insignificant differences between the two leaves.

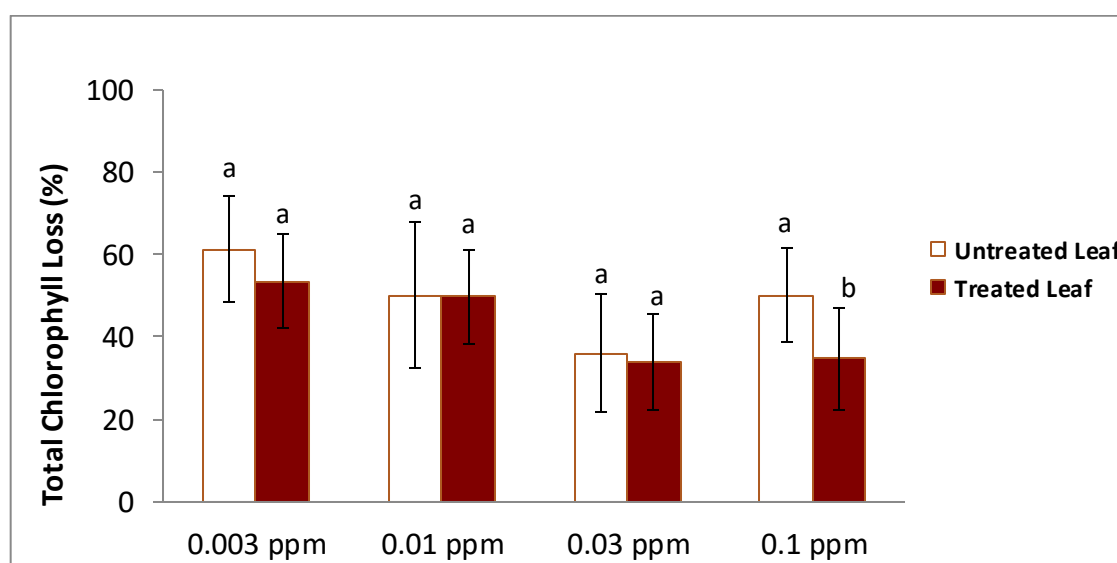


Figure 3.21: Percentage of total chlorophyll loss after 14 days following treatment with different concentrations of CPPU-loaded nanoemulsions, $n = 5$ plants. Bars are mean percentage of total chlorophyll loss \pm SD. Letters that differ within a treatment indicate significant difference between the treated and untreated leaves ($p < 0.05$).

Based on these results, CPPU concentrations from 0.1 to 30 ppm, when combined with the nanoemulsion delivery method effectively delay senescence. We have shown that for 3 ppm CPPU (Figure 3.20) the effect is potentially greater for CPPU-loaded nanoemulsions than CPPU water-based solutions. The final experiment required is to determine whether this difference is maintained as CPPU is decreased to the observed limit of 0.1 ppm CPPU in the CPPU-loaded nanoemulsions that produced a statistically significant response in the previous trial.

Hence CPPU water-based solutions were compared with the equivalent CPPU-loaded nanoemulsions in order to determine if there were any differences in the rate of senescence, caused by the increase or decrease of metabolic activities in the leaves treated with CPPU-loaded formulations at very low concentrations compared to CPPU water-based solutions of the same CPPU concentration. CPPU-loaded nanoemulsion formulations would be considered very effective if they could delay the senescence at a lower concentration than the minimum working concentration of a CPPU water-based solution.

Figure 3.22 shows the results of treatments with different CPPU concentrations. Statistical analysis was performed on each treatment and the results are shown in Table 3.5. Plants treated with CPPU-loaded formulations had statistically significant differences for chlorophyll loss between untreated and painted leaves from 1 ppm down to 0.1 ppm. On the other hand, treatment with CPPU water-based solutions gave a significant difference of % chlorophyll loss for its primary leaves at just 1.0 ppm. The difference was not statistically significant at concentrations less than 1.0 ppm.

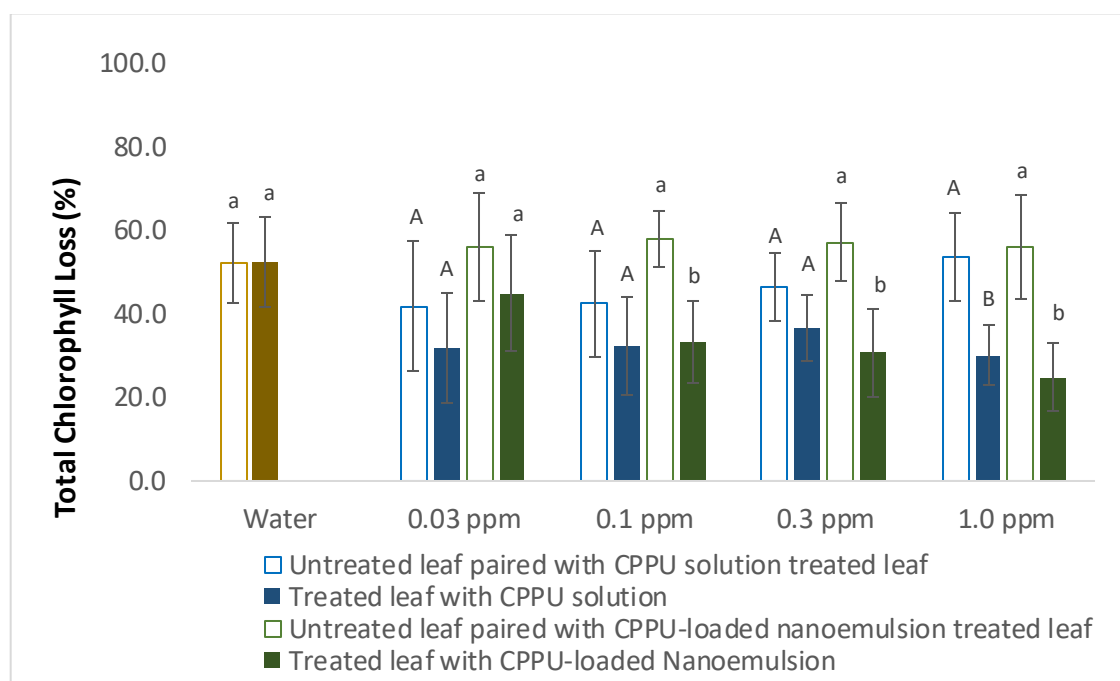


Figure 3.22: Percentage of total chlorophyll loss after 14 days following treatment with different concentrations of CPPU water-based solutions and CPPU-loaded nanoemulsions. $n = 12$ plants. Bars are mean percentage of total chlorophyll loss \pm SD. Letters that differ within a treatment indicate significant difference between the treated and untreated leaves ($p < 0.05$).

As mentioned already, the reduction in senescence of the painted leaf, and enhanced senescence of the paired leaf of explants treated with CPPU-loaded nanoemulsion formulation compared to explants treated with water, nanoemulsion solutions and CPPU solutions was a strong indicator of how effective the formulation was in delaying senescence of the treated leaf. To determine if the CPPU-loaded nanoemulsion formulations decreased the loss of chlorophyll at a lower concentration compared with the CPPU water-based solution, an additional test, a three-way mixed ANOVA was run to understand the effects of different treatments, concentration and treated and untreated leaves (refer to as leaf onwards) on the change in total chlorophyll loss for Figure 3.22. The change in chlorophyll was normally distributed, as assessed by Shapiro-Wilk's test ($p > 0.05$). There was homogeneity of variances for both painted and unpainted leaves, as assessed by Levene's test for equality of variances. The three-way interaction between leaf, treatment and concentration was trending towards statistical significance at the 5% significance level. The expected change in the total chlorophyll loss differs depending on the combination of treatment, concentration and leaf, $F(3,88)=2.523$, $p=0.063$.

To determine at each concentration, whether the CPPU solutions and CPPU-loaded nanoemulsions treatments are behaving differently with respect to total chlorophyll loss in the treated and untreated leaves at different concentrations, a two-way mixed ANOVA was used to determine if there were simple two-way interactions between the leaf and treatment at each concentration. As shown in Table 3.6 it was found that there was a statistically significant interaction between the leaf and treatment on the total chlorophyll loss at 0.3 and 0.1 ppm, ($p=0.000$ and $p=0.001$, respectively) which means that the total chlorophyll loss in both treated and untreated leaves on different treatments were significantly different at the two concentrations. At 1.0 ppm and 0.03 ppm, the total chlorophyll loss was not significantly different by the leaf and different treatments used on the plant. For control, the total chlorophyll loss was not significantly different with $p=0.999$.

Table 3.1: Comparison of the mean difference in chlorophyll loss (%) between treated and untreated leaves for plants treated with CPPU and plants treated with NE+CPPU

Control	Mean difference		p value
Water	0.3		0.999
CPPU concentration (ppm)	CPPU solution	CPPU loaded nanoemulsion	p value
0.03	10	11	0.886
0.1	10	25	0.001
0.3	10	27	0.000
1.0	24	31	0.128

This means that the CPPU loaded nanoemulsion was more effective at lower concentrations of CPPU compared with CPPU in water. The significant difference found in total chlorophyll loss between treated and untreated leaves on treatment with CPPU loaded nanoemulsion at 0.3 and 0.1 ppm compared to CPPU solutions at the same concentrations gave a good indication of the effectiveness of CPPU loaded nanoemulsions in affecting the senescence profile of the paired leaves at low concentrations.

CPPU is widely used to increase fruit size such as kiwifruit [16][17], watermelon [18], pear [19] and grape [20], as well as flowers [21]. The CPPU concentration range that has normally been used in these studies (between 5 – 25 ppm) results in increased fruit size and firmness. From the data presented in this chapter, using a nanoemulsion to deliver CPPU could significantly reduce the CPPU concentrations that might be effective in the field.

3.3 SUMMARY

Throughout the various experiments performed at the laboratory (n = 3) and at larger scales (n = 12), consistent results were obtained from the experiments. Explants treated with the new CPPU-loaded nano-formulation showed a significantly greater response over the 14 day experimental period compared to the CPPU alone. The bioassay data points to the successful development of a new nano-formulation. Statistical tests revealed that the CPPU loaded

formulation was more effective than CPPU solution in changing the senescence profile of the paired at low concentrations (0.3 ppm and 0.1 ppm).

To my knowledge, this study is the first describing the use of nanoemulsion technology in enhancing plant growth. Various experiments showed that this solution state formulation, the CPPU-loaded nanoemulsion, has good potential to serve as a nanocarrier whereby CPPU is effectively delivered to the plant. By using this new formulation, the efficacy has increased, allowing for an over 10-fold reduction in CPPU concentration, from 1 ppm to 0.1 ppm. This is a very successful outcome from these investigations. It hints that the formulation could be successfully used in on-farm treatments. Use of a lower CPPU concentration would decrease the risk of residue in food and reduce environmental impact[17].

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CHAPTER 4

HALLOYSITE CLAY NANOTUBE SYSTEM

Halloysite clay is a unique material. It is capable of entrapping active materials within the inner lumen of the clay nanotube structure. The lumen can act as a container for different molecules and has the capacity to then slowly release them to the surroundings. This exceptional capability of halloysite clay nanotubes has been documented in several recent research articles [1][2][3][4]. Halloysite has been described as being a viable nanoscale container for the encapsulation of biologically active molecules[2][4]. As mentioned in Chapter 1, previous literature does not report the testing of the method on using halloysite clay nanotubes for synthetic plant hormone delivery.

This method of controlled delivery of the active ingredient is the second variant explored in this thesis, extending beyond the nanoemulsion studies. As discussed in Chapter 1, two different types of carrier were explored, a solution form, the nanoemulsion system, and a dry form, the halloysite clay nanotubes. This latter dry agent is the focus of this chapter.

Switching to a dry delivery agent meant that a different delivery method, active material and plant model were required. Compared to the nanoemulsion bioassay studies, which were shown in Chapter 3 to be a successful formulation, carrying CPPU effectively to the leaves, direct application of the halloysite clay nanotube to leaves is not appropriate. The most well-known use of dry or solid-state formulations containing a synthetic plant hormone is auxin

in rooting powders where the explant is dipped directly into the synthetic hormone-containing powder[5][6].

Auxin is a plant hormone that plays an important role in the regulation of root growth and is widely known for its ability to stimulate adventitious root formation[7][8]. Previous studies have shown that application of auxin to the severed end of a young stem, without any roots, promotes root formation[6]. Based on this early observation, both natural (indole-3-acetic acid (IAA)) and synthetic auxins, (e.g. naphthalene acetic acid (NAA) and Indole-3-butyric acid (IBA)), have been used for many years to promote root formation from stem cuttings [9][10][11]. However, applying too much auxin causes an inhibitory action, reducing root elongation [7].

In an effort to explore the efficacy of a solid-state nanoformulation containing active materials that are known to affect plant growth and development, halloysite clay nanotubes were used as a carrier in the controlled release of 2,4-D, a synthetic auxin that stimulates root initiation and elongation[12]. Many earlier studies used auxins to stimulate root initiation on cuttings [6][10]. Natural auxins regulate lateral root emergence through signalling to the adjacent cells in the parental root, thus promoting root formation[13]. In this study, the 2,4-D was applied to the explant as a powder or in solid state trapped in a nanocarrier.

When used at concentrations of approximately $10^{-4}/10^{-3}$ M 2,4-D is toxic to plants. It is used as a herbicide to control weeds and is one of the most widely used growth inhibitors in agriculture[14]. At lower concentrations 2,4-D promotes root formation and around 10^{-6} M may be optimal for root initiation. However, this same concentration may be supraoptimal or even inhibitory for root elongation, as root elongation is promoted at even lower auxin concentrations.

Since only a low concentration is needed to promote root formation, the solid-state formulation using halloysite clay nanotubes containing 2,4-D inside the nanotube, might be able to supply this active material for longer as the 2,4-D

might be released slowly, and thus be more effectively applied to the plants over time. This could potentially reduce the problem of inhibiting root elongation. That is both the number of roots and the length of individual roots might be controlled using this formulation method. Furthermore, less of the active material may be able to be used as less will be lost to the surrounds. Additionally, the dry nanotubes or lumens inside the halloysite clay may trap the 2,4-D for a considerable time until it is exposed to water. On exposure to water, defect points on the clay would allow the water to get inside and reach the entrapped 2,4-D and thus release would be triggered. Slow release of 2,4-D would take place, thus inducing root initiation.

In this chapter, the initial laboratory-scale studies that were undertaken using 2,4-D as the active material entrapped within the nanotubes of halloysite are described. The method of constructing the halloysite clay nanotubes containing 2,4-D for controlled release was adapted from methods described by Veerabadran et al. (2007)[4] and Veerabadran et al. (2009)[3]. The resulting dry solid powder containing 2,4-D was then applied to plants with the technique based on the use of auxins as rooting powders: by dipping the stem end into the powder prior to the plant being placed in the growth medium.

In this experiment, mung beans (*Vigna radiata*) were used as the model plants. Mung bean cuttings, prepared from mung bean seedlings, have been commonly used as a model plant to examine adventitious root formation[10][11]. The advantages of using mung bean cuttings are because of their accessibility. Mung bean seeds are readily available, the seedlings can be prepared after 8-9 days and root formation can be observed a week after treatment. Adventitious root initiation was used essentially as a bioassay to determine the effectiveness of halloysite clay nanotubes in delivering 2,4-D from its tubule. A slow release of 2,4-D should give a greater chance for the active molecules to be effectively absorbed by the plants and provide a longer supply to the plants. Therefore, root initiation and elongation may show greater stimulation and sustained growth. Hence, both the number and length of the roots were monitored as a function of time, using a variety of treatments to allow the efficacy of the dry delivery system to be assessed.

4.1 MATERIALS AND METHODS

4.1.1 Materials

Natural hydrated and air milled halloysite clay was purchased from NaturalNano, Inc., New York. The clay was untreated prior to further use. Acetone was purchased from Sigma-Aldrich. The active ingredient, 2,4-dichlorophenoxyacetic acid (2,4-D) (97%), was also purchased from Sigma-Aldrich.

4.1.2 Loading of Halloysite Clay Nanotubes with 2,4-D.

As discussed in Chapter 1, only a very small amount of 2,4-D is needed to induce root formation. Providing excess of this synthetic plant hormone will inhibit root formation and root elongation. Therefore, low concentrations of 2,4-D are normally used (10^{-4} M and lower) to study the effect of this synthetic auxin on root formation and emergence[15][16][12]. 2,4-D solutions were prepared with various concentrations: 10^{-7} M, 10^{-6} M, 10^{-5} M, 10^{-4} M and 10^{-3} M. Loading of the halloysite nanotubes with 2,4-D was performed by following the procedure described by Veerabadran et al. (2007)[4]. This is briefly described below.

To entrap 2,4-D, halloysite was mixed as a dry powder within a 2,4-D solution (2,4-D + acetone) of different concentrations. The resulting suspension was then placed under vacuum. The slight fizzing of the suspension indicated that air was being removed from the core of the halloysite tubules and replaced with solution. This slurry was kept under vacuum for 1 h before it was cycled back to atmospheric pressure. This process was performed three times or more until the fizzing stopped due to all the air in the lumen being replaced by 2,4-D solution. Finally, halloysite nanotubes were separated from solution by centrifugation to produce a condensed pellet, the supernatant was then removed and the pellet was left to dry at room temperature before being ground

to a fine powder. This process is shown in Figure 4.1, and the product will be referred to as clay + 2,4-D + vacuum.

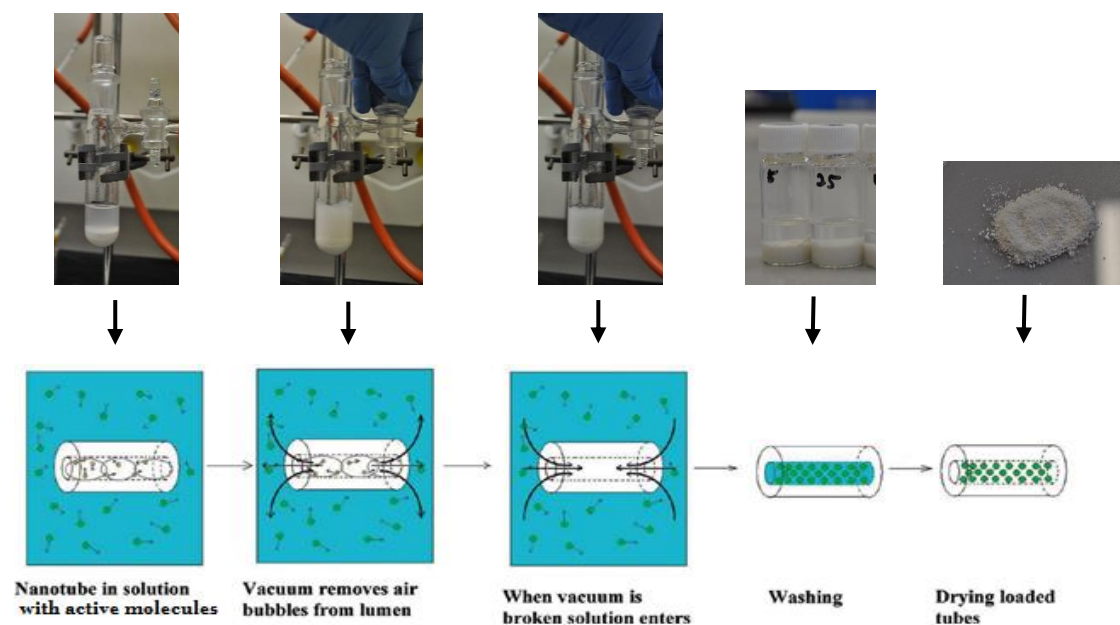


Figure 4.1: Loading procedure of halloysite nanotube with active molecules. The bottom schematic illustration is taken from [1].

4.1.3 Halloysite Clay Nanotube Control Formulations

Two controls were used in this experiment. The first consisted of the halloysite clay powder and the second halloysite clay exposed to the 2,4-D/acetone solution but not subjected to the vacuum/atmosphere cycles. For this second control, the 2,4-D was first dissolved in an appropriate quantity of acetone to make the same variation of concentrations as stated in Section 4.1.2. The solution was then mixed with the halloysite clay nanotube powder to form a slurry. The slurry was left to dry at room temperature and ground to a fine powder before being applied to the plant. This second control, whereby no or minimal entrapment of the 2,4-D within the halloysite nanotubes will have occurred allows the effect of surface adsorbed 2,4-D to be differentiated from entrapped 2,4-D, and will be referred to as clay + 2,4-D. The outcomes from the two controls of halloysite clay nanotube powder were compared to the outcomes obtained from the clay + 2,4-D + vacuum.

4.1.4 Plant Model

Mung bean seeds were purchased from Moore Wilson, Wellington, New Zealand. 100 mL of seeds were soaked in 200 mL bleach solution (1:10 dilution) for ten minutes. The seeds were then thoroughly rinsed three times and placed in a 1 L flask containing 500 mL water. The seeds were then aerated for 24 h at room temperature (25°C) and sown in trays containing perlite and/or vermiculite as the medium. The tray was watered with tap water and the seeds left to germinate for 10 days.

4.1.5 Halloysite Clay Nanotube Treatments

Plants were ready for the assay when the primary leaves were fully expanded and the trifoliate bud had not yet expanded. The seedlings were then harvested by severing the seedlings from their root systems and removing any attached cotyledons. The explants were prepared so that each cutting consisted of a hypocotyl, epicotyl, the primary leaves and the apical meristem. Explants of uniform size were collected to reduce variation. The working procedure is shown in Figure 4.2 (a – c).

The cut ends of the explants were slightly moistened with distilled water (often pre-moistened to enhance adhesion[5]) and then dipped into the clay + 2,4-D + vacuum or one of the two control treatments and then immediately placed into the moist medium as shown in Figure 4.2 (d – e). Five seedlings were used in each treatment with each of the different 2,4-D concentrations. The treatments are shown in Table 4.1. The moisture level of the medium was always maintained to ensure enough water was supplied to the beans. In particular, for the clay + 2,4-D + vacuum, water needed to reach the entrapped synthetic auxin to ensure that the active material would be released.

Table 4.1 The plants were observed for five days, the standard time that had been used previously[17]. However, after five days, there was no significant sign of root initiation in any of the test plants. The observation time was

therefore extended to 10 and 13 days. After 10 and 13 days, the length and number of the roots that had emerged for each of the seedlings were counted and recorded.

The roots were counted manually with the help of a microscope to count the small roots, particularly after the 10 day treatment where most of the roots were still small. The same technique was applied to record the root length where the roots were directly measured.

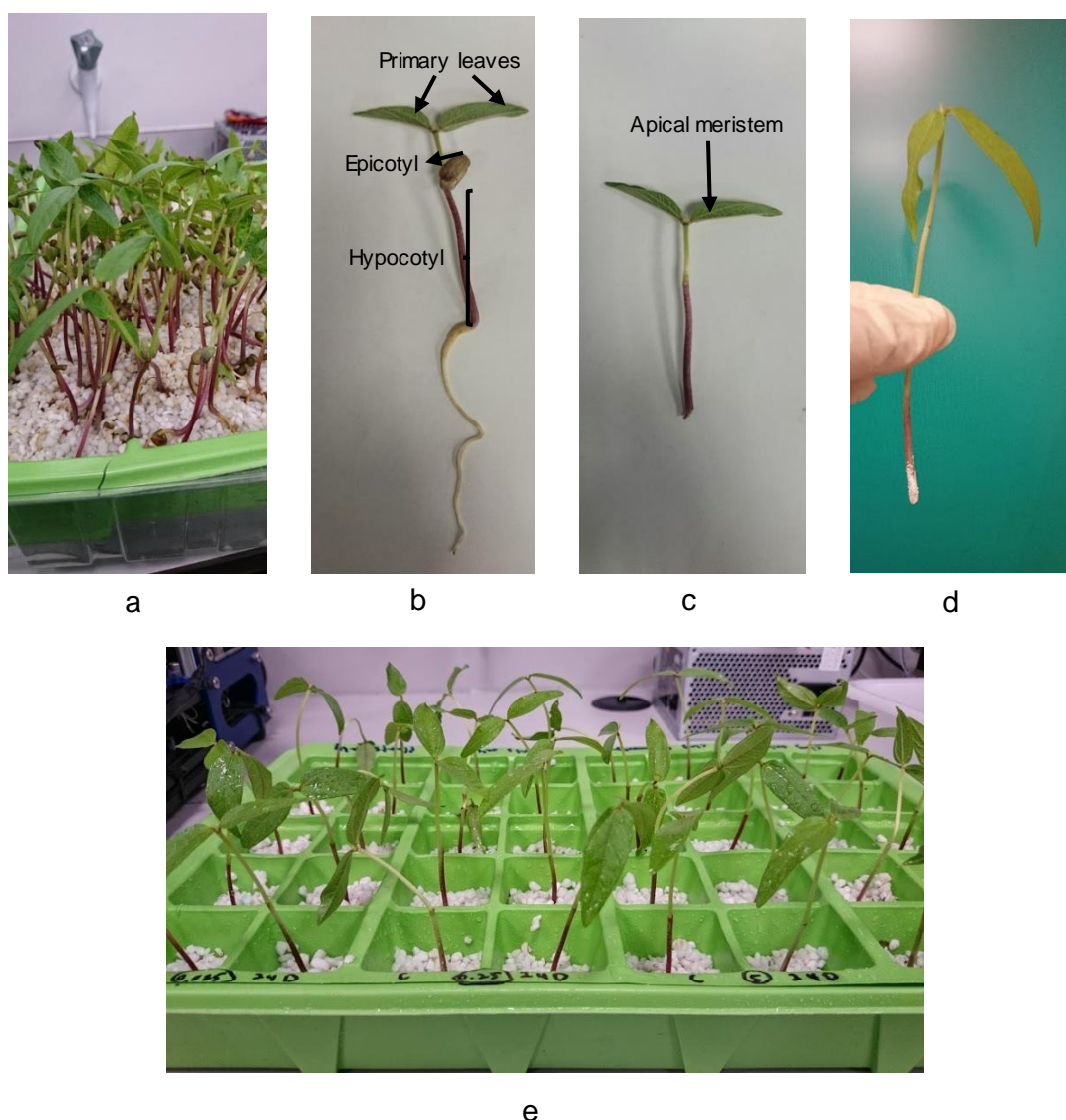


Figure 4.2: These images show a working procedure of the solid state formulation treatment. a) Mung bean seedlings in a perlite medium 10 days after germination which are ready to be harvested, b) a seedling before the root and cotyledon were removed, c) a seedling after the root and cotyledon have been removed, d) the seedling after being dipped into the halloysite clay nanotube powder and which is then immediately placed into the medium (e).

The moisture level of the medium was always maintained to ensure enough water was supplied to the beans. In particular, for the clay + 2,4-D + vacuum, water needed to reach the entrapped synthetic auxin to ensure that the active material would be released.

Table 4.1: Halloysite clay nanotube treatments

Treatments	A	B	C					D				
	Water	Halloysite clay nanotube only	Halloysite clay nanotube + 2,4-D					Halloysite clay nanotube + 2,4-D + vacuum				
Concentration of 2,4-D (M)	-	-	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}

4.1.6 Data Analysis

Statistical analysis was performed based on the five explants used per treatment. The data were analysed through a two-way mixed analysis of variance (ANOVA) to determine if there were simple two-way interactions between the days and treatments. Through this test, a comparison between the effect of different 2,4-D concentrations on the number of roots and root elongation of plants treated with halloysite clay nanotube +/- vacuum infiltration after different days of treatment was found. To test whether there was an interaction between the 2,4-D concentrations, vacuum treatments of halloysite clay and days of treatment on the root elongation and number of roots of sample plants, a three-way mixed ANOVA was used as another statistical test.

4.2 RESULTS AND DISCUSSION

Two types of measurements were performed on the mung beans cuttings. Firstly, root formation was scored as the number of emerged roots and, secondly, elongation of the roots emerging from the stem was measured. These measurements have been widely used in root formation research as they give a good indication of the effectiveness of the active material used in

stimulating root formation and root growth[12][18]. Two growth times were investigated: 10 and 13 days.

The roots emerging from the mung bean hypocotyl cuttings are called adventitious roots. Adventitious roots arise from an organ, usually stem, other than the root. The structure of these roots was short and thick.

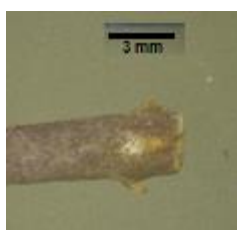
4.2.1 Plant Images

4.2.1.1 Root Formation and Elongation: 10 Days after Treatment

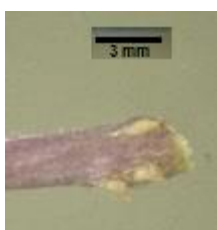
After 10 days of observation, magnified images of the explant stems were examined to observe the process of root formation using optical microscopy. Images of the plants were taken for analysis. Figure 4.3 shows examples of the stems that had been treated with halloysite clay nanotubes containing different 2,4-D concentrations, vacuum and non-vacuum treated or with no clay treatment or only clay treatment. The images were taken 10 days after the start of the treatments.

10 days after treatment, all explants treated with halloysite nanoclay + 2,4-D +/- vacuum showed root initiation. Those plants treated with water or only the clay showed no root emergence.

Considering the clay + 2,4-D +/- vacuum treatment starting from the lowest concentration of 2,4-D (10^{-7} M), small root protuberances can be seen at the cut end of the stems. The same event occurred in the explants treated with higher 2,4-D concentrations, until an inhibiting concentration, 10^{-3} M, was reached (Figure 4.3).



Treated with water

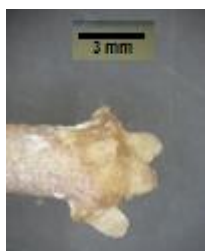


Treated with clay only

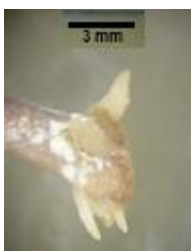
Halloysite clay nanotube + 2,4-D



10^{-7} M 2,4-D



10^{-6} M 2,4-D



10^{-5} M 2,4-D



10^{-4} M 2,4-D



10^{-3} M 2,4-D

Hallysite clay nanotube + 2,4-D + vacuum



10^{-7} M 2,4-D



10^{-6} M 2,4-D



10^{-5} M 2,4-D



10^{-4} M 2,4-D



10^{-3} M 2,4-D

Figure 4.3: Magnified images of the explant stems treated with water, clay and clay with different 2,4-D concentrations \pm vacuum and observed 10 days after treatment.

4.2.1.2 Root Formation and Elongation: 13 Days after Treatment

The same explants were again measured on day 13 after treatment. A different trend in the number of roots formed for clay + 2,4-D \pm vacuum treated clay is evident as the number of roots and root elongation has increased more without vacuum treatment than with vacuum treatment (Figure 4.4).



Treated with water only Treated with clay only

Halloysite clay nanotube + 2,4-D



10^{-7} M 2,4-D

10^{-6} M 2,4-D

10^{-5} M 2,4-D

10^{-4} M 2,4-D

10^{-3} M 2,4-D

Halloysite clay nanotube + 2,4-D + vacuum



10^{-7} M 2,4-D

10^{-6} M 2,4-D

10^{-5} M 2,4-D

10^{-4} M 2,4-D

10^{-3} M 2,4-D

Figure 4.4: Magnified images of the explant stems treated with water, clay and different 2,4-D concentrations with clay \pm vacuum, 13 days after treatment.

When the observation time was extended to 13 days, more adventitious roots emerged from the stems as shown in Figure 4.4, and the individual roots were longer. Rapid elongation of the roots occurred between days 10 and 13, noting that up to day 5 no root emergence had been evident. The data shown in Figure 4.4 indicate that for concentrations of 2,4-D less than 10^{-4} M the halloysite clay/2,4-D combination stimulated longer root formation compared

with water or clay alone. It should be noted that the number of roots formed and their length both varied across each of the five explants used for each treatment.

4.2.2 Data Analysis for Root Formation: 10 and 13 days After Treatment

The effect of the five different concentrations of 2,4-D on the number of roots formed after 10 days is shown in Figure 4.5. It was found that the bean explants treated with halloysite clay + 2,4-D + vacuum showed a stronger induction of adventitious roots compared to treatment with halloysite clay + 2,4-D, which had not been vacuum treated. Sample beans treated with water or clay only also produced fewer roots. These data indicate that there is a difference in the mean number of roots produced as the concentration of 2,4-D increases and also depending on whether the halloysite clay was vacuum or not vacuum treated. All concentrations of 2,4-D used stimulated the formation of adventitious roots except for the highest concentration, 10^{-3} M, that caused the end of the cutting stem to undergo necrosis irrespective of treatment.

The data illustrated in Figure 4.6, shows the data from 13 days, which has a similar pattern of response to increasing 2,4-D concentrations. Visual examination of the roots also suggested that after 13 days a similar response of root formation was obtained irrespective of whether vacuum treatment was applied or not for the halloysite/2,4-D treatments. The number of roots formed was reduced at the higher 2,4-D concentrations for both treatments.

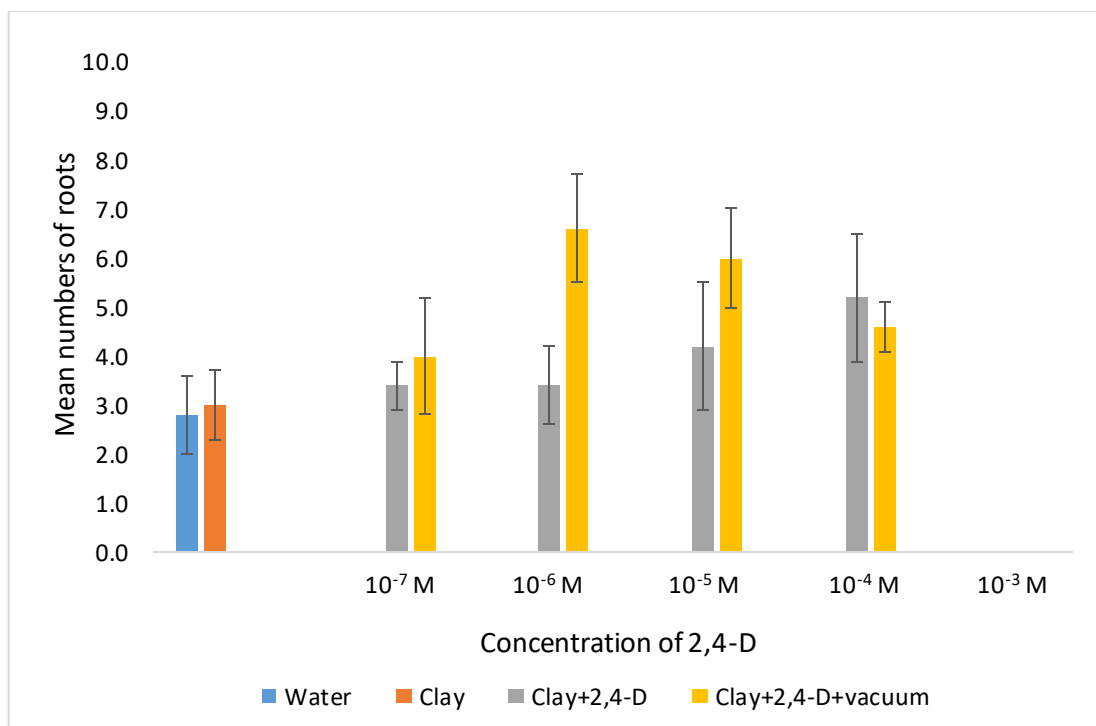


Figure 4.5: The effects of the different concentrations of 2,4-D and vacuum treatment on the number of roots formed 10 days after treatment. Bars are mean numbers of roots from five explants \pm SD.

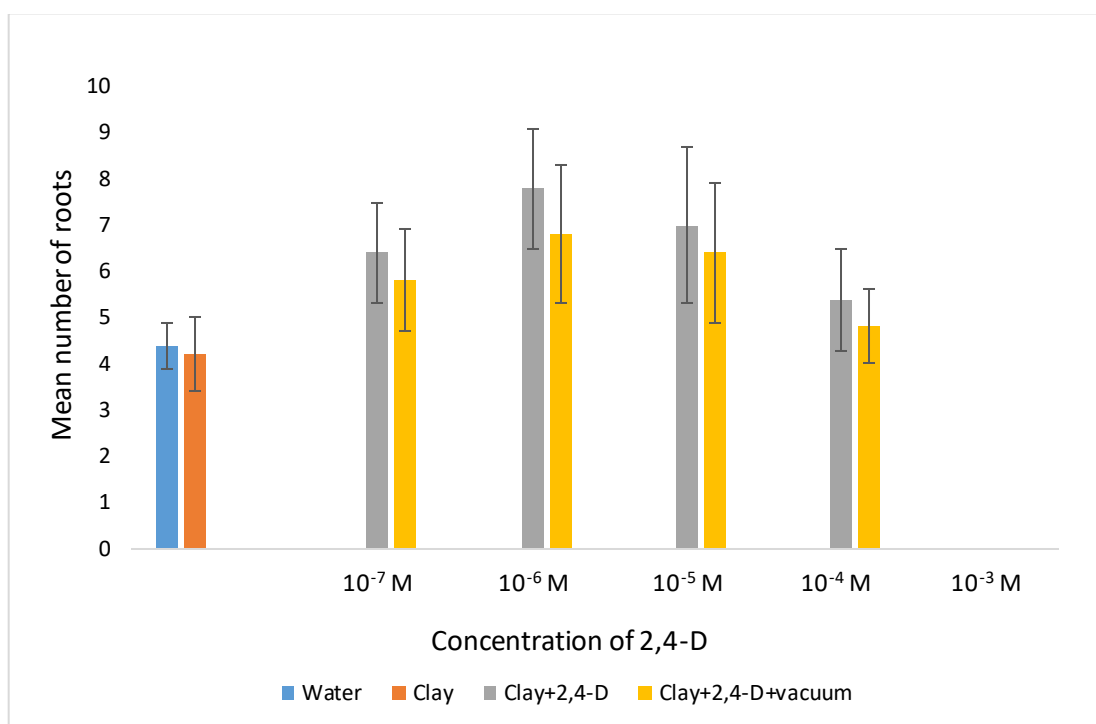


Figure 4.6: The effects of the different concentrations of 2,4-D and vacuum treatment on the number of roots formed 13 days after treatment. Bars are mean numbers of roots from five explants \pm SD.

A statistical analysis, three-way mixed ANOVA, was run to understand the effects of different treatments, concentration and days on the number of roots that emerged 10 days and 13 days after treatment. At each time point, the number of roots was normally distributed, as assessed by Shapiro-Wilk's test ($p > 0.05$). Homogeneity of variances was confirmed using Levene's test for equality of variances, and by visually assessing a scatterplot of the residuals versus predicted values. The three-way interaction between days, treatment and concentration was statistically significant at the 5% significance level; the number of roots differed depending on the combination of treatment, concentration and number of days after treatment, $F(5,48)=3.316$, $p=0.012$.

At each concentration, a two-way mixed ANOVA was used to determine if there were simple two-way interactions between the days and treatment. This analysis was to determine whether the clay + 2,4-D and clay + 2,4-D + vacuum were behaving differently with respect to the change in the number of roots emerged (from days 10 to 13) at each of the different concentrations. As shown in Table 4.2, it was found that there was a statistically significant interaction between the days and different treatments at 10^{-7} M and 10^{-6} M ($p=0.040$ and $p=0.008$, respectively). This means that the difference in the number of roots emerged between days 10 and 13 was significantly different between the two treatments at the 10^{-7} M and 10^{-6} M concentrations. Examination of the mean difference in the number of roots between 10 and 13 days after treatment (Table 4.2) indicated that there were significantly more roots formed in explants treated with clay + 2,4-D at 10^{-7} M and 10^{-6} M, compared to clay + 2, 4-D + vacuum. At 10^{-5} M, the number of roots is trending towards statistical significance with $p=0.068$. For controls (clay and water) and 10^{-4} M 2,4-D, the difference in the number of roots emerged between days 10 and 13 was not significantly different between treatments, with $p=0.999$ for all three. In other words: roots treated with clay + 2,4-D and clay + 2,4-D + vacuum at 10^{-4} M experienced the same change in the number of roots from 10 days to 13 days after treatment, as reported in Table 4.2

Table 4.2: Comparison of the mean difference in the number of roots emerged between 10 days after treatment and 13 days after treatment for plants treated with clay+2,4-D and plants treated with clay+2,4-D+vacuum.

Control	Mean difference		p value
Clay	1.6		0.999
Water	1.2		0.999
2,4-D Concentration (M)	Clay + 2,4-D	Clay + 2,4-D + vacuum	p value
10^{-7}	3	1.8	0.040
10^{-6}	4.4	0.2	0.008
10^{-5}	2.8	0.4	0.068
10^{-4}	0.2	0.2	0.999

The simple two-way analysis has shown a significantly greater number of roots for plants treated with clay + 2,4-D compared to clay + 2,4-D + vacuum at 10^{-6} M after 13 days of treatment. A possible reason why significantly fewer roots had been produced by plants treated with clay + 2,4-D + vacuum by 13 days was because the nanoclay may have continued to keep releasing the 2,4-D at a concentration greater than the optimal concentration needed for root initiation. The vacuumed clay formulation may actually be working as a slow release agent of the synthetic auxin.

4.2.3 Data Analysis for Root Elongation: 10 and 13 days After Treatment

The average root length of the mung bean explants 10 days after treatment is summarised in Figure 4.7. From these data, it can be seen that the bean explants show a longer average root length when treated with halloysite clay + 2,4-D + vacuum than clay + 2,4-D.

The average root length of the mung bean explants after 13 days is summarised in Figure 4.8. The data shown in the figure indicate that the bean explants treated with halloysite clay + different 2,4-D concentrations but not vacuum treated, have a longer average root length compared to the treatment with halloysite clay + different 2,4-D concentrations and vacuum treated. Both formulations, vacuum and not vacuum treated, showed that root elongation was inhibited for 2,4-D concentrations of 10^{-4} M and greater. Other 2,4-D concentrations, 10^{-5} M and lower, showed both root formation and elongation.

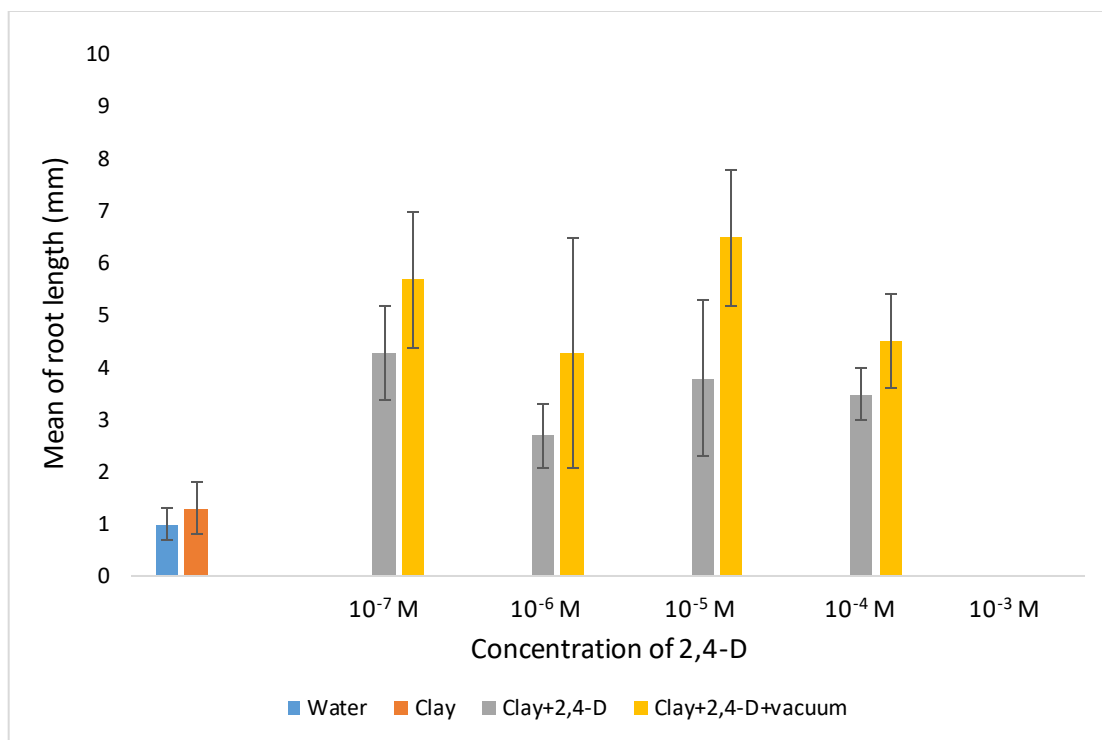


Figure 4.7: The effects of the different concentrations of 2,4-D and vacuum treatment on root elongation 10 days after treatment. Bars are mean numbers of roots from five ex plants \pm SD.

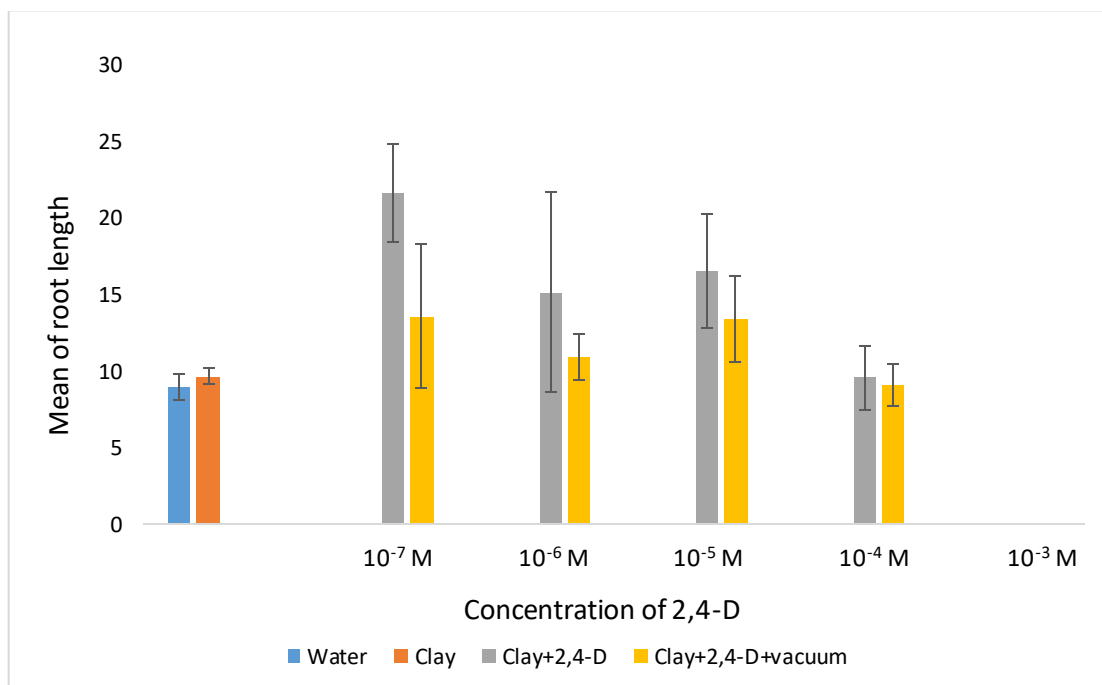


Figure 4.8: The effects of the different concentrations of 2,4-D and vacuum treatment on root length 13 days after treatment. Bars are mean numbers of roots from five explants \pm SD.

A three-way mixed ANOVA was also run to understand the effects of different treatments, concentration and days on the root elongation between 10 days to 13 days after treatment. At each time point, the number of roots was normally distributed, as assessed by Shapiro-Wilk's test ($p > 0.05$). Homogeneity of variances was confirmed using Levene's test for equality of variances, and by visually assessing a scatterplot of the residuals versus predicted values. The three-way interaction between days, treatment and concentration was statistically significant at the 5% significance level; the root length differed depending on the combination of treatment, concentration and number of days after treatment, $F(5,48)=3.515$, $p=0.009$.

For each concentration, a two-way mixed ANOVA was used to determine if there were simple two-way interactions between the days and treatment. This test determines whether the clay + 2,4-D and clay + 2,4-D + vacuum are behaving differently with respect to the change in root elongation (from days 10 to 13) at each of the different concentrations. It was found that there was a statistically significant interaction between the days and different treatments at 10^{-7} M and 10^{-5} M ($p=0.014$ and $p=0.018$, respectively), which means that the change in the root length between days 10 and 13 was significantly different for the two treatments, at 10^{-7} M and 10^{-5} M. The difference in root length between 10 and 13 days after treatment was not significantly different between plants treated with clay + 2,4-D and clay + 2,4-D + vacuum at 0 M, 10^{-6} M and 10^{-4} M (with $p>0.999$ $p=0.112$ and $p=0.303$, respectively). These statistical results are shown in Table 4.3. There was a greater increase in root length of explants treated with clay + 2,4-D at a concentration of 10^{-7} M compared to clay + 2,4-D + vacuum. These results may indicate that 10^{-7} M is promoting root elongation, and 10^{-4} M is a supraoptimal concentration for root elongation.

Table 4.3: Comparison of the mean difference in root elongation between 10 days after treatment and 13 days after treatment for plants treated with clay+2,4-D and plants treated with clay+2,4-D+vacuum.

Control	Mean difference (mm)		p value
Clay	8.4		0.999
Water	8.0		0.999
2,4-D Concentration (M)	Clay + 2,4-D	Clay + 2,4-D + vacuum	p value
10^{-7}	17.4	7.9	0.014
10^{-6}	12.5	6.7	0.112
10^{-5}	12.8	6.9	0.018
10^{-4}	6.1	4.6	0.303

Based on the understanding of auxin behaviour as described by de Klerk et al. (1999)[6], they concluded that during the initial stage of root formation, higher concentrations of auxin are needed to trigger root formation but much lower concentrations of auxin are required during the root elongation phase. High concentrations of auxin will inhibit root elongation. What may have happened during root formation and elongation 10 days after treatment was that enough 2,4-D was supplied to the sample plants that were treated with clay + 2,4-D + vacuum as the hormone was slowly released from the clay and effectively reached the plant roots. However, for sample plants treated with clay + 2,4-D, where the 2,4-D only covered the outside of the clay, which could easily be washed away thus causing the plants to receive reduced amounts of 2,4-D needed to trigger more roots to form. After 13 days, roots were continuing to elongate which could only happen in an environment of reduced auxin concentration. The results after 13 days observation showed significantly shorter roots for samples treated with clay + 2,4-D + vacuum compared to clay + 2,4-D. This may have happened because at this stage, the 2,4-D was still being slowly released by the clay and kept supplying the roots with a concentration of 2,4-D that was supraoptimal for elongation growth.

4.3 SUMMARY

The test of the second formulation, a dry/solid state formulation, was carried out using a mung bean rooting bioassay as a means of testing the release of the active ingredient, 2,4-D. The experimental procedure was according to Witham et al. (1986). Both root number and root elongation were measured. Based on the statistical analysis of the data only a few 2,4-D concentrations promoted root formation and root elongation.

Plant hormone behaviour and their roles in plant growth are very complicated processes. Many extensive studies have been performed to understand their behaviour in plant growth[6]. An analysis of whether the nanotube was slowly releasing 2,4-D was complicated by the fact that a concentration of 2,4-D that promotes root formation will inhibit root elongation.

In this study, the data indicate that the vacuum treated halloysite clay may be successful in slowly releasing the 2,4-D during the initial stage of root formation when higher concentrations of 2,4-D are needed to induce the root production. This was evident by the visual examination of the roots of plant samples after 10 days. After 13 days the explants treated with clay + 2,4-D + vacuum showed reduced elongation than those treated with the non-vacuum treated preparation.

Statistical tests revealed that both root number and root elongation differed depending on the combination of treatment, concentration and number of days after treatment. The critical comparison was the performance of the clay + 2,4-D and clay + 2,4-D + vacuum at difference concentrations. The statistical tests showed that at 10^{-7} M and 10^{-6} M, there was a significant difference in the number of roots emerged between days 10 and 13 between the two treatments. Tests on root elongation showed that there was a significant difference in root length from 10 days to 13 days after treatment with the clay + 2,4-D and clay + 2,4-D + vacuum at 10^{-7} M. It is therefore possible that the nano-formulation could be working in delaying the 2,4-D release to the surroundings,

After 13 days, it appears that the vacuum treated halloysite clay may have continued to release the 2,4-D trapped in its lumen which then caused inhibitory action on root elongation. From the laboratory data obtained in this study, halloysite clay nanotubes have a good potential to be used as a nanocontainer for active materials. However, its application on releasing active materials to plants still needs further testing. More replicates would be a good start to obtain less variable results, and even lower concentrations of 2,4-D should be tested as the optimal concentration for root elongation was not confirmed – this then might show an even stronger confirmation that the halloysite nanotube was releasing a consistent (and inhibitory to root elongation) amount of 2,4-D over time.

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CHAPTER 5

CONCLUSIONS AND FUTURE WORK

The main objective for the research reported in this thesis was to develop both a solution-based and a solid-state nano-formulation for delivering synthetic plant hormones more efficiently to plants compared with standard delivery methods and thereby serve as a proof-of-concept for the use of nanotechnology to enhance the agri-economy and potentially reduce the environmental impact of applying synthetic plant hormones. This initially required development of two formulations that were compatible with two of the classes of plant hormones, the cytokinins and auxins. In particular, two synthetic plant hormones were selected: N-phenyl-N'-(2-chloro-4-pyridyl)urea (CPPU) or forchlorfenuron, a synthetic cytokinin, and 2,4-dichlorophenoxyacetic acid (2,4-D), a synthetic auxin. For CPPU, delivery was via encapsulation within the oil droplets of a nanoemulsion, which was then applied to the leaves of a plant. For 2,4-D, a nanotube clay was used to trap the hormone within the interior of the tubes. The clay was then applied directly to the roots. The efficacy and effectiveness of the two methods was investigated using two different biological assays (bioassays): dwarf beans of *P. vulgaris* cv. Long John and mung beans (*Vigna radiata*), respectively.

The first delivery method investigated was the solution-based nanoemulsion formulation. The nanoemulsion system selected was water/polysorbate 80/glycerol/soybean oil coupled with CPPU as the active ingredient, which was incorporated into the nanoemulsion via the oil phase. The fabrication method for the nanoemulsion was the low-energy emulsification technique, where no

high energy input is used in any part of the formulation. Here a pre-concentrate was prepared which was then crash diluted to yield the final nanoemulsion. Nanoemulsions are created only when the concentrate is located in the bicontinuous microemulsion or oil-in-water (O/W) microemulsion regions of the phase diagram.

The development of the nanoemulsion delivery system is described in Chapter 2. Based on the literature[1] it was expected that either or both of a bicontinuous microemulsion and O/W microemulsion region(s) would be present in the pseudo ternary phase diagram of the water/polysorbate 80/glycerol/soybean oil system allowing for formation of nanoemulsions using the low energy method. This was found to be the case by exploring the full microemulsion and liquid crystalline regions of the phase diagram and generating the crash-diluted phase diagram. The nanoemulsion region was identified (the red region in Figure 2.10 in Chapter 2). Once the nanoemulsion region had been identified and a robust protocol established for their formulation the next stage was to ensure that CPPU could be incorporated into the oil phase and that nanoemulsions could still be successfully fabricated. This was achieved. Both the CPPU-loaded nanoemulsions and the nanoemulsions themselves were found to be stable for more than three days.

The critical part of the nanoemulsion study was to find the bicontinuous microemulsion region and/or the O/W microemulsion region ensuring that nanoemulsions could be produced. Nanoemulsions were produced and they were found to have droplets in the range of 30-100 nm, using dynamic light scattering measurements, hence it was assumed that the concentrate region was either an O/W or bicontinuous microemulsion. To be more certain about the bicontinuous and O/W microemulsion regions, further investigation into the structure of these regions could be performed. Instruments such as cryogenic transmission electron microscopy (cryo-TEM) could be used to observe the bicontinuous structure and morphology[2][3]. Using this instrument, a drop of the sample would be applied onto a carbon-coated film. The drop would be thinned into a film less than 300 nm thick, which would then be plunged into a cryogen and kept in liquid nitrogen before transfer to the microscope for

imaging studies. This method has been shown to produce good images of, in particular, the bicontinuous microemulsion structure[2].

It would also be useful, particularly from the perspective of practical farm-based use, to explore the stability data over a period longer than three days from a quantitative perspective compared with the qualitative visual observation used here. Nanoemulsions can be produced that stay stable for more than a month, or even a year because of their characteristic kinetic stability[4]. There is high probability, based on the qualitative investigation undertaken here, that the droplet size and size distribution of the nanoemulsions produced in this study would be stable for longer than three days.

Once the full fabrication process had been established for the formulation of CPPU-loaded nanoemulsions, the nanoemulsions were investigated from the perspective of whether they were able to deliver CPPU effectively to plant leaves. Dwarf beans were used as the model plant. CPPU is a phenylurea derivative that shows strikingly strong cytokinin-like activity in plants[5], including delaying senescence[6]. It is relatively insoluble in water, but is soluble in organic solvents. CPPU has been widely applied to fruits such as kiwifruit and table grapes, delivered via a water-based solution made possible through the addition of a small amount of organic solvent to aid the water solubility of the CPPU[7]. CPPU load levels of 5-10 ppm are routinely used on farm. Nanoemulsions were considered to be potentially ideal delivery agents for facilitating CPPU transfer to the plants since their size correlates well with the nanoscale surface features of leaves, thereby potentially achieving significantly greater total contact area between the oil droplets and the leaves as compared with the standard water delivery treatment. As such it was hypothesized that the total amount of CPPU that had to be used in the delivery to elicit the same response in the plants could be reduced when using the nanoemulsion delivery method.

To explore this, a leaf senescence bioassay was conducted to test the senescence-delaying effect of the plants following treatment with CPPU-loaded nanoemulsions. Through this bioassay, the nanoemulsions were applied

directly to the leaves of the sample plant. A chlorophyll assay was conducted to quantitatively assess the amount of chlorophyll present in the leaves. A laboratory-scale trial was first conducted and the results indicated that CPPU-loaded nanoemulsions enhanced the effectiveness of CPPU in delaying leaf senescence compared with the control and comparator experiments, including direct application of CPPU. Based on these results, large-scale experiments were then designed to apply the solution to a larger number of explants to enhance the power of the statistical analysis. Statistically significant results were obtained indicating that a greater than 10-fold reduction in CPPU concentration, from 1 ppm to 0.1 ppm, when the CPPU was loaded into the nanoemulsions prior to delivery compared with the standard water-based delivery method, was achieved.

To achieve this result extensive preparative work was required to be carried out in order to obtain sufficient plant samples to conduct the bioassay investigation. To be able to be used in the nanoemulsion delivery study, primary leaves of the explant must be fully expanded, close to the same size as each other, green, and present a uniform flat surface. However, achieving this was very challenging because of the difficulties in getting such 'perfect' plant leaves. Germination and growth protocols were developed which eventually led to a 50% yield routinely being able to be achieved.

Even once these protocols were in place, as expected when working with plants, it was found that there was high variability in the measured chlorophyll content of individual leaves. The weight of the sample leaves was also very different between plants, despite taking standardised samples using a hole punch, indicating the density and/or thickness of the leaves was highly variable. Together these resulted in high variability in the measured total chlorophyll loss between individual plants within a single test.

The challenges of the bioassay, notwithstanding, the data presented in this research indicate that the nanoemulsion delivery method has significantly greater efficacy than the standard water-based delivery method for the CPPU test case explored. Furthermore, significantly lower concentrations of CPPU

were shown to affect the senescence of the explants. The nanoemulsion carrier system formulated here could be a good generic delivery system for other active ingredients. Any plant hormones, pesticides, herbicides or even drugs that are soluble in soybean oil at the required concentrations for optimal activity could be considered to replace the CPPU.

The second nanosystem delivery method investigated for delivering synthetic plant hormones to plants is presented in Chapter 4. In this case a solid-state technique was investigated and in particular a nanotube clay known as halloysite. Such a delivery system is best applied directly to the plant roots. Here, a preliminary laboratory-based plant trial was conducted. A synthetic auxin, 2,4-D, was used as the active ingredient. 2,4-D has been widely used as a herbicide and for fruit thinning[8]. Synthetic auxins are commonly used in rooting powders. However, it has been shown that excess use of 2,4-D results in inhibition of root growth and, when used as a herbicide, plant death.

In this study, halloysite clay nanotubes were loaded with 2,4-D, following a literature formulation procedure[9]. The 2,4-D loaded halloysite powder was applied to the plant roots using a dipping method. Mung beans were used as the model plants. The number of roots and root elongation after 10 days and 13 days were recorded. It was found that application of 2,4-D loaded nanotubes to the cut end of a young stem, without roots, stimulated greater numbers of roots to be formed and greater root elongation compared with other treatments 10 days post treatment application. In contrast, after 13 days, the average number of roots formed and their elongation was greater for samples treated with clay + 2,4-D compared to clay + 2,4-D + vacuum. At different concentrations, there was a significant difference in the number of roots emerged between days 10 and 13 between the two treatments at 10^{-6} M and 10^{-7} M of 2,4-D. Additionally, the root lengths of explants treated with clay + 2,4-D and clay + 2,4-D + vacuum were significantly different at 10^{-7} M 2,4-D between days 10 and 13. During this time, when roots were elongating only a very, very low concentration of 2,4-D was needed. The retardation of root elongation, relative to controls after 13 days, potentially indicated continued slow release of the active ingredient from the nanotubes, thus continuously exposing the roots to 2,4-D and reducing root

elongation. For future work, lower concentrations of 2,4-D should be used to identify an optimal concentration for root elongation. It is, therefore, possible that the vacuum treated nanoclay could be working in delaying the 2,4-D release to the surroundings.

In this study, the morphology of the clay nanotube before or after being subjected to 2,4-D, with or without vacuum treatment, was not shown. It could not be fully ascertained that the 2,4-D was actually trapped inside the nanotubes or if it was whether it fully utilised the interior of the nanotubes. A full release study was not performed, hence the release profile of 2,4-D to the medium is not known, including how much of the 2,4-D is being released over what time profile and how long it takes to fully release the trapped 2,4-D. However, despite not knowing the full release profile, the bioassay performed for clay + 2,4-D + vacuum and clay + 2,4-D provided good initial results where there was a significant difference with the data from the sample plants treated with clay + 2,4-D + vacuum and clay + 2,4-D. The data imply that the 2,4-D was actually trapped inside the nanotube and has been slowly released throughout the observation time and that the two treatments provided different outcomes.

Extension of the studies that have been undertaken on the halloysite clay nanotube containing 2,4-D could include investigating the release process. This would allow determination of the amount of 2,4-D trapped inside the halloysite nanotubes, and also the time taken for the synthetic hormone to be fully released from the nanotube. These data would be helpful when performing the biological studies. The release study would be carried out in deionized water where the sample is constantly stirred and the supernatants are collected periodically. Typical release times vary but, based on literature studies, times of more than 15 h would be expected[10]. The concentration of released active material would be determined by using UV-vis spectrophotometry[10]. However, the very small concentrations of synthetic plant hormones used here might present a practical limitation from an instrument perspective in order to give accurate readings of the concentration of the hormone ($\sim 10^{-6}$ M and below). A more sensitive instrument that could read to a very low concentration would be very useful in such a study. A morphological study of the clay

nanotubes before and after vacuum treatment could also be performed to visually investigate the effectiveness of vacuum treatment in trapping the 2,4-D in its nanotube.

Moving forward, this study could be extended into larger scale experiments where a greater number of sample plants could be used, in order to obtain less variable results and even lower concentrations of 2,4-D should be tested to confirm the optimal concentration for root elongation and a consistent release of 2,4-D over time.

The use of other plant hormones for this test could also be considered for future work. The naturally occurring auxin, indole-3-acetic acid (IAA) is another plant hormone that could be explored using this halloysite nanotube technique. IAA is the main auxin in plants, controlling many important physiological processes including cell enlargement and division during both root and shoot development[6]. IAA is rapidly broken down by soil microorganisms, which is why this hormone is not used in rooting powders. By using the clay nanotube, it is possible that the IAA would not be as readily degraded as it would be trapped in the tube and could work better in initiating root formation by being slowly released from the nanotube.

Finally, both of the formulations developed in this study have shown considerable potential to become widely-used methods in effectively delivering synthetic plant hormones to plant leaves and roots in real-world applications. Nanoemulsions have been used previously as drug delivery agents[11] and the application of halloysite clay nanotubes in various fields is gaining interest[12]. However, neither technique has been used in the delivery of plant hormones as active materials to plants. Hence the work undertaken here has opened up both of these methods as being viable for plant hormone delivery to plants.

The collective results presented in this thesis unequivocally show that the use of CPPU and 2,4-D, coupled with nanoemulsions and nanotubes, respectively, served as proof of concept investigations showing the potential of nanotechnology to aid in the release of plant growth regulators and more

generally its potential impact in the agri-sector. Further study is clearly needed before either of these formulations could be applied in real farming activities, including undertaking field studies.

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