Development of a plant root cell model to study the perception of elicitors

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ABSTRACT

The plant plasma membrane is a very complex structure, mainly composed of lipids and proteins, that determines cell boundaries and controls the entry and exit of molecules. In plants, the plasma membrane is involved in the perception of elicitors, such as surfactin. This molecule is able to induce the plant defense response which makes it a potential alternative to conventional pesticides. The molecular mechanism behind this perception process is little-known. To investigate this process, biomimetic models, including lipid monolayers, supported bilayers and liposomes, and biological models, including protoplasts are required. A protoplast is a cell which is delimited by the biological plasma membrane but that does no longer have its cell wall, it is a reliable model because it has a composition similar to the biological membrane.

This master's thesis aimed to develop and optimize the isolation of tomato root protoplasts and to optimize isothermal titration calorimetry (ITC) parameters for measuring the thermodynamics of binding events with protoplasts. After these optimization steps, interaction of protoplasts with surfactin was thermodynamically characterized with ITC and their reactivity in terms of defense response in presence of surfactin was analyzed by reactive oxygen species measurement.

First, to optimize the isolation of tomato root protoplasts, the effect of four factors, including time and speed of centrifugation, the age of roots, the time of incubation with enzymes and the agitation speed during the incubation with enzymes, on protoplast production yield and percentage of viability was investigated. It was shown that all parameters had an influence on protoplast production yield and the protoplast suspension with the highest yield was obtained from 7-day-old roots that were incubated for 17 hours without agitation and that were purified with a centrifugation at 600 rcf for 6 minutes. It was also shown that flow cytometry on tomato root protoplasts is complicated due to the high number of debris in protoplast suspension. Its use would require an improvement of the purification steps of the protoplast suspension.

Secondly, it was determined that the best configuration for the ITC measurements is to put the protoplasts in the measuring cell with a low agitation. In this case there was no decrease of the protoplast concentration and of their percentage of viability during a measurement. In presence of surfactin, a binding event was observed. However, optimization of the concentration of protoplasts is still required in order to obtain an optimal profile of heat flow for the determination of the thermodynamic parameters.

Production of reactive oxygen species (ROS) was also observed in presence of surfactin confirming the good reactivity of protoplasts in terms of defense response.

In conclusion, this thesis paves the way to produce and use root protoplasts in biophysical experiments such as ITC to better understand the perception of elicitors in plants.

RÉSUMÉ

La membrane plasmique végétale est une structure très complexe, principalement composée de lipides et de protéines, qui détermine les limites de la cellule et qui contrôle l'entrée et la sortie de molécules. Chez les plantes, la membrane plasmique est impliquée dans la perception d'éliciteurs, tel que la surfactine, qui est capable d'induire la réponse de défense dans la plante et qui en fait une alternative potentielle aux pesticides conventionnels. Le mécanisme moléculaire à l'origine de ce processus de perception est peu connu. Pour étudier ce processus, des modèles biomimétiques, tels que les monocouches lipidiques, les bicouches lipidiques supportées et les liposomes, et des modèles biologiques, tel que les protoplastes, sont nécessaires. Un protoplaste est une cellule qui est délimitée par la membrane plasmique biologique mais qui ne dispose plus de sa paroi cellulaire. C'est un modèle fiable parce qu'il a une composition similaire à celle de la membrane biologique.

Cette thèse a pour but de développer et d'optimiser l'isolation des protoplastes de racines de tomates ainsi que d'optimiser les paramètres de titrage calorimétrique isotherme (ITC) pour mesurer la thermodynamique des événements dûs à une interaction avec les protoplastes. Après ces étapes d'optimisation, l'interaction des protoplastes avec la surfactine a été caractérisée thermodynamiquement avec l'ITC et leur réactivité en termes de réponse de défense en présence de surfactine a été analysée par la mesure des espèces réactives de l'oxygène.

Premièrement, pour optimiser l'isolation des protoplastes de racines de tomates, l'effet de quatre facteurs, dont le temps et la vitesse de centrifugation, l'âge des racines, le temps d'incubation avec les enzymes et la vitesse d'agitation pendant l'incubation avec les enzymes, sur le rendement en protoplastes et le pourcentage de viabilité a été étudié. Il a été démontré que tous les paramètres avaient une influence sur le rendement en protoplastes et la suspension de protoplastes ayant le rendement le plus élevé a été obtenue à partir de racines de 7 jours qui ont été incubées pendant 17 heures sans agitation et qui ont été purifiées par une centrifugation à 600 rcf pendant 6 minutes. L'utilisation de la cytométrie de flux s'est révélée difficile suite à la présence d'un nombre importants de débris racinaires dans la suspension de protoplastes. Pour pouvoir employer la cytométrie, une amélioration des étapes de purification de la suspension de protoplastes est requise.

Ensuite, il a été déterminé que la meilleure configuration pour les mesures à l'aide de l'ITC est de placer les protoplastes dans la cellule de mesure avec une faible agitation. Dans ce cas, il n'y a eu aucune diminution de la concentration en protoplastes et de leur pourcentage de viabilité au cours de la mesure. En présence de surfactine, un événement dû à une interaction a été observé. Cependant, l'optimisation de la concentration en protoplastes est toujours nécessaire afin d'obtenir un profil optimal du flux thermique pour la détermination des paramètres thermodynamiques.

La production d'espèces réactives de l'oxygène (ROS) a également été observée en présence de surfactine, ce qui a permis de confirmer que les protoplastes ont une bonne réactivité en termes de

réponse de défense.

En conclusion, ce mémoire pose les bases pour l'obtention et l'utilisation de protoplastes de racines dans des expériences biophysiques tel que l'ITC, afin de mieux comprendre la perception des éliciteurs chez les plantes.

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Nomenclature

%	Percentage
Ara	Arabinose
ASG	Acylated Steryl Glycoside
BSA	Bovine serum albumin
cal	Calories
Cer	Ceramide
cmc	critical micelle concentration
DAMP	Damage-Associated Molecular Pattern
DCFDA	Dichlorofluorescin Diacetate
DGDG	Digalactosyldiacylglycerol
DGlcCG	Diglucosyldiacylglycerol
DIM	Detergent Insoluble Membrane
DMSO	Dimethyl Sulfoxyde
ES	Enzymatic Solution
FDA	Fluorescein Diacetate
FL1	Forward Light 1
FSC	Forward Scatter Channel
g	Grams
Gal	Galactose
GIPC	Glycosyl Inositolphosphoceramide
GlcA	Glucuronic Acid
GlcCer	Glucosylceramide

GlcN Glucosamine

GlcNAc N-acetyl-glucosamine

GUV Giant Unilamellar Vesicle

HSE Hydroxycinnamate Sterol Ester

IPC Inositol Phosphoryl Ceramide

ITC Isothermal Titration Calorimetry

L Litre

$LaCl_3$ lanthanum chloride

LCB Long Chain Based

Ld Liquid-Disordered

Lo Liquid-Ordered

LUV Large Unilamellar Vesicle

M Molar concentration

m Milli

MAMP Microbe-Associated Molecular Pattern

Man Mannose

MES 2-(N-morpholino)ethanesulfonic acid

MGDG Monogalactosyldiacylglycerol

MGlcCG Monoglucosyldiacylglycerol

min Minutes

MLV Multilamellar Lipid Vesicle

MS Murashige and Skoog

n Nano

NS Not Significative

PA Phosphatidic Acid

PAMP Pathogen-Associated Molecular Pattern

PC Phosphatidylcholine

PE Phosphatidylethanolamine

PG Phosphatidylglycerol

PI Phosphatidylinositol

PM Plasma Membrane

Pmg Phytophthora megasperma glycinea

PR Pathogenesis-related

PrI Propidium Iodide

PRR Pattern-recognition receptors

PS Phosphatidylserine

rcf Relative Centrifugal Field

RGM Red-to-green ratio of membrane fluorescence

ROS Reactive Oxygen Species

S1 Saline Washing Solution

S2 Conservation Solution

SALB Solvent-Assisted Lipid Bilayer

SE Sterol Ester

sec Seconds

SG Steryl Glycoside

SLB Supported Lipid Bilayer

SQDG Sulfoquinovosediacylglycerol

SSC Side Scatter Channel

SUV Small Unilamellar Vesicle

ULV Unilamellar Lipid Vesicle

°C Celsius Degree

µ Micro

Chapter 1

INTRODUCTION

1.1 Context

The plasma membrane is essential, it ensures the viability of the cell and acts as a barrier between the inside and the outside of the cell, determining cell boundaries. Hence, the plasma membrane is the site of many reactions and exchanges and is involved in the recognition of some molecules.

The plasma membrane, and more particularly its lipid fraction, is implicated in the perception of some molecules called elicitors. Some of these elicitors are produced by microorganisms. It is the case of surfactin produced by several *Bacillus subtilis* strains. Elicitors stimulate the plant defense response against several aggressors. Therefore, they represent a potential alternative to conventional pesticides. The understanding of the molecular mechanism behind the perception process of these elicitors by the plant plasma membrane is far to be accomplished while it is rather essential in order to develop eliciting products with the highest performance.

The only way to get valuable insights at a molecular level is to use plant plasma membrane models. They have to be a compromise between the reality of the membrane that they mimic and the level of complexity which has to be simple enough to allow correct data interpretation. In this context, lipid vesicles prepared from main lipids representative of the plant plasma membrane are simple models suitable to get molecular details of their interaction with exogenous molecules. Nevertheless, these models do not contain any proteins and present only a restricted number of lipids. Therefore, they do not account for the high complexity of the plant plasma membrane, which is known to play a crucial role in the recognition of the exogenous molecule and transduction of the signal. This is why protoplast, which is a plant cell that no longer has its cell wall, can be a more reliable membrane model with a higher complexity. The combination of the information coming from lipid vesicles and from protoplasts should lead to a more complete view of the mechanism.

In this master's thesis, the objective is to contribute to the understanding of the perception mechanism of surfactin by the plasma membrane of root cells. More specifically, it will be focused on the development of an optimized protocol for the production of root protoplasts and the preparation of experimental procedures to use protoplasts in biophysical studies.

1.2 Plant plasma membrane organization

The plasma membrane (PM) is a complex structure that defines the spatial identity of cells and that separates intracellular and extracellular media (Simons and Sampaio, 2011). Therefore, the PM controls the entry and exit of molecules through the cell and is involved in cellular signal transduction events (Buchanan et al., 2015; Mamode Cassim et al., 2019). The PM is structured in a lipid bilayer whose lipids interact with proteins by hydrophobic and Coulomb forces (Casares et al., 2019). Lipids are amphiphilic molecules, which means that they have distinct hydrophobic and hydrophilic domains (Simons and Sampaio, 2011). Therefore, the lipid bilayer is organized in such a way that polar heads are facing the aqueous phase and non-polar tails are facing each other on the inside of the bilayer, minimizing their contact with aqueous environment.

Membrane lipids are less studied than membrane proteins, even though they are more abundant. As the average molecular mass of proteins is much higher than lipid one, the lipid-to-protein molar ratio in the plant PM ranges from 50 : 1 to 100 : 1 (Furt et al., 2011). Therefore, lipids are one of the major components of the PM.

Lipids are involved in many properties of the plant PM. Firstly, balance and bending movements of their hydrocarbon chains control the fluidity of the membrane by making it elastic. Lipids also preserve the structure of the PM by keeping its organization. Finally, some lipids are negatively charged, which causes the membrane to be electrostatically charged (Mamode Cassim et al., 2019).

1.3 Lipid composition in the plant plasma membrane

Three main classes of lipids constitute the plant PM: glycerolipids, sphingolipids and sterols (**Figure 1.1**).

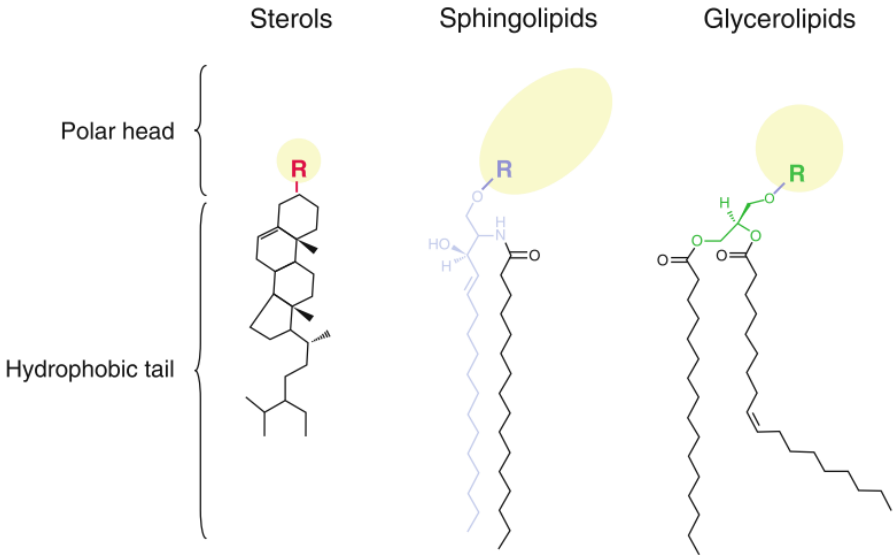

Figure 1.1: **Chemical structure of the three main classes of lipids in the plant plasma membrane.** (Furt et al., 2011)

Glycerolipids consist of a glycerol backbone carrying substitutes. This group is divided in two classes based on the composition of substitutes. There are phospholipids and non-phosphorous glycerolipids (Jouhet, 2013). Phospholipids consist of a glycerol backbone with two non-polar substitutes, forming the non-polar tail, and one polar substitute containing a phosphate group, forming the polar head. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the two major phospholipids, corresponding to 68-80 % of all plant structural phospholipids where palmitic (16:0) and linoleic (18:2) acids are the most abundant fatty acids (Donato et al., 2013; Furt et al., 2011). Phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidic acid (PA) represent all minor plant structural phospholipids (**Figure 1.2**). Major non-phosphorous glycerolipids are monogalactosyldiacylglycerol (MGDG), monoglucosyldiacylglycerol (MGlcDG), digalactosyldiacylglycerol (DGDG), diglucosyldiacylglycerol (DGlcDG), and sulfoquinovosediacylglycerol (SQDG) (Jouhet, 2013).

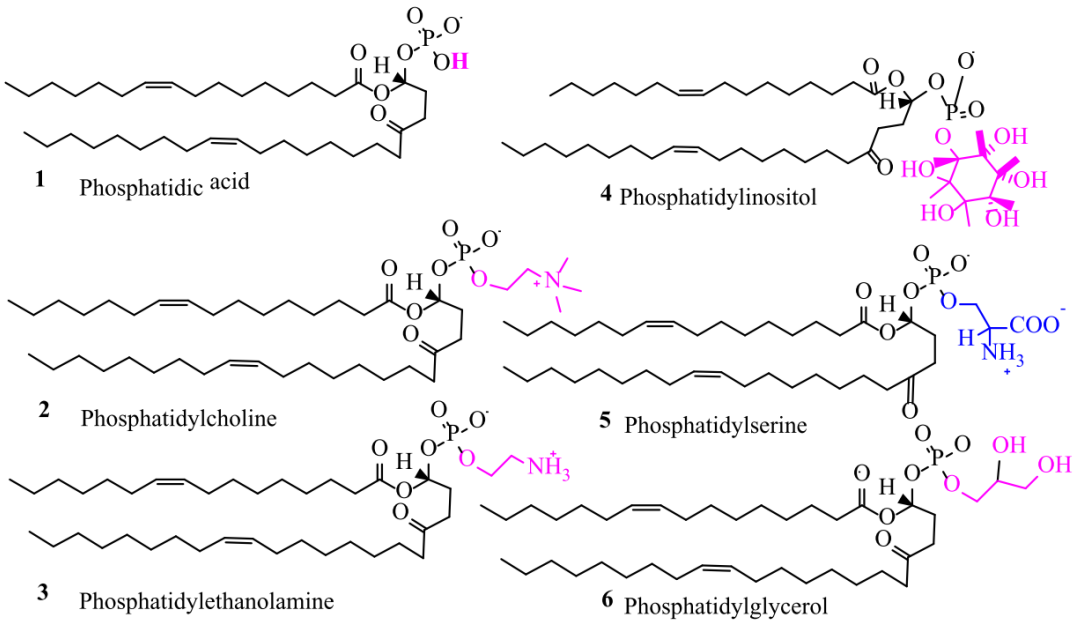

Figure 1.2: **Chemical structure of phospholipids.** 1. Phosphatidic acid (PA); 2. phosphatidylcholine (PC); 3. phosphatidylethanolamine (PE); 4. phosphatidylinositol (PI); 5. phosphatidylserine (PS); 6. phosphatidylglycerol (PG). (Alagumuthu et al., 2019)

Plant sphingolipids are amphiphilic molecules containing two hydrophobic chains and a polar head (Chen and B. Cahoon, 2009). They are formed with a long chain based (LCB), or sphingoid base, amidated by a fatty acid, generally in position 2 (Furt et al., 2011; Michaelson et al., 2016; Mongrand et al., 2010). This structure is denominated ceramide (Cer) and can be modified, resulting in more complex molecules (**Figure 1.3**). The complex polar head can contain up to 13 sugar moieties (Mongrand et al., 2010; Simon-Plas et al., 2011). The most common LCB in plants is phytosphingosine (Donato et al., 2013; Michaelson et al., 2016). Chain length of fatty acids of plant sphingolipids range from 16 to 26 carbon atoms, while plant LCBs contain 18 carbon atoms and two or three hydroxyl groups (Chen and B. Cahoon, 2009; Michaelson et al., 2016). There are among 500 different plant molecular species of sphingolipids (Furt et al., 2011), including free forms of LCBs and Cers and their more complex forms, also called complex sphingolipids, which are glucosylceramides (GlcCers) and glycosyl inositolphosphoceramides (GIPCs) (Chen and B. Cahoon, 2009; Michaelson et al., 2016). GlcCers and free Cers are considered as neutral molecules, while GIPCs are considered as charged molecules due to the charge of the phosphorous group (Chen and B. Cahoon, 2009). In GlcCers, the primary carbon of the LCB carries between 1 and 4 glycosyl residues. They represent approximately 34 % of total sphingolipids in *Arabidopsis thaliana* (Mamode Cassim et al., 2019). In GIPCs, the inositol phosphoryl ceramide (IPC) backbone consists of a phosphoinositol group attached to the primary carbon of the Cer backbone through a phosphoester bond (Chen and B. Cahoon, 2009). IPC is linked to a glucuronic acid (GlcA), which is linked to a sugar unit, forming series A GIPCs. Series B to G GIPCs can be formed with the addition of respectively three to seven sugar moieties such as glucosamine (GlcN), N-acetyl-glucosamine (GlcNAc), arabinose (Ara), galactose (Gal) and mannose (Man) (Mamode Cassim et al., 2019). GIPCs contain mostly saturated or mono-saturated fatty acid chains composed of 22-26 carbon atoms (Mongrand et al., 2010). In *Arabidopsis thaliana* leaves,

GIPCs are the most abundant sphingolipids, accounting for 60-65 % of total sphingolipids and they are not detected in animal cells (Chen and B. Cahoon, 2009; Furt et al., 2011; Yu et al., 2020).

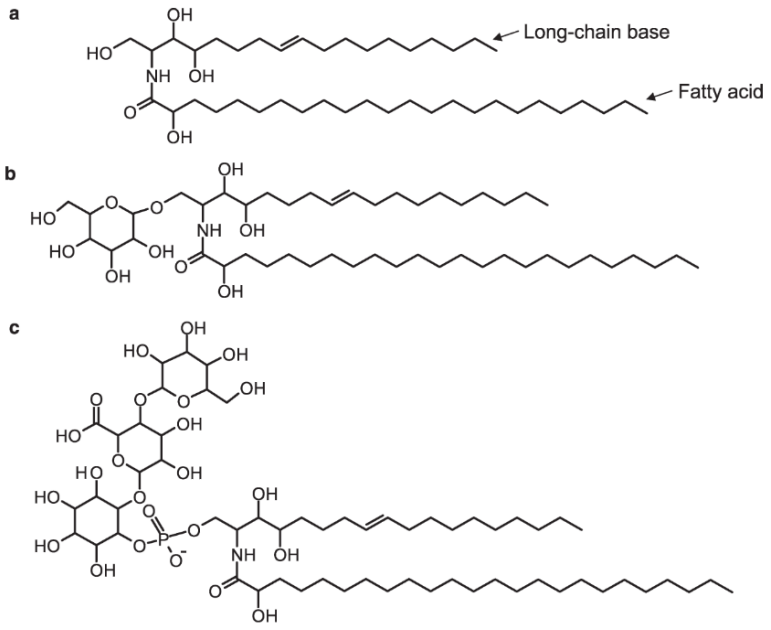

Figure 1.3: **Chemical structure of sphingolipids.** Chemical structure of (a) ceramide backbone, including a fatty acid and a C18 long chain base (LCB); (b) a glucosylceramide (GlcCer), including a glucose bound to the ceramide backbone; (c) hexose-hexuronic acid-inositolphosphoceramide, the major glycosyl inositolphosphoceramides (GIPCs) of *Arabidopsis thaliana*. (Chen and B. Cahoon, 2009)

Sterols belong to the family of isoprenoids (Furt et al., 2011; Hartmann, 1998). They represent approximately 30 mol % of total PM lipids (Mamode Cassim et al., 2019).

Sterols are organized in four rigid rings, formed by a cyclopentaperhydrophenanthrene moiety that carries a hydroxyl group at position 3, forming a polar head (Furt et al., 2011). The ring system also carries a side chain containing 8 to 10 carbon atoms attached to carbon 17 (Ferrer et al., 2017; Hartmann, 1998) (**Figure 1.4**). In opposition to animal and yeast membranes that contain only one species of sterols, respectively cholesterol and ergosterol (Furt et al., 2011), there are different sterol species in plant membranes. Those species differ according to the composition of the lateral chain and the number and the position of double bonds in the ring system (Furt et al., 2011).

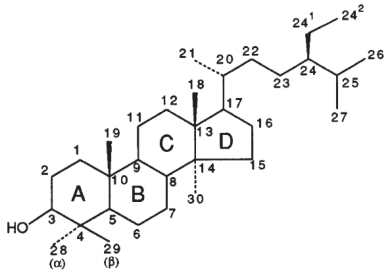

Figure 1.4: **Sterol backbone**. (Hartmann, 1998)

The majority of sterols present in the plant PM are free sterols, representing 70 to 90 % of total sterols (Furt et al., 2011). Sitosterol is a free sterol and is the most abundant sterol in most plant species, including *Arabidopsis thaliana* (Mamode Cassim et al., 2019), followed by stigmasterol and campesterol (Ferrer et al., 2017; Moreau et al., 2018) (**Figure 1.5**). Stanols are a subgroup of phytosterols and they are characterized by a saturated ring structure (Moreau et al., 2018) (**Figure 1.5**).

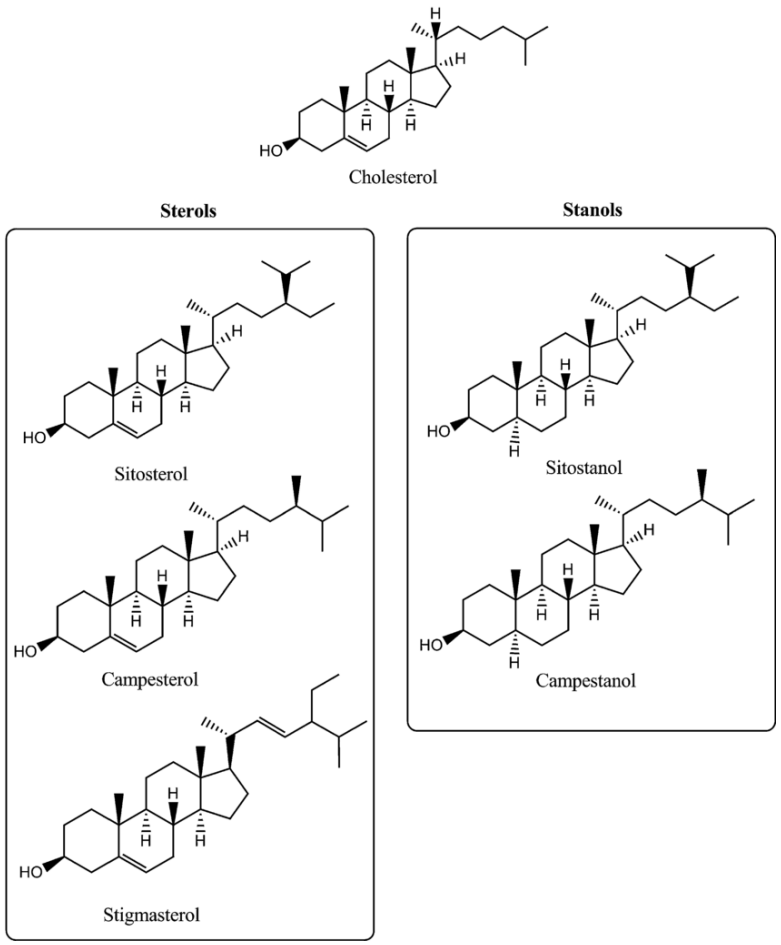

Figure 1.5: **Chemical structure of major plant sterols and stanols.** (Santas et al., 2013)

Phytosterols can also be modified to form more complex sterols. Steryl glycosides (SGs) are formed by acylation of phytosterols by a sugar, generally glucose, and acylated steryl glycosides (ASGs) are formed by acylation of SGs (Cacas et al., 2012; Furt et al., 2011). Sterol esters (SE) and hydroxycinnamate sterol esters (HSE) can be formed by esterification (Moreau et al., 2018) (**Figure 1.6**).

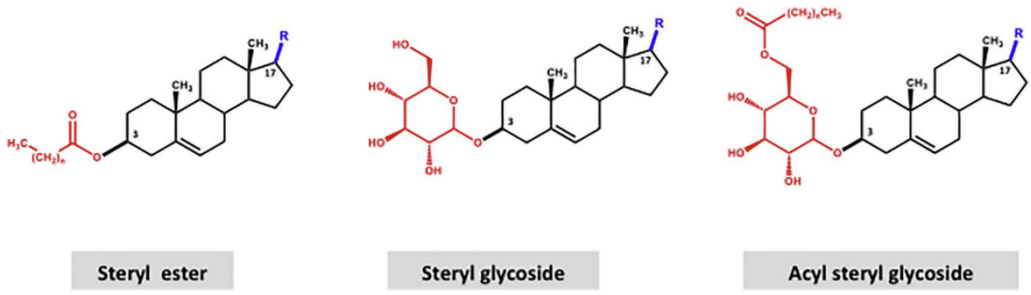

Figure 1.6: **Chemical structure of complex sterols** Chemical structure of (a) Steryl ester (SE), (b) Steryl glycoside (SG), (c) Acyl steryl glycoside (ASG). (Ferrer et al., 2017)

1.4 Lipid organization in the plant plasma membrane

In addition to the high molecular diversity of lipids in the PM, there are both lateral and transversal heterogeneity in the PM due to differences in the concentration and organization of proteins and lipids (Mamode Cassim et al., 2019).

First, the PM is laterally heterogeneous due to specific domains enriched with sphingolipids packed with sterols, called microdomains, present in both the inner and the outer leaflets (Furt et al., 2011; Mongrand et al., 2010).

The hydrophobic cores of sterols interact with aliphatic chains of phospholipids and sphingolipids (Cacas et al., 2012). Sterols and sphingolipids form subdomains in liquid-ordered (Lo) phase where sphingolipids are associated through polar and non-polar interactions with other sphingolipids and sterols, that allow the regulation of the fluidity and the permeability of the membrane (Furt et al., 2011; Hartmann, 1998). Grosjean et al. (2015) has shown that the relative amounts of each phytosterols strongly influence the plant membrane order. Stigmasterol, a planar unsaturated sterol, weakly promotes Lo phase formation and induces an increase of plant membrane fluidity and permeability, while campesterol provides greater plant membrane order (Grosjean et al., 2015). Lo phase is surrounded by a continuous liquid-disordered (Ld) phase, which is mostly composed of unsaturated phospholipids in interaction with sterols by Van der Waals bonds (Cacas et al., 2012; Furt et al., 2011).

Lo phase is highly compacted, which prevents detergent molecules from being inserted into the membrane (Mongrand et al., 2010). Therefore, Lo phase is also called detergent insoluble membrane (DIM). Compared to the PM, DIMs are enriched in SG, ASG, GIPCs, sterols and polyphosphoinositides, a minor phospholipid, even if DIMs are globally depleted in glycerolipids (Simon-Plas et al., 2011) (**Figure 1.7**). Also, there is an increase of saturated fatty acyl chains in DIMs in comparison to PM (Simon-Plas et al., 2011).

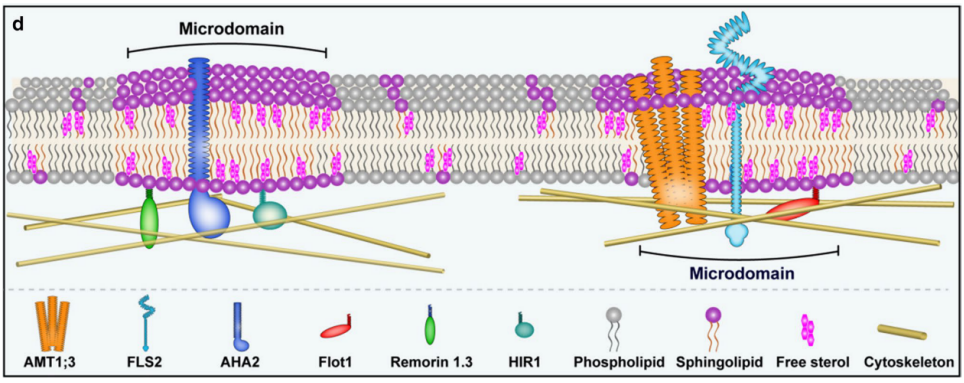

Figure 1.7: **Lateral heterogeneity in the plant plasma membrane.** (Yu et al., 2020)

Beside to the lateral heterogeneity, the PM has a difference in composition between the inner and the outer leaflets, also called transversal asymmetry (Mamode Cassim et al., 2019). The transversal asymmetry is well known in animal cell membranes, where the outer leaflet is composed of most of PC, sphingolipids and sterols, and the inner leaflet is composed of most of PS, PE, PI and minor phospholipids (Fujimoto and Parmryd, 2017; Mamode Cassim et al., 2019). Due to this asymmetry, the two leaflets display different physical properties. The outer leaflet has the highest average viscosity (Mamode Cassim et al., 2019). But only few publications discuss the asymmetry of lipids in the plant PM. It has been shown that the inner leaflet of the plant PM contains all of DGDG and most of phospholipids while the outer leaflet contains approximately 60 % of total sterols and most of sphingolipids, including GluCers (Cacas et al., 2016; Mamode Cassim et al., 2019). In addition, it has been supposed that GIPCs would be exclusively located in the outer leaflet and that PS and PI are exclusively present in the inner leaflet (Cacas et al., 2016; Mamode Cassim et al., 2019) (**Figure 1.8**).

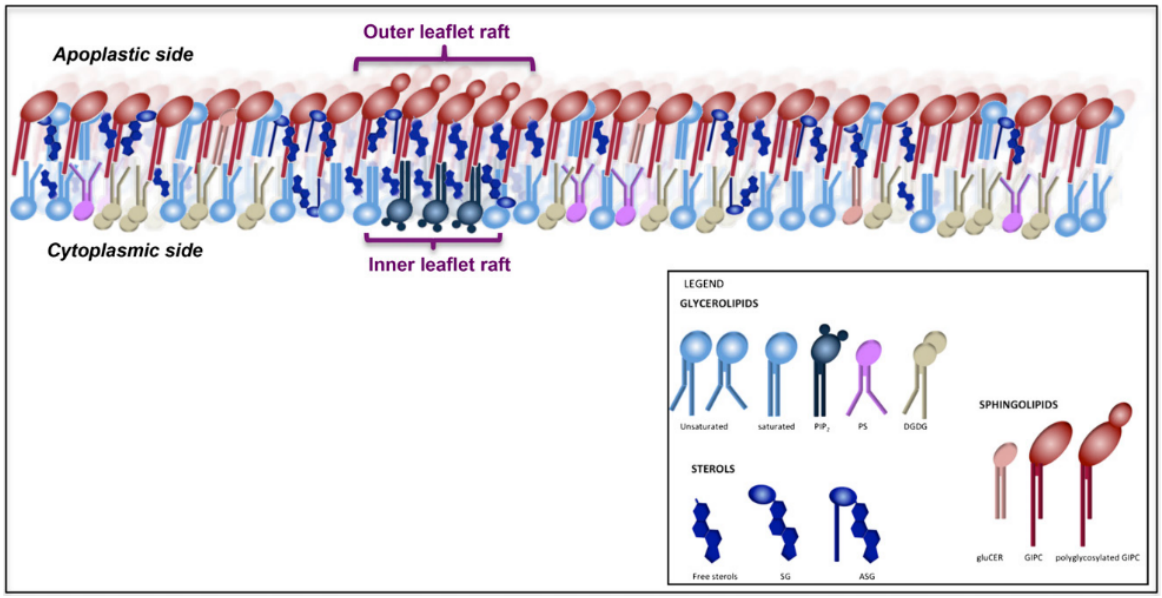

Figure 1.8: **Representation of the transversal asymmetry of the plant PM** Model for the organization of lipids in tobacco PM presenting transversal asymmetry. (Cacas et al., 2016)

1.5 Membrane models

Membranes are highly organized and contain a wide variety of lipids and proteins, which makes them very complex. Therefore, studying membrane properties and conducting experiments on membranes can be difficult, and biomimetic models are required. Biomimetic models are artificial simplified membrane models used to mimic biological membranes, with similar lipid composition and organization (Deleu et al., 2014; Eeman and Deleu, 2010).

Lipid monolayers, supported bilayers and liposomes are the most widely known biomimetic models (**Figure 1.9**).

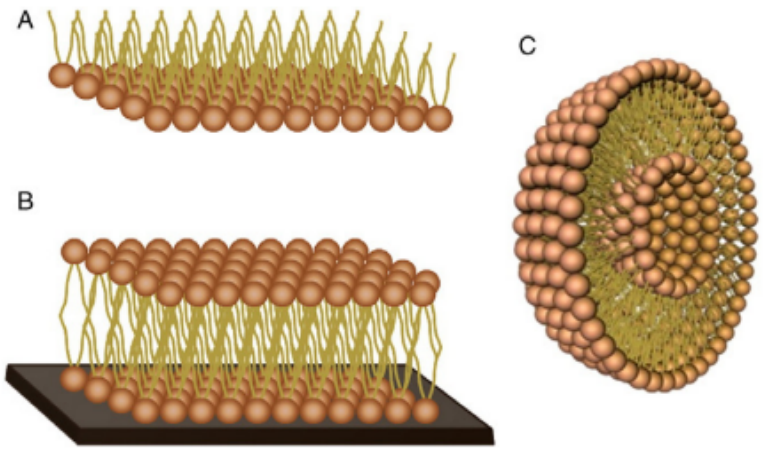

Figure 1.9: **Representation of biomimetic models** of (A) a lipid monolayer, (B) a supported lipid bilayer, and (C) a liposome. (Deleu et al., 2014)

1.5.1 Lipid monolayers

Lipid monolayers consist in half the bilayer of biological membranes (Deleu et al., 2014; Kök et al., 2019) (**Figure 1.9 A**). They are formed by spreading lipids, amphiphilic molecules, at an air/water interface (Eeman and Deleu, 2010; Kök et al., 2019). They are generally used to study the interaction between lipids of the membrane outer layer and exogenous molecules. They are also useful to study the insertion of amphiphilic compounds into the membrane (Eeman and Deleu, 2010). One of the main advantages of this model is that biological conditions of the membrane, including pH, ionic strength, temperature and lipid composition, can be easily mimicked even if they do not reflect the complexity of biological membrane structure (Deleu et al., 2014; Eeman and Deleu, 2010; Kök et al., 2019).

1.5.2 Supported lipid bilayers

Supported lipid bilayers (SLBs) consist of a lipid bilayer deposited on a solid surface, such as mica, glass or silica wafers (Eeman and Deleu, 2010) (**Figure 1.9 B**). Many techniques exist to create SLBs, such as Langmuir-Blodgett/Schäfer deposition, vesicle fusion, solvent-assisted lipid bilayer (SALB) method or spin coating (Hardy et al., 2012, 2013; Kilic and Kok, 2016; Ferhan et al., 2019). For the Langmuir-Blodgett/Schäfer deposition technique, a lipid monolayer is firstly transferred from the

air-water interface of a Langmuir trough onto a solid support. Then, the same support is immersed a second time through a second monolayer to form a lipid bilayer fixed on a solid support (Eeman and Deleu, 2010; Hardy et al., 2013; Kök et al., 2019) (**Figure 1.10 a**). The vesicle fusion technique consists briefly in the adsorption of a lipid vesicle on a solid surface through hydrophobic interactions followed by the vesicle rupture and fusion, leading to the formation of a lipid bilayer fixed on the surface (Eeman and Deleu, 2010; Hardy et al., 2013; Kök et al., 2019) (**Figure 1.10 b**). In SALB method, lipids are added in an organic solvent on a solid support. Then, the organic solvent is exchanged with an aqueous buffer, leading to the formation of micelles and lipid vesicles, forming a lipid bilayer on the surface by hydrophobicity (Ferhan et al., 2019) (**Figure 1.11**). In the spin coating technique, membrane lipids are dissolved in an organic solution, which is applied on a spin-coater, forming a lipid bilayer on the solid support (Mennicke and Salditt, 2002).

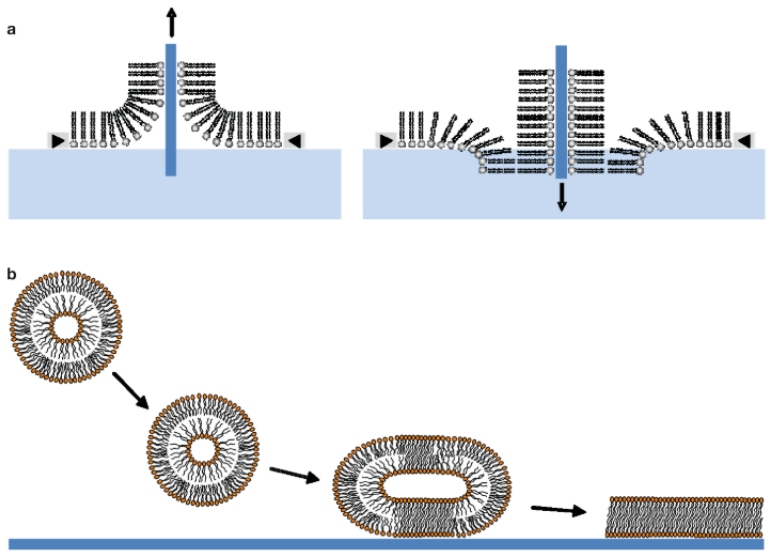

Figure 1.10: **Supported lipid bilayer formation processes.** SLBs formation process using (a) Langmuir-Blodgett/Schäfer deposition technique and (b) vesicle fusion technique. (Eeman and Deleu, 2010)

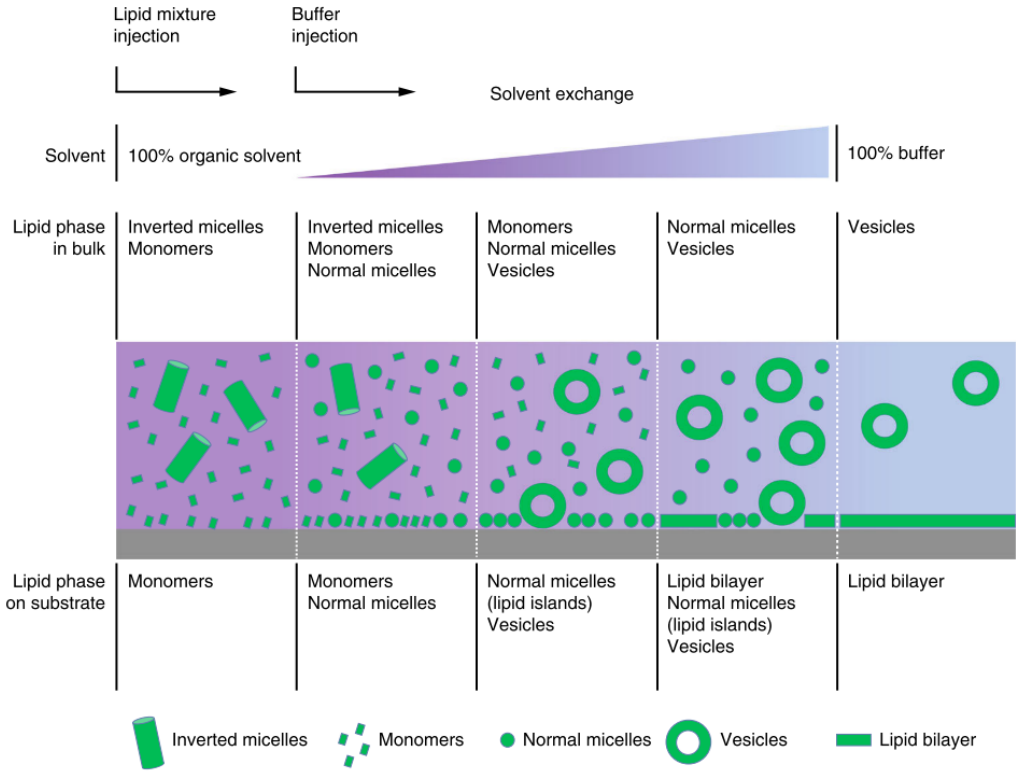

Figure 1.11: **Supported lipid bilayer formation process using solvent-assisted lipid bilayer (SALB) method.** (Ferhan et al., 2019)

SLBs are preferred over lipid monolayers due to their structure and their ability to better mimic biological membranes bilayers (Kök et al., 2019). Their preparation is easy and stable (Hardy et al., 2013; Eeman and Deleu, 2010). In addition, as they are fixed on a solid surface, their characterization is easier than floating vesicles and can be measured with more sensitive techniques (Deleu et al., 2014; Eeman and Deleu, 2010; Hardy et al., 2013). But one of the main drawbacks of this technique is that the proximity between the lipid bilayer and the solid surface may affect the membrane properties of the model, such as the mobility of membrane lipids and proteins or the incorporation of transmembrane proteins (Eeman and Deleu, 2010). In addition, SLBs are not suitable for the incorporation of large transmembrane proteins (Kilic and Kok, 2016).

SLBs are commonly used to investigate molecular organization of biological membranes and interactions between membrane lipids and other compounds, such as drugs (Eeman and Deleu, 2010; Kök et al., 2019).

1.5.3 Liposomes

Liposomes, also called lipid vesicles, consist in a membrane bilayer organized in a spherical vesicle containing a small aqueous compartment, similarly to cell membranes (Deleu et al., 2014; Eeman and Deleu, 2010) (**Figure 1.9 C**). They can contain one or more bilayers, and they are respectively called unilamellar (ULV) and multilamellar (MLV) lipid vesicles (**Figure 1.12**). ULVs are classified in three classes according to their size: (i) small unilamellar vesicles (SUV) with a size ranging from 20 to

100 nm, (ii) large unilamellar vesicles (LUV) with a size ranging from 100 to 1000 nm and (iii) giant unilamellar vesicles (GUV) with a size bigger than 1000 nm. MLVs are vesicles with a size bigger than 500 nm (Deleu et al., 2014; Isalomboto Nkanga et al., 2019).

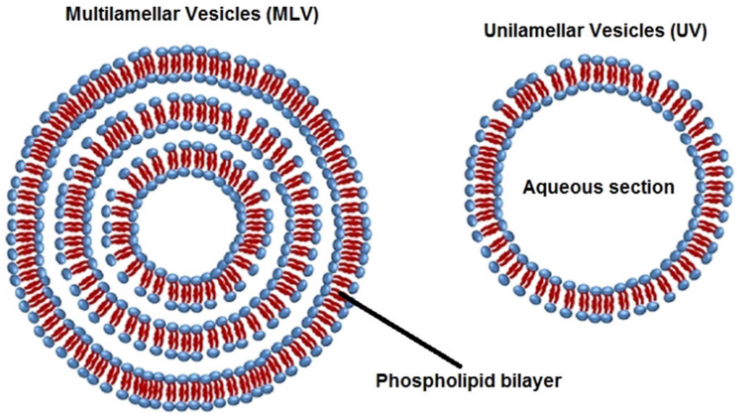

Figure 1.12: **Representation of multilamellar and unilamellar lipid vesicles.** (Sheikhpour et al., 2017)

In order to form liposomes, firstly lipids are dissolved in organic solvents, then the solution is dried, leading to the formation of a film, which is either gently or harshly hydrated, leading to the formation of GUVs or MLVs respectively (Isalomboto Nkanga et al., 2019). From obtained MLVs, SUVs or LUVs can be formed by sonication or extrusion. Beforehand, the size of MLVs can be reduced and homogenized by performing freeze-thaw cycles or by prefiltering the suspension (Eeman and Deleu, 2010). GUVs can also be obtained with the electroformation method, where an external electric field is applied on a dry lipid film (Pereno et al., 2017).

In general, liposomes are used to study membrane behaviors and interactions between membrane molecules and other compounds (Kök et al., 2019). In addition, liposomes are widely used in the biomedical field, for example as drug delivery systems due to its ability to encapsulate molecules (Eeman and Deleu, 2010; Isalomboto Nkanga et al., 2019).

The formation of symmetrical liposomes is an important drawback of the above techniques, while a lipid asymmetry is found in biological membranes. In addition, the final lipid composition can be slightly different from the initial lipid mixture used for vesicles formation. Furthermore, liposomes are not stable and aggregation, fusion or the formation of two separated phases may occur (Eeman and Deleu, 2010). More realistic models can be produced by forming asymmetric vesicles, using methyl-B-cyclodextrin- or cyclodextrin-mediated lipid exchange (Doktorova et al., 2018) , emulsion technique or microfluidics systems (Deshpande and Dekker, 2018; Lu et al., 2015). Despite their difficulty in preparation, they can better mimic the biological membrane (Kök et al., 2019).

1.5.4 Limitations of biomimetic models

Firstly, it is important to keep in mind that biomimetic systems are simplified models of the biological membrane and they do not represent the whole complexity of the membrane. Therefore, results of analysis on biomimetic membranes may differ slightly from those of biological membranes (Eeman and Deleu, 2010).

Secondly, the number of components, including lipids and proteins, that can be included in biomimetic membranes is limited compared to biological membranes. In addition, it is relatively difficult to reconstitute proteins in biomimetic models (Eeman and Deleu, 2010). However, some biomimetic models contain proteins able to conserve their biological activity, they are called proteoliposomes (Li et al., 2011).

Another important limitation is that lipid asymmetry between the two leaflets as in biological membrane has not been fully mimic while it plays an important role in the structure of the membrane and in the signaling transduction (Eeman and Deleu, 2010).

1.5.5 Protoplasts

Definition

As biomimetic models have some limitations, it is interesting to carry out experiments directly on biological membranes to obtain additional information and to confirm some results obtained with biomimetic models. The two types of models are therefore useful and complementary.

In plants, all cells have a cell wall, which protects the PM. Living cells that no longer have their cell walls but still have their PM are called protoplasts (Aoyagi, 2011). Protoplasts are equivalent to cultured cells but one of the differences is that protoplasts are totipotent, which means that they are undifferentiated (Davey et al., 2005).

Preparation

Plant protoplasts can be isolated from a large variety of tissues and organs, such as leaves, petals, roots or fruits (Fowke and Constabel, 1989). In order to isolate protoplasts, cell walls must firstly be removed, either with mechanical or enzymatic methods. With the mechanical method, cell walls are removed by dissection but the yield is generally insufficient. Therefore, the enzymatic method is the most common one. In that case, the tissue is brought into contact with the enzymatic solution, containing, among other things, pectinase and cellulase (Fowke and Constabel, 1989). Since cells are no longer protected by cell walls, the wall pressure must be replaced by an osmotic pressure to prevent cell bursting. Osmotic pressure can be induced by adding mannitol, sorbitol, glucose or sucrose to solutions (Bengochea and Dodds, 1986; Fowke and Constabel, 1989).

After protoplast isolation, protoplasts must be purified to separate them from cellular debris. The solution containing the plant material and enzymatic solution is filtrated through nylon mesh to remove large debris, consisting of undigested tissues and cell clumps (Bhojwani and Razdan, 1996;

Fowke and Constabel, 1989). Then the solution is centrifuged. Intact protoplasts are kept in the pellet while the supernatant containing small debris and enzymes is discarded. Afterwards, protoplasts are washed with a salt solution to remove the rest of enzymes, debris and broken protoplasts (Bhojwani and Razdan, 1996; Fowke and Constabel, 1989).

Viability characterization

Once protoplasts are isolated and purified, viability tests must be performed to test the ability of protoplasts to work successfully and to be regenerated into an entire plant. Different kinds of experiments are used to test protoplast viability.

The first type is dye-based methods. Fluorescein diacetate (FDA) is a dye which accumulates in PM of viable protoplasts and which can be detected by fluorescence microscopy (Fowke and Constabel, 1989). As described by Grimm et al. (2013), FDA is a non-fluorescent molecule able to cross the biological membrane. It is an enzyme substrate for esterases, living inside living cells. Once FDA has been hydrolyzed, the molecule become fluorescent (**Figure 1.13**). It is one of the most frequently used methods to measure protoplasts viability. Evans blue and phenolsafranine are dyes which can enter broken and dead protoplasts (Fowke and Constabel, 1989). Dead protoplasts turn red in presence of phenolsafranine (Bengochea and Dodds, 1986). Calcofluor white is a dye able to stain cell walls of cell-wall-regenerating protoplasts (Pilet, 1985). It is characterized by a ring of fluorescence around the PM (Bengochea and Dodds, 1986). Other fluorochromes can be used to detect the presence or absence of nuclei, the initiation of the cell cycle or other typical living cell phenomena (Pilet, 1985).

Secondly, respiratory metabolism can be estimated by measuring oxygen uptake by using oxygen electrodes, which gives an indication of protoplast viability (Bhojwani and Razdan, 1996).

Thirdly, metabolism activity can be estimated by microscopically observing cytoplasmic streaming (Bhojwani and Razdan, 1996). It is a movement of cytoplasm assisting the delivery of nutrients, metabolites and other materials to all parts of the cell and, therefore, allowing the evaluation of protoplast viability (Liu et al., 2017).

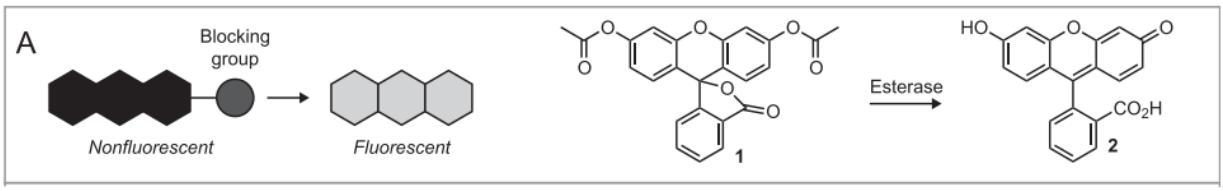

Figure 1.13: **Mode of fluorescence of fluorescein diacetate (FDA).** (Grimm et al., 2013)

It is important to know that some factors affect yield and viability of protoplasts (Bhojwani and Razdan, 1996). The first one is the source of materials. Leaves are the best sources of plant protoplasts because they can provide a large number of protoplasts without necessarily killing the plant. The second factor is the presence of pre-enzyme treatments, including peeling the epidermis of leaves or cutting tissue into small strips. It facilitates the penetration of enzymatic solution into the tissue, which increases protoplast yield. It has also been proven that agitation of the mixture during enzyme

treatment improves protoplast yield. The third factor relates to the enzyme treatment. The protoplast yield depends strongly on the nature and concentration of enzymes. Two enzymes are used: cellulase, which can digest the cellulosic cell wall, and pectinase, which degrades the middle lamella which joins cell walls of different cells together. Finally, protoplast viability depends strongly on the osmolarity of media.

Culture methods

The culture of isolated protoplasts can be difficult because protoplasts are fragile and require an adapted adjustment of osmolarity in the culture medium and cannot be shaken too much (Fowke and Constabel, 1989). Different culture systems have been developed to meet the needs of protoplasts. They use liquid and semi-solid media.

In the liquid category, the most common method is the liquid culture in Petri dishes. In this method, protoplasts are suspended in a small volume of liquid medium and placed in Petri dishes sealed with Parafilm® to reduce water losses (Fowke and Constabel, 1989).

A second type of liquid culture is the drop culture consisting of small drops (40 to 200 µL) of protoplast suspension placed on the inner side of the lid of a Petri dish (**Figure 1.14 d**). Therefore, drops are turned upside down (Fowke and Constabel, 1989). The Petri dish contains mannitol solution and is sealed with Parafilm® to prevent medium evaporation (Bengochea and Dodds, 1986).

A third one is the microchamber culture, very similar to the drop culture. In this case, a small volume of protoplast suspension is added in microchambers on slides for protoplast culture. They offer a better optical view and an adequate oxygen supply (Fowke and Constabel, 1989). Lots of chamber designs are possible but the simplest consists in dropping a droplet between two cover slips sealed with a mineral oil ring, preventing medium evaporation (Bengochea and Dodds, 1986) (**Figure 1.14 c**).

A fourth type of liquid culture is the multiple drop array technique using the same concept as drop cultures, except that drops are smaller (40 µL), which allows to place up to 50 drops per Petri dish (Fowke and Constabel, 1989).

A last one is the microdroplet culture also using the same concept as drop culture but the drop volume is reduced to 0,25 to 0,50 µL so that each drop contains only one protoplast (Fowke and Constabel, 1989).

Besides the liquid culture, there are also cultures using two types of semisolid media. The first one uses agar as gelling agent and is the most common. Agar is directly added to protoplast suspension in appropriate concentration to create a soft agar gel (Fowke and Constabel, 1989). Second, agarose or alginate can be used as gelling agent, which has demonstrated similar plating efficiency as agar. Agarose has shown the best results in terms of retention of viability and secondary product production compared to other gelling agents, probably due to the more neutral charge of agarose polymers. Protoplasts can be directly applied on solidified agarose in Petri dishes (Fowke and Constabel, 1989) (**Figure 1.14 a**). Finally, liquid and solid media can be combined. In gel embedded protoplast cultures, agarose plating and bead cultures are combined, which improves plating efficiency of protoplasts. Protoplasts are incorporated into the culture medium, then small blocks of gelled agar or agarose are transferred into the liquid culture medium (Fowke and Constabel, 1989) (**Figure 1.14**

b).

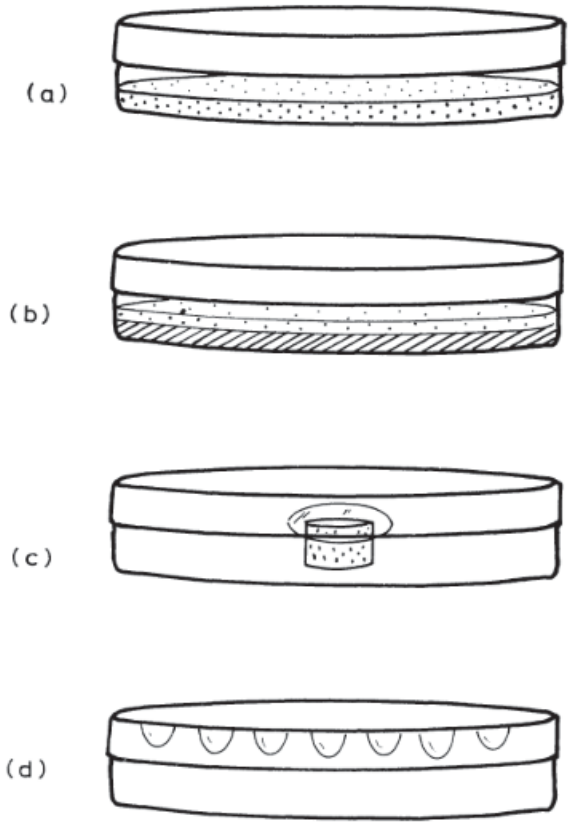

Figure 1.14: **Different methods of protoplast culture** (a) Agar embedding; (b) Liquid culture poured over culture media; (c) Microchamber culture constructed of a small plastic ring sealed with oil and a cover slip; (d) Hanging drop culture. (Bengochea and Dodds, 1986)

Applications

Plant protoplasts have several applications. First, due to their totipotency properties, plant protoplasts are capable of regenerating an entire organism from a single cell (Davey et al., 2005).

In addition, protoplast fusion technique can be used, where protoplasts from two different plant species are fused, leading to a novel somatic hybrid. This fusion can be induced chemically, by using fusogens such as polyethylene glycol (PEG), or electrically, by applying a current. Those transformed protoplasts can then regenerate whole organisms presenting the induced genetic modifications (Davey et al., 2004).

Furthermore, protoplasts are commonly used as genetic tools. DNA can be introduced into plant protoplasts by using PEG, electroporation or microinjection (Newell, 2000), therefore able to regenerate an entire organism presenting this modification.

Another important application of plant protoplasts is their ability to produce useful metabolites. Plant cells have already been used for this purpose, but metabolites that are produced are stored

within the cells. It means that cells must be disintegrated to extract and purify metabolites, which makes the process complex and expensive. To overcome those problems, plant protoplasts can be used. The advantage of metabolites production by protoplasts is that compounds are directly released in the culture medium due to the absence of cell walls, so downstream processing is much easier. The main drawbacks of this technique are that protoplasts are very fragile and that they cannot be used for long-term production because cell walls are easily regenerated. Those problems can be countered by using an "immobilization matrix", which is an artificial cell wall that provides stability to protoplasts in addition to an inhibitor of cell walls synthesis (Aoyagi, 2011).

Protoplasts can also be used to isolate and characterize the PM. Once cells are disrupted, the PM will rearrange to form vesicles which can be separated from other cellular components by centrifugation (Fowke and Constabel, 1989).

And finally, most importantly in the context of this master's thesis, protoplasts are useful to study different biological processes. For instance, protoplasts are used to understand the effects of an increase of salt concentration in extracellular solution on membrane channels (Jiang et al., 2019; Fuchs et al., 2005). Protoplasts were also used to understand the early signaling pathway of some receptors in presence of microbe-associated and pathogen-associated molecular patterns (MAMPs and PAMPs), such as flagellin (Asai et al., 2002; Gong et al., 2019) and their effects on membrane channels and ion influxes and effluxes (Gong et al., 2019; Kurusu et al., 2013; Wu et al., 2020).

Jouanneau et al. (1991) measured the defense responses of tobacco protoplasts when they were in contact with exogenous elicitors from the cell wall of *Phytophthora megasperma glycinea* (Pmg). Defense reactions are evaluated by measuring β-glucanase synthesis. β-glucanase is an enzyme that digests glucans. It is a pathogenesis-related (PR) protein, which is able to defend plants against fungal pathogens by hydrolyzing the cell walls of fungal pathogens (Balasubramanian et al., 2012). Using protoplasts, Jouanneau et al. (1991) have also demonstrated that the expression of β-glucanase induced by elicitors depends on auxin concentration.

The study of Bach et al. (1993) about the effect of a fungal elicitor prepared from the culture medium of *Pythium aphanidermatum* on protoplasts of carrot cells is another example of the usefulness of protoplasts for plant defense study. When protoplasts were in contact with the elicitor, they released 4-hydroxy-benzoic acid (4-HBA) in the culture medium. In addition, a fast increase in Ca^{2+} influx and in K^+ efflux precedes this secretion.

1.5.6 Protoplast limitations and similarities between cell and protoplast membranes

Even though protoplasts are useful models to study plant stresses, they have some limitations. First, the model is not fully comparable to a normal cell, because cell walls and plasmodesmata have been removed and cell-cell interactions in the tissue have been interrupted (Sheen, 2001). In addition, it has been demonstrated that, in *Arabidopsis thaliana* leaves, the lipid composition of protoplasts

membranes differs slightly from the lipid composition of cells, which may impact biological processes (Browse et al., 1988).

Nevertheless, several studies have compared the biological processes and responses taking place in complete plant cells and protoplasts. The observation of a response to stress in protoplasts similar to the response observed in intact plant is important to indicate that protoplasts are relevant to study biological processes in plants despite their small differences with complete cells.

Such observations were noted when comparing kinase activation profiles in *Arabidopsis* cell suspension, seedlings and protoplasts under hyperosmotic and saline stresses (Boudsocq et al., 2004). The three different cell types presented similar profile in the activated protein kinases following hyperosmotic stress, confirming the suitability of protoplasts to study response to osmotic stress.

Regarding the immune responses due to elicitation, several studies also highlighted the ability of protoplasts to respond to several elicitors comparably to intact plant cells. In terms of gene expression, treatment of *Arabidopsis* protoplasts with flg22, a fragment of the bacterial flagellin (Jelenska et al., 2017), induced the expression of specific genes also induced in leaf cells (Asai et al., 2002). Similar behavior between protoplasts and intact plants has also been observed concerning the phosphorylation of CERK1, a pattern-recognition receptors (PRR) co-receptor, when plants and protoplasts were exposed to flg22 peptide, elf18 peptide or pep1 (Gong et al., 2019). Concerning the production of hydrogen peroxide, the response to COS-OGA, an elicitor able to induce plant defense-signaling pathways, in cell suspensions and protoplast suspensions both resulted in a strong increase in hydrogen peroxide production after elicitation (Ledoux et al., 2014). Lastly, the calcium signature observed in *Arabidopsis thaliana* leaf protoplasts was shown to be representative of the one observed in seedlings (Maintz et al., 2014). Indeed, when comparing the kinetics of cytoplasmic calcium concentration after induction with flg22, elf18, Pep1 or chitin, the obtained curves in protoplasts and seedlings were comparable even though some small variations were observed with Pep1 and chitin. However, after the elicitation with lipopolysaccharides, the variation of cytosolic calcium did not followed the same trend in seedlings and protoplasts but these differences might be explained by the different age of seedlings and leaves. In addition, Maintz et al. (2014) studied the effect of two channel blockers, lanthanum chloride ($LaCl_3$) and staurosporine, on the variation of cytoplasmic calcium in protoplasts and seedlings. In both cases, a typical sigmoidal dose-response curve was observed but protoplasts showed a tendency to be more sensitive to lower inhibitor concentrations, leading to a lower half-maximal inhibitory concentration. Nevertheless, the authors considered that protoplasts are suitable models to investigate MAMP-/DAMP- (damage-associated molecular pattern) induced changes in cytosolic calcium concentration. Their use may reduce the experimental variation and produce more reproducible results as protoplasts are more homogeneous and adjustable and less complex than the entire organism.

Finally, (Grosjean et al., 2018) demonstrated that the cell wall did not affect the dynamics and the ordered domain organization of the plant PM. The red-to-green ratio of membrane fluorescence (RGM) was similar between tobacco protoplasts devoid of cell wall and tobacco protoplasts carrying a freshly

regenerated cell wall. In addition, similar sizes of ordered domains between tobacco protoplasts, before and after cell wall regeneration, were measured.

In conclusion, even if protoplasts are not complete cells, they can have similar stress responses as intact cells in terms of activation of phosphorylation cascades, reactive oxygen species (ROS) production and calcium signature.

1.6 One example of elicitor : Surfactin

As mentioned above, the PM controls the entry and exit of molecules into and out of the cell and is involved in cellular signal transduction events (Buchanan et al., 2015; Mamode Cassim et al., 2019). Thanks to membrane models, entrance mechanism of molecules, including elicitors, can be investigated.

Surfactin is an elicitor that belongs to one of the main classes of lipopeptides produced by several *Bacillus subtilis* strains (Eeman et al., 2006; Henry et al., 2011; Cawoy et al., 2014; Deleu et al., 2003). It is synthesized by non-ribosomal peptide synthetases (NRPS) (Henry et al., 2011). Surfactin is a cyclic heptapeptide linked to a C12 to C16 fatty acid chain by a lactone ring (Debois et al., 2015; Eeman et al., 2006; Deleu et al., 2013). This molecule is known for its antiviral, antibacterial and haemolytic activities (Debois et al., 2015; Deleu et al., 2003, 2013). More interestingly in the context of this master's thesis is that surfactin was demonstrated to induce the defense-related early responses in plants (Henry et al., 2011; Deleu et al., 2013; Debois et al., 2015; Cawoy et al., 2014).

Henry et al. (2011) studied the interaction of surfactin with plant PM and its little-known recognition process. The induction of the defensive state was verified by the accumulation of ROS extracellularly and in the cytoplasm of induced cells. It was demonstrated that the recognition of surfactin depended on the negative charge, the structure of the peptide cycle and the length of the fatty acid chain of surfactin. In addition, by using isothermal titration calorimetry (ITC), it was demonstrated that surfactin has a strong affinity for membrane lipids, due to their hydrophobic interactions. Among these lipids, it was also shown that surfactin does not have a particular affinity for plant sterols, probably because they restrict the access to the inner side of the membrane.

Other studies focused on the interaction of surfactin with the PM. Shen et al. (2010) suggested that surfactin can enter the outer leaflet and solubilize phospholipids, only if surfactin concentration is higher than its critical micelle concentration (cmc). Surfactin can remove phospholipids and form pores through the PM. Eeman et al. (2006) studied the ability of surfactin to enter lipid monolayers. It was demonstrated that the nature of phospholipids, including the polar head charge and the chain length, and the nature of the lipopeptide, including the nature of the peptide moiety and the chain length, has an influence on surfactin penetration into the lipid monolayer. Maget-Dana and Ptak (1995) investigated the ability of surfactin to penetrate monolayers constituted of different phospholipids. It would appear that the ability of surfactin to penetrate the monolayer depends on the charge of phospholipids. Negatively charged phospholipids would prevent the peptide cycles of surfactin from being close to the phospholipids head groups. Similarly, Razafindralambo et al. (2009) studied the

effect of structural changes of surfactin on its binding affinity to phospholipid vesicles. It was demonstrated that the binding affinity between surfactin and lipid vesicles increases when surfactin is cyclic rather than linear, when the number of negative charges decreases or when the chain length increases. However, the entrance mechanism of surfactin remains little-known and further experiments must be carried out in order to better understand this mechanism.

Chapter 2

OBJECTIVES

The complexity of biological processes, like the perception of exogenous molecules by plant cells, requires the use of membrane models to decipher the mechanism at a molecular level. Among membrane models, protoplasts are one of the most reliable as they retain the complexity of the biological PM comprising lipids and proteins while being devoid of the cell wall (Aoyagi, 2011) which could complicate the interpretation of the results. Therefore, protoplasts are particularly suitable to investigate the perception process of elicitors by the PM.

Surfactin, a well-known elicitor, is a lipopeptide produced by the soil bacteria *Bacillus subtilis*. It is known to induce the plant defense response when it is in contact with the roots of the plant (Henry et al., 2011; Cawoy et al., 2014). It could therefore represent an interesting alternative to conventional pesticides. However, the entrance mechanism of this molecule within the cell through the membrane is not widely known. It has been suggested that surfactin could enter the cells via an uncommon mechanism involving the lipid fraction of the PM and not via a proteic receptor as it was shown for other elicitors like flagellin. The use of protoplasts to further explore the particular mechanism of the perception of surfactin by the root cells is of particular interest to obtain information about the potential lipid specificity of the interaction and about the effect of surfactin on the PM organization and how it is related to the triggering of the plant defense response.

The first objective of this master's thesis is to develop and optimize a protocol for tomato root protoplast isolation. This protocol should lead to a high yield of production of protoplasts with a high viability. Once this protocol will be optimized, a methodology will be developed to perform isothermal titration calorimetry (ITC) measurements on protoplasts. Indeed to the best of our knowledge, ITC has never been applied to living cells or protoplats. Since protoplasts are fragile cells, prone to explode, it is important to find the best condition parameters not to alter the viability of protoplasts during the ITC measurement. In addition, protoplasts will be used to perform flow cytometry measurements.

After the development of the different methods, the second objective of this master's thesis is to use protoplasts to first characterize their interaction with surfactin, by using ITC and secondly to analyze their reactivity in terms of early defense response by measuring the ROS generated upon their interaction with surfactin.

Chapter 3

MATERIALS AND METHODS

3.1 Plant material

3.1.1 Plant specie choice

Tomato (*Solanum lycopersicum*) was selected as plant model due to its short cycle of crop, its capacity to produce a significant quantity of biological material, its ease of in vitro culture and because tomato plants are well studied and used as a plant model (Zorzoli et al., 2007).

3.1.2 Tomato plant culture

Seed sterilization

First, tomato seeds are placed in a Falcon tube containing 5 mL of 75 % ethanol and held under agitation for 2 minutes. Then, the ethanol solution is discarded. A bleach solution is prepared with 1.7 mL of bleach, 8.3 mL of sterile water and one drop of tween-20. Then, 5 mL of this bleach solution is added on seeds, which are held under agitation for 10 minutes. The bleach solution is discarded and seeds are rinsed with sterile water at least 3 times until there is no more foam formation (**Figure 3.1**).

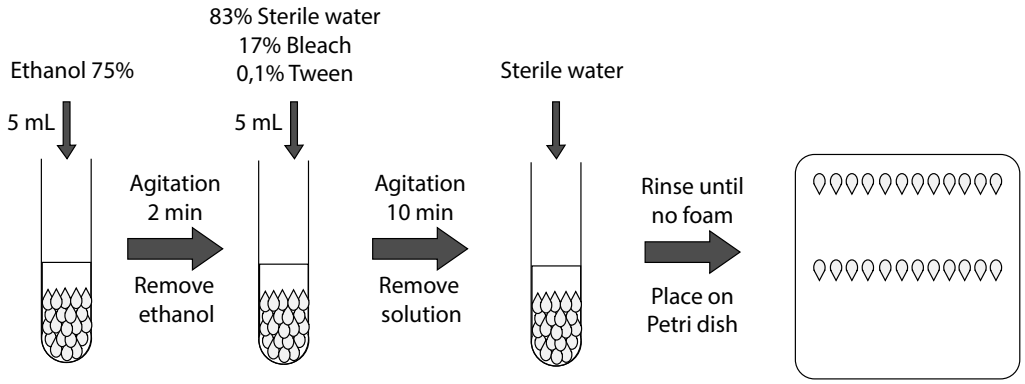

Figure 3.1: **Seed sterilization method**

Culture on Petri dishes

20 sterilized tomato seeds are deposited on square Petri dishes containing a solid agar solution, composed of Murashige and Skoog (MS) medium (Duchefa Biochemie), including 2,15 g/L of vitamins, 10 g/L of sucrose and 10 g/L of agar. Then, Petri dishes are placed under lights with a photoperiod of 12 hours at room temperature.

3.2 Tomato root protoplast isolation

3.2.1 Preparation of solutions

Enzymatic solution

Enzymatic solution (ES) was prepared by dissolving 400 mM of mannitol, 20 mM of KCl, 10 mM of $CaCl_2$, 0.1% of BSA, 1.5% of cellulase (Onozuka R-10)(Duchefa Biochemie) and 0.4% of pectinase (macerozyme R-10)(Duchefa Biochemie) in 20 mM MES buffer at pH 5.7 (Evrard et al., 2012; Mastuti and Rosyidah, 2018). The solution is prepared just before the experiment.

Saline washing solution

The saline washing solution (S1) was prepared by dissolving 154 mM of NaCl, 5 mM of KCl and 125 mM of $CaCl_2$ in 2 mM MES buffer at pH 5.7 (Mazarei et al., 2008) The solution is stored at 4°C.

Conservation solution

The conservation solution (S2) was prepared by dissolving 400 mM of mannitol and 15 mM of $MgCl_2$ in 4 mM MES buffer at pH 5.7 (Mazarei et al., 2008). The solution is stored at 4°C.

3.2.2 Tomato root protoplast isolation

Tomato root collection and isolation of protoplasts

7- or 9-day-old roots of tomato plants were collected into a petri dish and cut into 0.5-1 mm slices with a sharp razor blade. Root pieces were then incubated in 10 mL of ES for 3h30 - 17h in the dark at room temperature. During incubation, solutions were unshaken or shaken at 70 rpm on a rotary shaker. After incubation, the ES was diluted with 5 mL of saline washing solution and filtered through a 70 µm cell trainer. After, the solution was centrifuged at 200 to 700 relative centrifugal field (rcf) for 2 to 6 minutes. Then, the supernatant was discarded, the pellet was resuspended in 5 mL of saline washing solution and the solution was centrifuged again at 200 to 700 rcf for 2 to 6 minutes. The supernatant was removed and the pellet was resuspended in 2 to 5 mL of the conservation solution, depending on protoplast density (**Figure 3.2**).

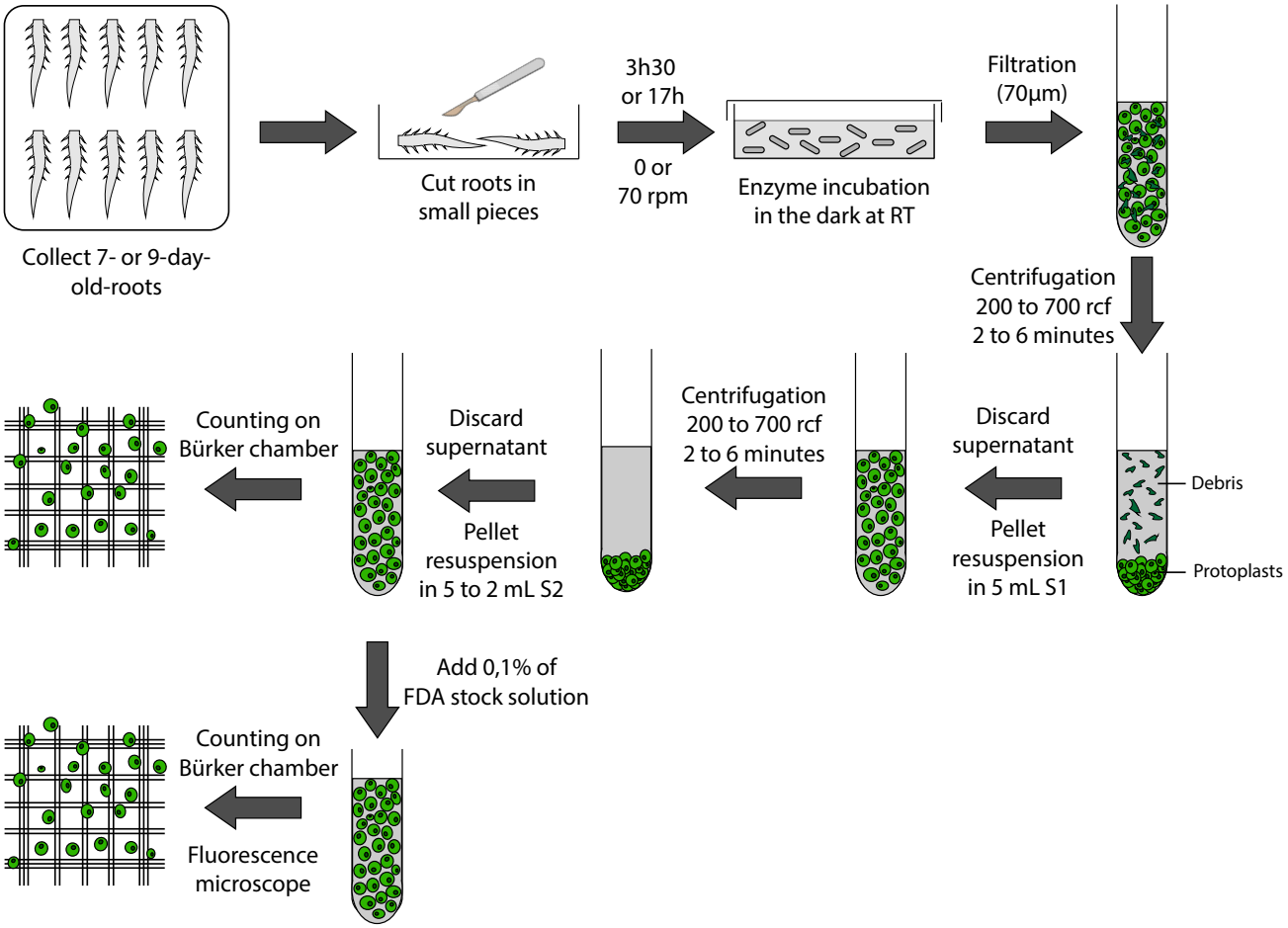

Figure 3.2: **Protoplast isolation method and determination of protoplast concentration and viability**

Optimization of tomato root protoplast isolation

In order to optimize protoplast isolation protocol, several factors were tested, including the age of roots, the incubation time with the enzymes, the agitation speed during this incubation and the time and speed of centrifugation.

Protoplast quantification and viability

After isolation, protoplasts were quantified by counting, using a Bürker chamber (**Figure 3.2**). The concentration of protoplasts per gram of roots was obtained by using this formula :

$$\text{Protoplasts production yield} = \left(\frac{\text{Average number of protoplasts} \times \text{Solution volume}}{\text{Number of squares} \times \text{Volume of a square} \times \text{Weight of roots}} \right)$$

The counting was repeated 3 times on the same solution and the average number of protoplasts was calculated. Solution volume corresponds to the volume of the conservation solution where the protoplast pellet was resuspended after centrifugation. The volume of a small square in a Bürker

chamber is 0,1 µL.

In addition, viability of the protoplasts was assessed by using FDA. An FDA stock solution was prepared by dissolving 5 mg/mL of FDA in acetone and stored at 0°C (Sangra et al., 2019; Cheng and Bélanger, 2000). Then, the FDA stock solution was added to protoplast suspension to obtain a final concentration of 0.1 %, or 1 µL of FDA stock solution in 1 mL of protoplast suspension (Cheng and Bélanger, 2000). The mix was incubated for 5 min at room temperature before observation with fluorescent microscope (**Figure 3.2**) (Zeiss Axioskop 2 Plus Ergonomic Trinocular Microscope, with Colibri 7 as blue light source and Axiocam ICM1 as camera). Finally, the percentage of viable protoplasts was calculated by using this formula (Sangra et al., 2019) :

$$\text{Percentage of viability} = \left(\frac{\text{Number of viable cells counted}}{\text{Total of cells counted}}\right) \times 100$$

Flow cytometry on protoplasts

Flow cytometry (BD Accuri™ C6 Plus Flow Cytometer) was performed on protoplast suspension after incubation with 0,1% of FDA stock solution for 5 minutes. The protoplast fluorescence was measured through flow cytometry. For each sample, 20 000 events were analyzed with the software C Flow Plus Analysis.

3.3 Isothermal titration calorimetry on tomato root protoplasts

ITC measurement was performed on protoplast suspension, obtained as described in the previous section. The different components of ITC are presented on **figure 3.3**.

The first experiment was performed on protoplasts that were washed twice with the saline washing solution and resuspended in the saline washing solution containing 1 µL/mL of dimethyl sulfoxyde (DMSO). Before using ITC, gases in milliQ water, protoplast suspension and saline washing solution containing 1 µL/mL of DMSO and 10 µM of surfactin were removed in a vacuum chamber. Degassed protoplast suspension was placed in the injection syringe and degassed saline washing solution containing 1 µL/mL of DMSO and 10 µM of surfactin was placed in the measuring cell and the reference cell was filled with degassed milliQ water. The same ITC measurement was performed without surfactin in saline washing solution to be used as a blanco.

The second experiment was performed on protoplasts that were washed once with the saline washing solution and twice with the conservation solution and that were finally resuspended in the conservation solution containing 1 µL/mL of DMSO. Gases in milliQ water, protoplast suspension and the conservation solution containing 1 µL/mL of DMSO and 10 µM of surfactin were removed in a vacuum chamber. Degassed protoplast suspension was placed in the measuring cell and degassed saline washing solution containing 1 µL/mL of DMSO and 10 µM of surfactin was placed in the injection syringe and the reference cell was filled with degassed milliQ water. The same ITC measurement was performed without surfactin in the conservation solution to be used as a blanco.

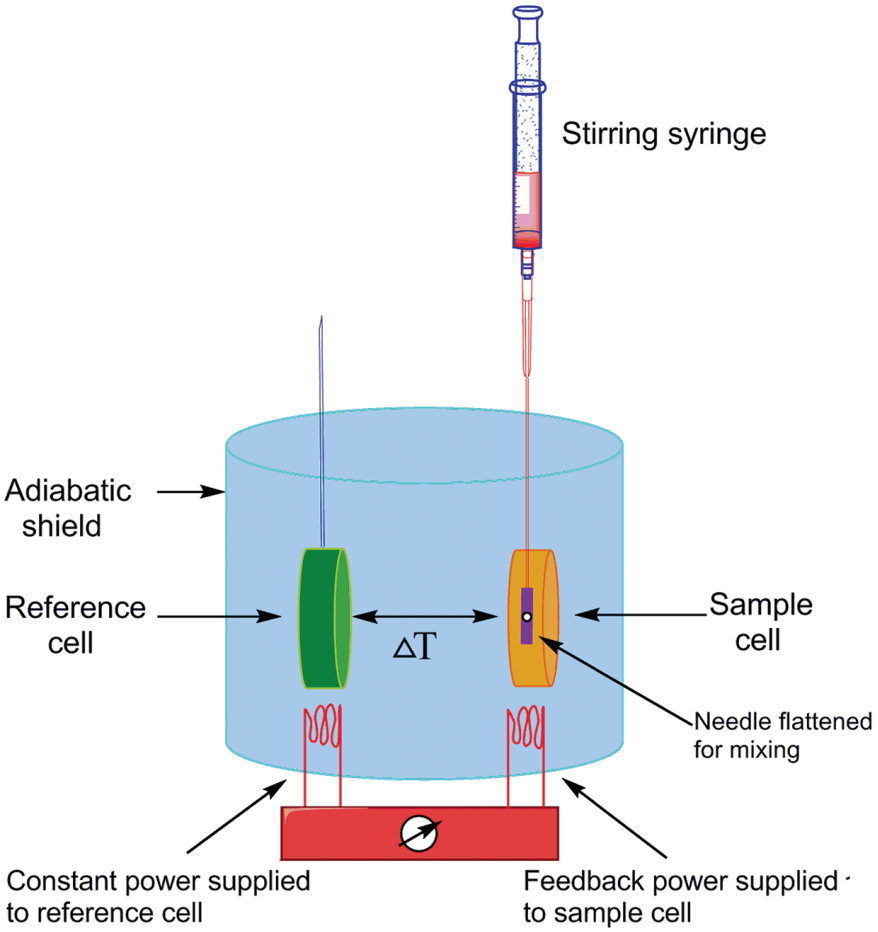

Figure 3.3: **Different components of isothermal titration calorimetry.** Schematic representation of isothermal titration calorimeter including the reference cell on the left, the stirring syringe, or the injection syringe, which is inserted into the sample cell, or the measuring cell. Solution in the measuring cell is constantly agitated by the needle of the injection syringe. The heat flow is measured in the measuring cell, while the heat flow is constant in the reference cell. (Song et al., 2015)

Measurement parameters selected for both ITC measurement are presented in **table 3.1** and injection parameters are presented in **table 3.2**. Once parameters were set, the experiment was started. ITC measurement was performed on VP-ITC Microcalorimeter (Microcal, Northampton USA). Data were processed using the software (ORIGIN 7, Originlab, Northampton, USA).

Table 3.1: Measure parameters for ITC

Measure parameters	
Number of injections	29
Cell temperature	26°C
Reference power	15 µCal/sec
Initial delay	300 sec
Stirring speed	260 rpm

Table 3.2: Injection parameters for ITC

Injection parameters				
Number of the injection	**Volume of injection**	**Duration of injection**	**Spacing between injections**	**Filter period**
1	2 µL	4 sec	250 sec	1 sec
2 to 29	10 µL	14.3 sec	500 sec	1 sec

3.4 Measurement of ROS production in tomato root protoplasts in presence of surfactin

A protoplast suspension was prepared from 7-day-old roots incubated for 17 hours without agitation. The solution obtained after incubation was filtrated, centrifuged at 700 rcf for 6 minutes and washed once with saline washing solution and twice with the conservation solution. After each wash, the solution was centrifuged at 700 rcf for 6 minutes and the supernatant was discarded. Finally, the pellet was resuspended in the conservation solution.

A solution was prepared to dissolve dichlorofluorescin diacetate (DCFDA), a dye able to measure ROS activity in cells. This solution is composed of the conservation solution containing 1 µL/mL of DMSO with 100 µM of DCFDA and the solution was kept in the dark. This solution was mixed with protoplast suspension to obtain a total concentration of DCFDA of 5 µM. Therefore, 50 µL/mL of DCFDA solution were added to the protoplast suspension. The solution was incubated for 10 minutes at room temperature in the dark.

Another solution composed of ethanol with 10 mg/mL of surfactin was prepared. Then, this solution was dissolved in the conservation solution with a concentration of 30 µL/mL, therefore the solution contained 300 µM of surfactin.

Then, 100 µL of both solutions were mixed in four wells of a 96 well plate. Then, four wells were also prepared, containing control solutions, composed of 100 µL of the same protoplast suspension incubated with DCFDA and 100 µL of the conservation solution containing 30 µL/mL of ethanol, without surfactin. ROS production by protoplasts was then measured on Tecan Spark ® microplate reader.

Before starting the measurement, the 96-well plate was agitated for 5 secondes at 270 rpm. Then, every 2 minutes the probe fluorescence emission was measured in 5 positions in all wells. The excitation and emission wavelengths of DCFDA are respectively 485 and 530 nm, both with a bandwidth of 20 nm.

3.5 Statistical analysis

The statistical analysis of data was performed on the software R studio. For each test, the normality of the populations has been assumed since it cannot be verified on populations of only three replicates.

Equality of variances was verified with Levene's test. Once this condition was verified, ANOVA test was performed to compare means, followed by Tukey's test.

Chapter 4

RESULTS

4.1 Optimization of tomato root protoplast isolation

The general schema to obtain protoplasts from plant roots is first to incubate root fractions in a solution containing cellulase and pectinase. Then, the solution is filtrated and centrifuged. The pellet is recovered and rinsed first with a solution containing NaCl, KCl and $CaCl_2$ in MES buffer at pH 5.7, in order to purify the protoplasts, and after with a solution containing mannitol and $MgCl_2$ in MES buffer at pH 5.7, in order to conserve protoplasts by maintaining good osmotic pressure with mannitol. In order to increase the yield of protoplast production, their purity and their viability, the protocol can be optimized at different steps. In this master's thesis, we focused on the age of roots, the incubation time with the enzymes and the agitation speed during this incubation and the time and speed of centrifugation. The optimization was done on tomato roots because tomato plants are easy to cultivate, have a short cycle of crop and produce a significant quantity of biological material within a short period of time.

The time and speed of centrifugation were first investigated. For the first tests, protoplast suspensions were centrifuged at 100 rcf, 300 rcf, 400 rcf or 500 rcf for 2 minutes. For each test, the protoplasts remained in suspension and it was impossible to proceed to the washing without wasting a significant number of protoplasts. Then, protoplast suspensions were centrifuged at 500 rcf for 4 minutes or at 600 rcf for 2 minutes and a pellet was obtained but it was easily resuspended in the solution. Finally, centrifugation at 600 rcf and 700 rcf for 6 minutes were performed on solutions and the pellets that were obtained remained stuck to the wall of the centrifugation tube. Therefore, centrifugation parameters that have been chosen for subsequent optimization experiments 600 rcf for 6 minutes because there is less chance that protoplasts explode than at 700 rcf.

The different treatments for the optimization of protoplast isolation, presented in **table 4.1**, were tested, performing for each treatment centrifugation at 600 rcf for 6 minutes. The average number of protoplasts per gram of root for each treatment is shown in **figure 4.1**.

Table 4.1: Different conditions for the optimization of protoplast isolation

Treatments						
	1	**2**	**3**	**4**	**5**	**6**
Age of roots (days)	7	7	7	7	9	9
Incubation time (hours)	3.5	3.5	17	17	3.5	3.5
Shaker speed (rpm)	0	70	0	70	0	70

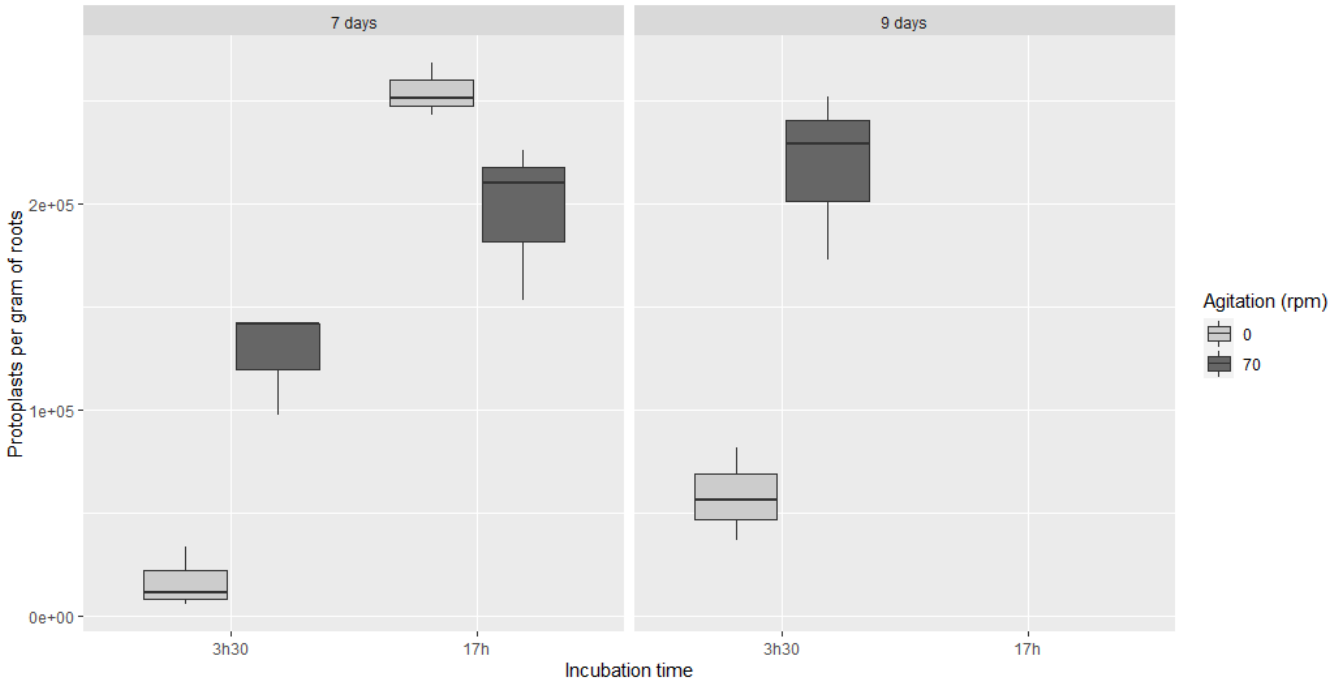

Figure 4.1: **Average number of protoplasts isolated from 1 gram of tomato root for the optimization of protoplast isolation protocol.** The box plot represents the average number of protoplasts per gram of roots grouped according to the three factors studied. For each box plot, three counts from the same protoplast suspension were taken into account. The line in the box plot represents the median. The graph is divided according to the age of the roots. There is the protoplast production yield for 7-day-old roots on the left side and the protoplast production yield for 9-day-old roots on the right side. The x-axis is the incubation time, while the color refers to the agitation during incubation.

A three-factor ANOVA was performed on the data presented in the box plot above. The equality of the variance was verified by Levene's test (p-value = 0.858). Results are presented in **table 4.2**.

In **table 4.2** it first can be noticed that the interaction between time and agitation have an influence on protoplast production yield, while the interaction between age and agitation does not. P-values for the interaction between age and time and for the triple interaction are not given. The reason for this lack of data is that for 9-day-old roots, there were tests only for a 3h30 incubation and not for a 17-hour incubation, so the p-value for the interaction between age and time and for the triple interaction could not be calculated.

Table 4.2: P-values of the three-factor ANOVA performed on the average protoplast production yields of the six treatments for the optimization of protoplast isolation protocol. NR : No Results because the protoplast production yield for 9-day-old root protoplasts with an incubation time of 17h was not calculated.

	p-value	p-value < 0.05
Age	0.003	*
Time	0.000000594	*
Agitation	0.000166	*
Age*Time	NR	NR
Age*Agitation	0.15	
Time*Agitation	0.000214	*
Age*Time*Agitation	NR	NR

As the interaction between time and agitation has a significant influence on protoplast production yield, a two-factor ANOVA was performed. The analysis was performed by grouping data according to the time of incubation. As for 9-day-old roots, there is no data for a 17-hour incubation, the analysis was only performed on 7-day-old roots data. The equality of the variance was verified by Levene's test (p-value = 0.218). Results of this analysis are presented in **table 4.3**.

Table 4.3: P-values of the two-factor ANOVA performed on protoplast production yields of the interaction of the factors time and agitation (i.e. Time*Agitation).

Time of incubation	Effect	p-value	p-value < 0.05
3h30	Agitation	0.003	*
17h	Agitation	0.067	

In **table 4.3**, it is shown that the agitation during incubation has an influence on protoplast production yield for an incubation time of 3h30 while it has not for an incubation time of 17h.

Then, in **table 4.2** it can be observed that each factor (i.e. Age, Time and Agitation) separately has an influence on protoplast production yield. First, by focusing on the time factor, it can be seen that 17 hours of incubation provides a higher protoplast production yield than 3h30 (**Figure 4.1**). Then, by focusing on the agitation factor, in **table 4.3** it is shown that agitation during incubation provides a higher protoplast production yield for a 3h30 incubation, while the agitation has no influence on protoplast production yield for an incubation of 17 hours. As a 17-hour incubation provides a larger protoplast production yield, the agitation during incubation has no influence. Therefore, as agitation during incubation is not necessary, the incubation solution will not be agitated. Finally, in **table 4.2**, it can be seen that the age of roots has an influence on the protoplast production yield. 9-day-old roots provide higher protoplast production yields for equivalent incubation time and agitation. However, protoplasts can be efficiently isolated from 7-day-old roots within two days less than 9-day-old roots. So, 7-day-old roots will be preferred over 9-day-old roots.

Finally, it can be seen on **figure 4.1** that protoplast suspension made from 7-day-old roots incubated for 17 hours without agitation and protoplast suspension made from 9-day-old roots incubated for 3h30 with agitation show similar protoplast production yield. By performing a Tukey's test on all different treatments, it was shown that there was no significant difference between protoplast production yield of protoplast suspension made from 7-day-old roots incubated for 17 hours without agitation and protoplast suspension made from 9-day-old roots incubated for 3h30 with agitation (p-value = 0.616). As 7-day-old roots can be obtained more quickly, they will be preferred over 9-day-old roots. Therefore, the protoplast suspension selected has the one which provides the larger protoplast production yield is the one made from 7-day-old roots incubated for 17 hours without agitation.

However, the protoplast production yield is not the only important factor to look at during protoplast isolation. Another important factor is their viability. Protoplast viability can be assessed by using FDA, which is a dye able to enter both viable and dead protoplasts. However, only viable protoplasts can produce esterases, which is able to hydrolyze FDA inducing the production of fluorescence. To perform the viability test, FDA stock solution is added to protoplast suspension and the mix is incubated for 5 minutes at room temperature. Then, protoplast suspensions can be observed under a fluorescent microscope, under blue light. Viability of protoplasts made from 7-day-old roots incubated for 17 hours without agitation was observed and viable protoplasts were detected (see **Figure 4.2** for an example of image). Two or more protoplasts were sometimes stuck together, even if the majority of the protoplasts were isolated.

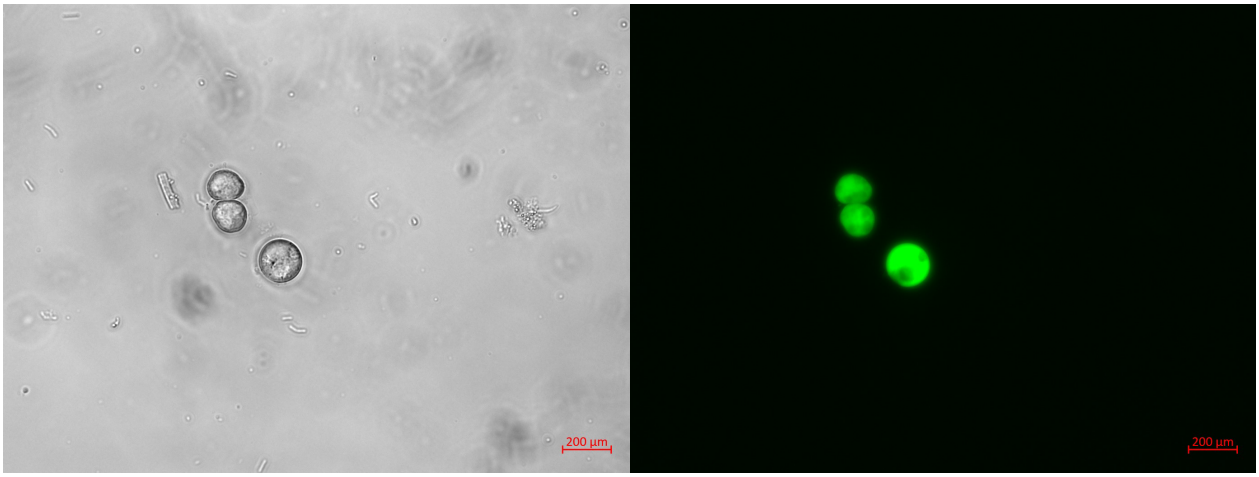

Figure 4.2: **Fluorescent microscope image of protoplasts, prepared from 7-day-old roots incubated for 17 hours without agitation, stained with FDA.** Protoplasts are observed under normal light on the left picture and under blue light on the right picture.

The percentage of viability for each treatment was calculated by using the following formula and is shown in **figure 4.3** :

$$\text{Percentage of viability} = \left(\frac{\text{Number of viable cells counted}}{\text{Total of cells counted}}\right) \times 100$$

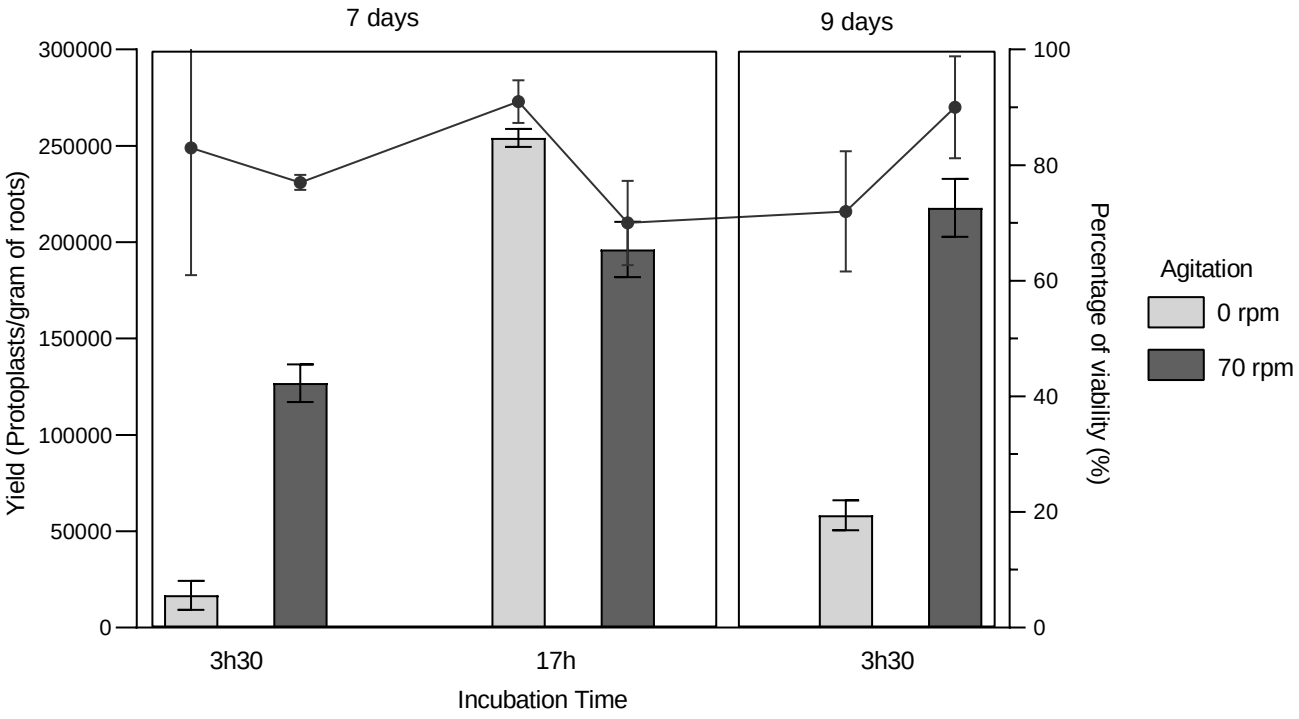

Figure 4.3: **Average number of protoplasts isolated from 1 gram of tomato root and average percentage of viability for the optimization of protoplast isolation protocol.** The bar chart represents the average number of protoplasts per gram of roots for each treatment. Each value represents the mean of three counts from the same protoplast suspension and the standard error is shown on each bar chart for each mean. The dots on the curve represent the average percentage of viability for each treatment. Each value represents the mean of three percentages of viability obtained from the same protoplast suspension and the standard error is shown on each dot for each mean.

First, after observing the different suspensions, it can be concluded that there was quite a large quantity of debris in each protoplast suspension, such as root fragments or cell organites, as shown in **figure 4.2** in the normal light image. Then, on **figure 4.3**, it first can be observed that for all treatments, the percentage of viability range between approximately 70 % and 90 %, which means that the percentage of viability of protoplasts in every protoplast suspension is quite high. It can be seen that the percentage of viability of protoplast suspension made from 7-day-old roots incubated for 17 hours with agitation is lower than the percentage of viability of protoplast suspension made from 7-day-old roots incubated for 17 hours without agitation. Therefore, it is preferable that the solution is not agitated. In addition, it can be seen that protoplast suspension made from 7-day-old roots incubated for 17 hours without agitation and protoplast suspension made from 9-day-old roots incubated for 3h30 with agitation show similar percentage of viability. Therefore, both treatments provide similar protoplast production yields with similar percentages of viability. The two treatments are therefore both equally efficient. As said above, the treatment "7-day-old roots incubated for 17 hours without agitation" was privileged over the "9-day-old roots incubated for 3h30 with agitation" to quicker obtain protoplasts.

4.1.1 Optimization of the purification of the protoplast suspension

Before proceeding to further experiments, including ITC measurement on protoplasts, it is important to make sure that protoplast suspension contains as little debris as possible to avoid biasing the results obtained. One way to get rid of debris is to wash the protoplasts with a saline washing solution that will allow removing debris and enzymes without disturbing the osmotic balance within the protoplasts. Therefore, protoplast production yield was measured on protoplasts obtained from 7-day-old roots after 17 hours of incubation, without agitation, when protoplast pellets, obtained by centrifugation at 700 rcf for 6 minutes, were washed one, two or three times with the saline washing solution (**Figure 4.4**).

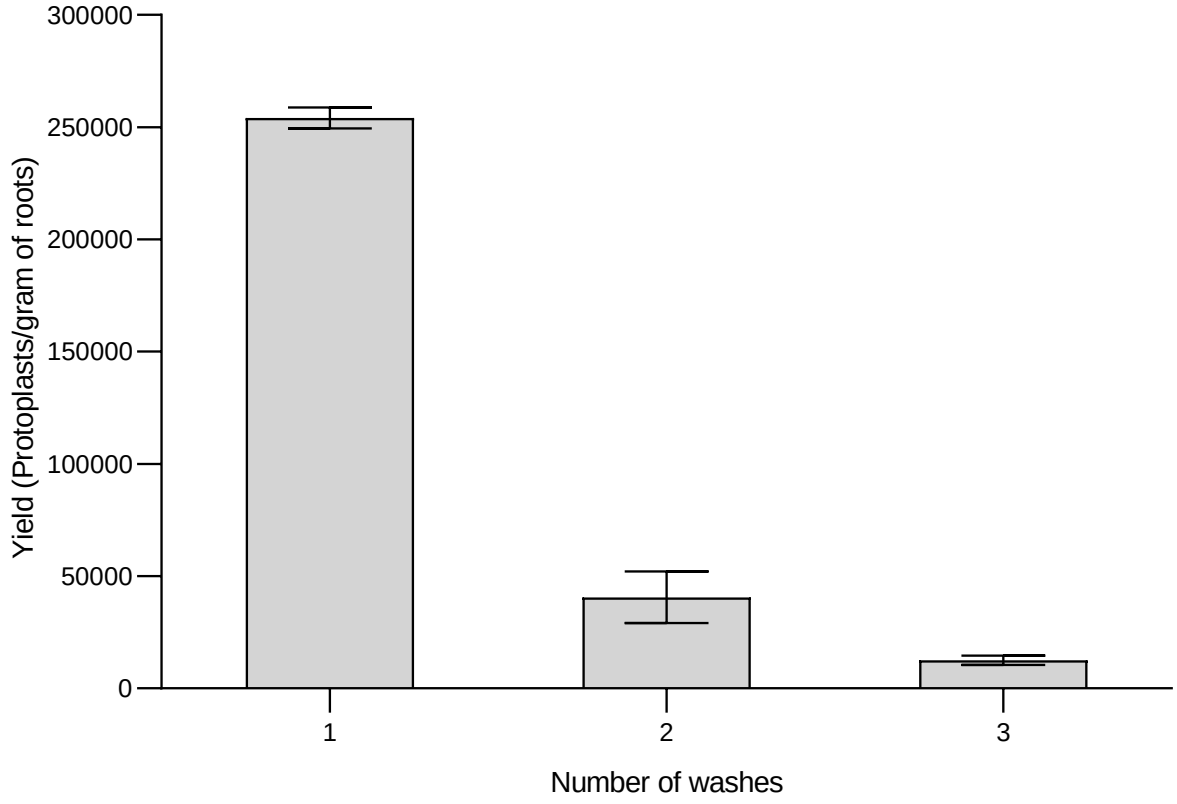

Figure 4.4: **Observation of the effect of washes with saline washing solution by calculating the average number of protoplasts isolated from 1 gram of tomato roots.** The effect of the number of washes with saline washing solution was compared on protoplasts from 7-day-old roots obtained after 17 hours of incubation, without agitation. The bar chart represents the average number of protoplasts per gram of roots for each treatment. Each value represents the mean of three counts from the same protoplast suspension and the standard error is shown on each bar chart for each mean.

A Tukey's test was performed to investigate the effect of washing on protoplast production yield. The equality of the variance was verified by Levene's test (p-value = 0.252).

Both on **figure 4.4** and **table 4.4**, it can be seen that the protoplast production yields for protoplasts washed two and three times are not significantly different, while they are both highly significantly lower than the protoplast production yield for protoplasts washed one time. When observing the dif-

Table 4.4: P-values of Tukey's test made on average protoplast concentrations of protoplasts from 7-day-old roots obtained after 17 hours of incubation, without agitation, to evaluate the influence of washing on protoplast production yield. P-values : NS (Not significative) $> 0.05; * \leqslant 0.05; ** \leqslant 0.01; * * * \leqslant 0.001; * * ** \leqslant 0.0001$. P-values are given in the appendix (**table 7.1**).

	1 wash	2 washes
2 washes	****	-
3 washes	****	NS

ferent solutions under the microscope, there was a clear reduction in the number of debris when the pellet was washed three times with the saline washing solution. Despite the reduction of protoplast production yield, further experiments were performed on protoplast suspension that was washed two times with the saline washing solution. This way, there is as little debris as possible without wasting a too large quantity of protoplasts.

4.2 Optimization of isothermal titration calorimetry measurement on protoplasts

ITC is a technique used to measure the heat exchange during a chemical reaction, in this context, between a surfactant and lipids of the PM of protoplasts. From the heat flow, a binding constant can be calculated, representing the binding affinity between a macromolecule and a ligand (Martinez et al., 2013). There are several factors that can bias the results obtained by ITC. Firstly, bubbles should not be injected inside the measuring cell as this could disturb the measurement of the heat flow. Secondly, no molecules other than those being studied should be injected. Here, the suspension of protoplasts must be as pure as possible and therefore the presence of debris that could interact with surfactin must be avoided. Finally, it is important that the composition of the solution containing the protoplasts and the solution containing surfactin is as similar as possible in order to avoid measuring a heat flow that is due to a change in the composition of the medium and not due to an interaction between the protoplasts and surfactin.

Two types of experiment can be performed on ITC. First, the protoplast suspension can be placed in the injection syringe and the solution containing surfactin can be injected in the measuring cell. Or, the protoplast suspension can be injected in the measuring cell and the solution containing surfactin can be placed in the injection syringe.

4.2.1 Evaluation of tomato root protoplasts behavior during isothermal titration calorimetry preparation and measurement when protoplast suspension is placed in the injection syringe

As protoplasts are very fragile and as ITC was never performed on protoplasts, it is important to evaluate how protoplasts resist to each preparation step of ITC. Protoplast production yields and percentages of viability were calculated after each step of ITC preparation.

A first experiment was performed where the protoplast suspension was placed in the injection syringe. The protoplast suspension was obtained from 7-day-old roots after 17 hours of incubation, without agitation. Then, protoplast suspension was centrifuged at 700 rcf for 6 minutes and the pellet was washed two times with the saline washing solution and resuspended in the saline washing solution containing 1 µL/mL of DMSO after the last centrifugation. Then, protoplast suspension was degassed in a vacuum chamber, transferred in the injection syringe and was injected in the measuring cell and held under agitation during ITC measurement. Protoplast concentration and percentages of viability were determined for protoplasts before ITC measurement, for degassed protoplasts and for protoplasts after passing through the injection syringe (**Figure 4.5**) and for protoplasts in the measuring cell of ITC at the end of the experiment.

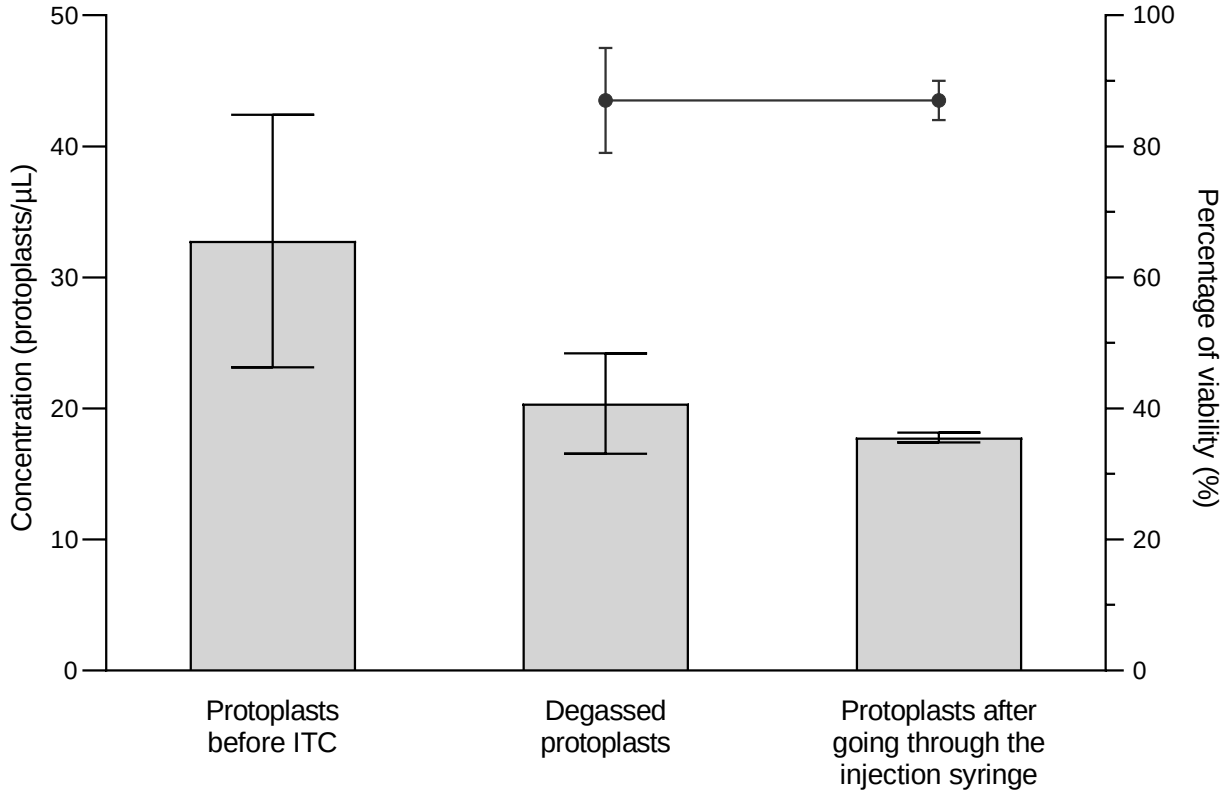

Figure 4.5: **Average concentration of tomato root protoplasts and average percentage of viability before and after ITC measurement where protoplast suspension is placed in the injection syringe.** The ITC measurement was performed on protoplasts from 7-day-old roots obtained after 17 hours of incubation, without agitation. The bar chart represents the average protoplast concentration for each step. Each value represents the mean of three counts from the same protoplast suspension and the standard error is shown on each bar chart for each mean. The dots on the curve represent the average percentage of viability for each treatment. Each value represents the mean of the percentage of viability obtained from the same protoplast suspension and the standard error is shown on each dot for each mean. Percentage of viability of protoplasts before ITC is not shown in the graph because the suspension volume was too small to make three counts. The protoplast production yield and the percentage of viability for protoplasts in the measuring cell of ITC is not shown in the graph because the solution was too diluted and the counts were unreliable.

A Tukey's test was performed to investigate the effect of the different steps of ITC preparation and measurement on the protoplast concentration when protoplast suspension is placed in the injection syringe. The equality of the variance was verified by Levene's test (p-value = 0.445).

Table 4.5: P-values of Tukey's test made on average concentrations of protoplasts before and after ITC measurements where protoplast suspension was placed in the injection syringe. P-values : NS (Not significative)$> 0.05; * \leqslant 0.05; ** \leqslant 0.01; * * * \leqslant 0.001; * * ** \leqslant 0.0001$. P-values are given in the appendix (**table 7.2**)

	Protoplasts before ITC	Degassed protoplasts
Degassed protoplasts	NS	-
Protoplasts after going through the syringe	NS	NS

In **table 4.5**, it is shown that the average concentration of protoplasts before ITC measurement, degassed protoplasts and protoplasts that went through the injection syringe of ITC are all not significantly different. It means that the average concentration of protoplasts does not significantly decrease during ITC preparation. It means that ITC measurements do not alter protoplasts, even if it should be ensured that the average concentration of protoplasts at the end of the ITC measurement does not decrease. Moreover, the percentage of viability of degassed protoplasts and protoplasts that went through the injection syringe of ITC are both around 87 % (**Figure 4.5**). The protoplast concentration and the percentage of viability for protoplasts in the measuring cell of ITC is not shown in the graph because the solution was too diluted and the counts could not be performed. In addition, it is important to notice that the concentration of protoplasts before ITC is unexpectedly much lower than the one of protoplasts prepared from 7-day-old roots incubated for 17 hours without agitation shown in **figure 4.3**, while it is the same treatment. Moreover, the percentage of viability of protoplasts before ITC measurement is not shown in the graph because protoplast suspension volume was too small to make three counts.

Isothermal titration calorimetry measurement of the interaction between protoplasts and surfactin when protoplast suspension is placed in the injection syringe

ITC was performed on protoplast suspension that was placed in the injection syringe. The protoplast suspension was obtained as described above in 4.2.2. The degassed saline washing solution containing 1 µL/mL of DMSO and 10 µM of surfactin was placed in the measuring cell and the degassed protoplast suspension was transferred in the injection syringe. 10 µL of protoplast suspension were successively injected 28 times in the measuring cell and held under low agitation during ITC measurement. The same ITC measurement was performed with the saline washing solution containing 1 µL/mL of DMSO without surfactin instead of the saline washing solution containing 1 µL/mL of DMSO and 10 µM of surfactin, serving as a control. Results are presented in **figure 4.6**.

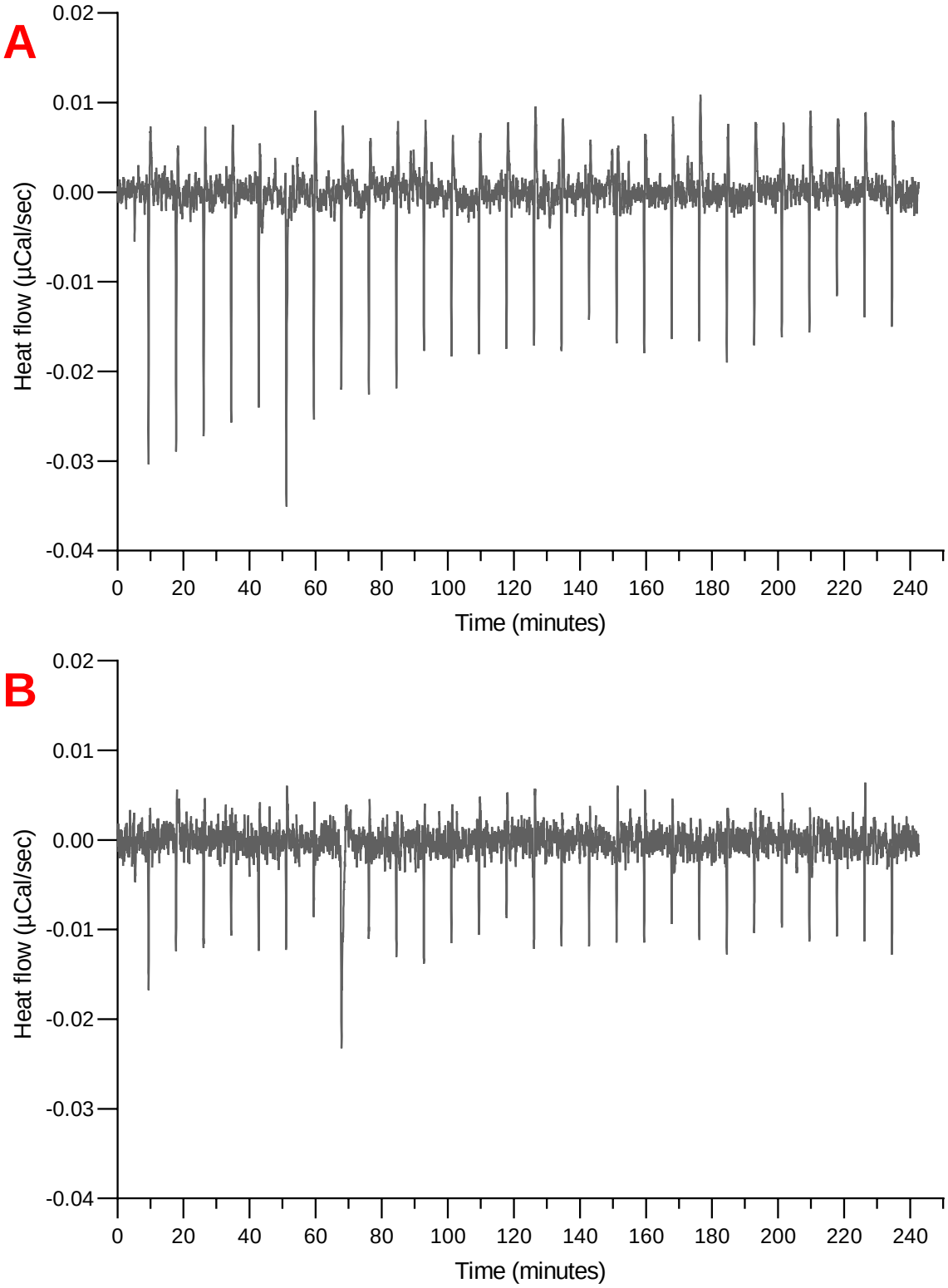

Figure 4.6: **ITC raw data of ITC measurement performed on protoplast suspension, made from 7-day-old roots incubated for 17 hours without agitation, placed in the injection syringe.** (A) ITC raw data where the saline washing solution containing 1 µL/mL of DMSO is placed in the measuring cell and the protoplast suspension is placed in the injection syringe, with an average concentration of 32.8 protoplasts/µL (B) ITC raw data where the saline washing solution containing 1 µL/mL of DMSO and 10 µM of surfactin is placed in the measuring cell and the protoplast suspension is placed in the injection syringe, with an average concentration of 17.8 protoplasts/µL

It can be observed on the second graph on **figure 4.6** that there is a low heat flow during ITC measurement. The first graph on **figure 4.6** is the control and it is similar to the second one with longer peaks. The control is used to see the kind of profile obtained when there are no interactions between protoplasts and surfactin as there was no surfactin in the measuring cell. Peaks on both graphs are in the same range, but peaks for the control are longer because the protoplast concentration for ITC measurement with surfactin was almost twice as low as for ITC measurement without surfactin. It can therefore be supposed that there was no interaction between protoplasts and surfactin. One way to standardize the experiment is to estimate the surface area of the protoplasts and adapt their concentration in order to have the same surface area from one experiment to another.

Calculation of total surface area of tomato root protoplasts during ITC measurement

To calculate the total lipid surface area of tomato root protoplasts exposed during ITC measurement, the two following formulas were used :

$$\text{Total number of protoplasts} = (\text{Protoplast concentration} \times \text{Total volume})$$

$$\text{Total surface area} = (4 \times \pi \times (\frac{d}{2})^2 + 4 \times \pi \times (\frac{d}{2} - h)^2) \times \text{total number of protoplast}$$

Where d is the average diameter of protoplasts and h is the thickness of the lipid bilayer.

When ITC measurement was performed on protoplasts in the presence of surfactin (**figure 4.6 B**), protoplast concentration was 17.8 protoplasts per µL and the volume of the injection syringe is 282 µL. Therefore, the total number of protoplasts in the measuring cell during ITC measurement is 5020.

Assuming that the average diameter of tomato root protoplasts is 50 µm, knowing that *Lotus japonicus* root protoplast size is 30-50 µm (Jia et al., 2018) and by neglecting the thickness of the bilayer, the total surface area of tomato root protoplasts exposed during ITC measurement is approximately 0.0000788 m^2.

Comparatively, successful ITC measurements can be achieved on liposomes in a concentration of 5 mM (Lebecque et al., 2019), where 282 µL of liposome suspension are placed in the injection syringe. Assuming that the average diameter of liposomes is 100 nm and that the head area of a lipid is 0.71 nm^2 (Israelachvili et al., 1975) and by neglecting the thickness of the bilayer, the total surface area of liposomes exposed during ITC measurement is approximately 0.602 m^2, which is ten thousand times higher than the surface area of protoplasts for this experiment.

4.2.2 Evaluation of *Arabidopsis* leaf protoplasts behavior during isothermal titration calorimetry preparation and measurement when protoplast suspension is placed in the injection syringe

ITC measurement was also performed on *Arabidopsis* leaf protoplasts. As for root protoplast isolation, leaves were incubated for 17 hours without agitation. Then, protoplast suspension was centrifuged at 250 rcf for 4 minutes and the pellet was washed two times with the saline washing solution and

resuspended in the saline washing solution containing 1 µL/mL of DMSO after the last centrifugation. Then, the degassed protoplast suspension was transferred in the injection syringe and was injected in the measuring cell and held low under agitation during ITC measurement. Protoplast concentration and percentages of viability were calculated for freshly degassed protoplasts and for fresh protoplasts after passing through the injection syringe. Then, those two suspensions were stored at 4°C for 5 hours, which is approximately the time required to perform ITC measurement, and protoplast concentration and percentages of viability were calculated once more.

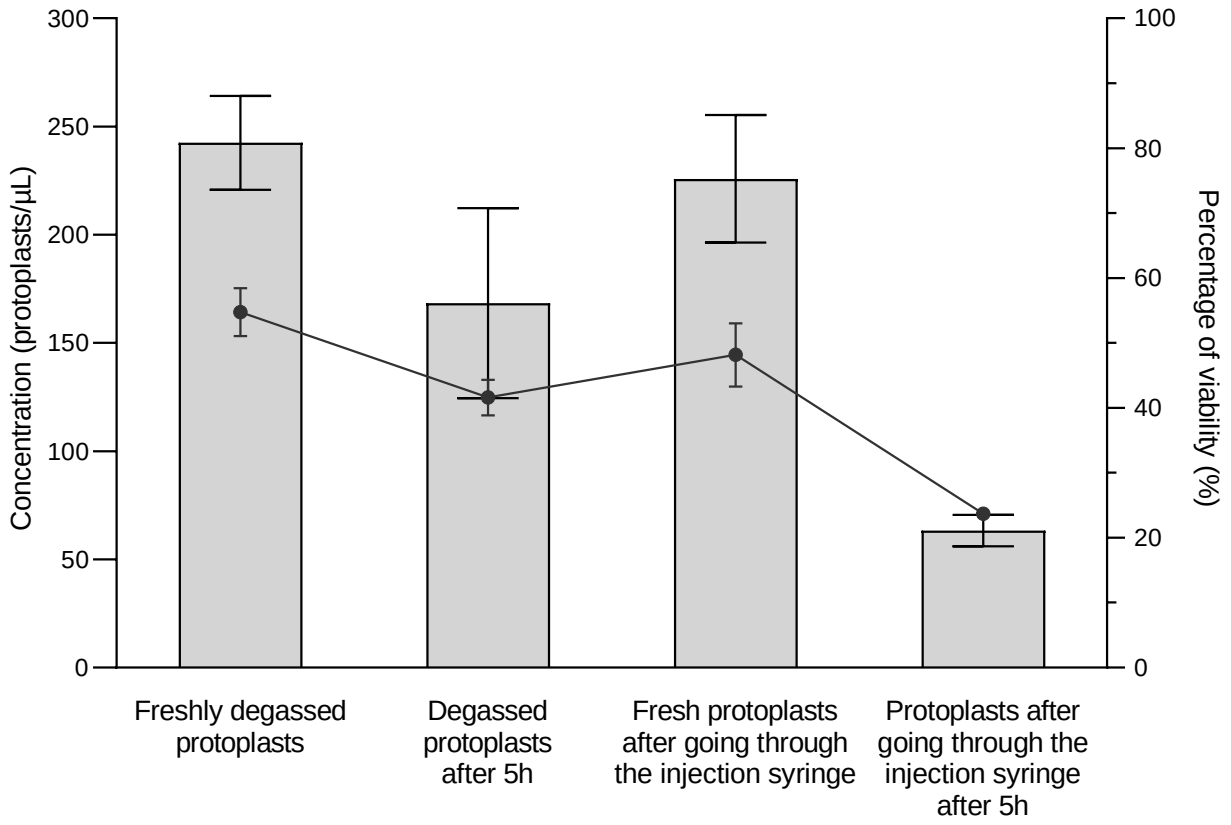

Figure 4.7: **Average concentration of *Arabidopsis* leaf protoplasts and average percentage of viability for degassed protoplasts and protoplasts that went through the injection syringe before and after 5 hours.** The ITC preparation steps were performed on *Arabidopsis* leaf protoplast suspension obtained after 17 hours of incubation, without agitation. The bar chart represents the average number of protoplasts per µL. Each value represents the mean of three counts from the same protoplast suspension and the standard error is shown on each bar chart for each mean. The dots on the curve represent the average percentage of viability for each treatment. Each value represents the mean of the percentage of viability obtained from the same protoplast suspension and the standard error is shown on each dot for each mean.

A Tukey's test was performed to investigate the resistance of protoplasts stored in the saline washing solution for 5 hours. The equality of the variance was verified by Levene's test (p-value = 0.661).

Table 4.6: P-values of Tukey's test made on average protoplast concentrations obtained for degassed protoplasts and protoplasts that went through the injection syringe before and after 5 hours on *Arabidopsis* leaf protoplasts obtained after 17 hours of incubation, without agitation. P-values : NS (Not significative)$> 0.05; * \leqslant 0.05; ** \leqslant 0.01; * * * \leqslant 0.001; * * ** \leqslant 0.0001$. P-values are given in the appendix (**table 7.3**)

	Freshly degassed protoplasts	Degassed protoplasts after 5 hours	Fresh protoplasts after going through the syringe
Degassed protoplasts after 5 hours	NS	-	-
Fresh protoplasts after going through the syringe	NS	NS	-
Protoplasts after going through the syringe after 5 hours	**	*	**

First, the protoplast concentration after ITC was not calculated because protoplasts clogged the syringe and the entire suspension couldn't be injected.

Then, it is shown on **figure 4.7** and on **table 4.6** that there is no reduction in protoplast concentration between freshly degassed protoplasts and fresh protoplasts after going through the injection syringe. It means that the injection through the syringe does not cause the leaf protoplasts to explode. Then, the effect of time on protoplast yield and viability was investigated by comparing the average protoplast concentration and percentage of viability of both suspensions before and after 5 hours. For degassed protoplasts, a decrease of both the percentage of viability and the protoplast concentration can be observed but the decrease of the protoplast concentration is not significant between freshly degassed protoplasts and degassed protoplasts after 5 hours. Similarly, for protoplasts that went through the syringe, there is a decrease of the percentage of viability when protoplast suspension is stored for 5 hours. There is also a highly significant decrease of the protoplast concentration in this case. It is important to know that leaf protoplasts are more likely to explode than root protoplasts. Usually, protoplasts are preserved in the conservation solution rather than the saline washing solution. The higher concentration of mannitol in the conservation solution is able to maintain osmotic pressure in protoplasts and therefore prevents them for exploding, so the conservation solution seems more appropriate to perform ITC on protoplasts.

4.2.3 Evaluation of tomato root protoplasts behavior during isothermal titration calorimetry preparation and measurement when protoplast suspension is placed in the measuring cell

As protoplasts do not seem to preserve well in the saline washing solution, an ITC measurement was performed on protoplasts resuspended in the conservation solution rather than the saline washing solution. In addition, as the total surface area of protoplasts was very low when protoplast suspension was placed in the injection syringe, ITC measurement was performed on protoplast suspension placed in the measuring cell. In this way, protoplast concentration may be higher because the dilution rate

will be lower.

For this experiment protoplast suspension was obtained from 7-day-old roots after 17 hours of incubation, without agitation. Then, protoplast suspension was centrifuged at 700 rcf for 6 minutes and the pellet was washed once with the saline washing solution and twice with the conservation solution and resuspended in the conservation solution containing 1 µL/mL of DMSO after the last centrifugation. Then, degassed protoplast suspension was transferred with a glass syringe in the measuring cell and held under low agitation during ITC measurement. Protoplast concentrations and percentages of viability were calculated for protoplasts before ITC measurement, for degassed protoplasts, for protoplasts after passing through the glass syringe and for protoplasts in the measuring cell of ITC.

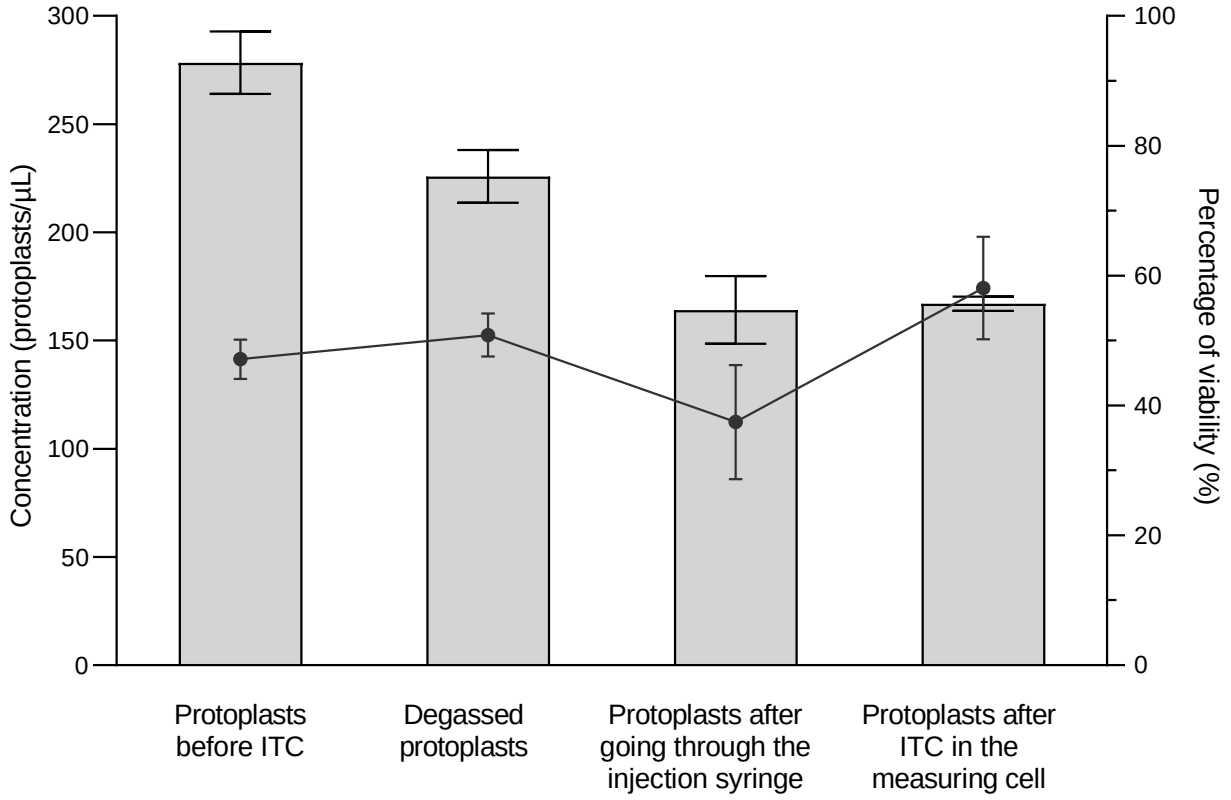

Figure 4.8: **Average concentration of tomato root protoplasts and average percentage of viability before and after ITC measurement where protoplast suspension is placed in the measuring cell.** The ITC measurement was performed on protoplasts from 7-day-old roots obtained after 17 hours of incubation, without agitation. The bar chart represents the average concentration of protoplasts for each step. Each value represents the mean of three counts from the same protoplast suspension and the standard error is shown on each bar chart for each mean. The dots on the curve represent the average percentage of viability for each treatment. Each value represents the mean of the percentage of viability obtained from the same protoplast suspension and the standard error is shown on each dot for each mean. The concentration of protoplasts after ITC measurement was corrected by the dilution factor, with a value of 1,2.

A Tukey's test was performed to investigate the effect of the different steps of ITC preparation

and measurement on protoplast concentration when protoplast suspension is placed in the measuring cell. The equality of the variance was verified by Levene's test (p-value = 0.723).

Table 4.7: P-values of Tukey's test made on average protoplast concentrations of protoplasts before and after ITC measurements where protoplast suspension was placed in the measuring cell. P-values : NS (Not significative) $> 0.05; * \leqslant 0.05; ** \leqslant 0.01; * * * \leqslant 0.001; * * ** \leqslant 0.0001$. P-values are given in the appendix (**table 7.4**).

	Protoplasts before ITC	Degassed protoplasts	Protoplasts after going through the glass syringe
Degassed protoplasts	*	-	-
Protoplasts after going through the glass syringe	***	**	-
Protoplasts after ITC in the measuring cell	***	*	NS

First, it is important to note that the protoplast isolation was performed on a large quantity of root material and that the protoplasts were washed three times. The initial average protoplast production yield had an approximate value of 250 000 protoplasts per gram of roots, which is very close to the value obtained for protoplasts that were washed once in 4.1.1., suggesting that the number of washes does not affect protoplast production yield.

It is shown in **figure 4.8** and **table 4.7** that degassing protoplast suspension significantly decrease the protoplast concentration. Similarly, the protoplast concentration also significantly decreases when degassed protoplasts go through the glass syringe. But the concentration of protoplasts that went through the glass syringe and the one of protoplasts after ITC measurement in the measuring cell are statistically equal. Therefore, there is a strong decrease in the number of protoplasts under the degassing phase and during the introduction of the protoplast sample into the measuring cell. But the most important is that there is no significant decrease of protoplast concentration during the ITC measurement.

Pictures of protoplasts stained with FDA after each step of ITC preparation and measurement are shown in **figure 4.9**. Protoplasts are shown under normal light and under blue light on the pictures on the right and on the left respectively. Viable protoplasts present bright green fluorescence, when excited by blue light, while dead protoplasts do not present green fluorescence, or a low green fluorescence because protoplasts without the presence of FDA dye present a low natural fluorescence. At each step of ITC preparation and measurement, there were viable and dead protoplasts. Also, on pictures under normal light, it can be seen that there were some debris, which could influence the heat flow measurement.

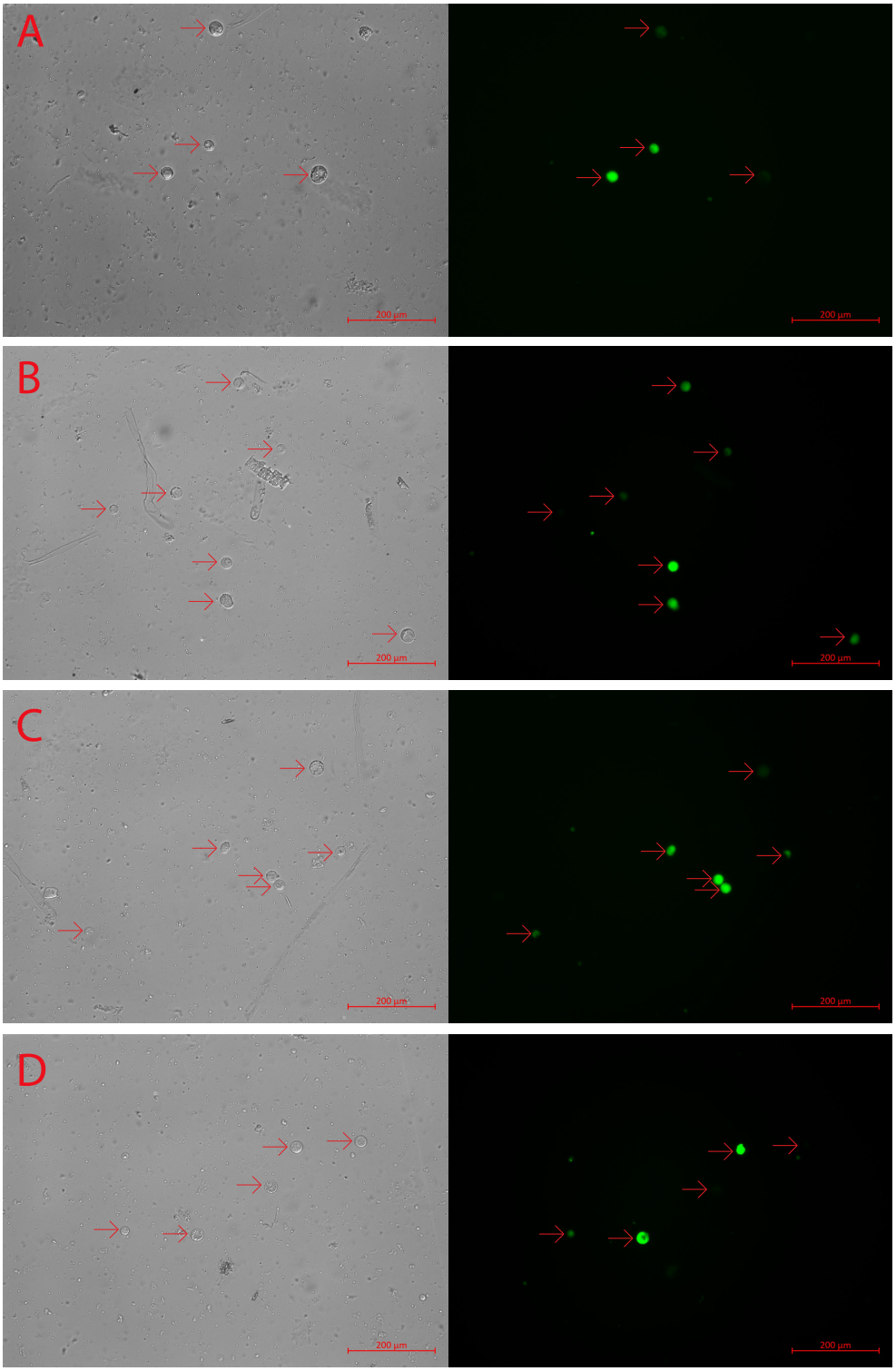

Figure 4.9: **Image of tomato root protoplasts stained with FDA under normal light and blue light** (A) after isolation and before ITC preparation steps, (B) after degassing protoplast suspension, (C) after going through the glass syringe and (D) at the end of ITC measurement, in the measuring cell. Viable protoplasts present bright green fluorescence, when excited by blue light, on the picture on the right, and dead protoplasts do not present green fluorescence or present a low green fluorescence.

Isothermal titration calorimetry measurement of the interaction between protoplasts and surfactin when protoplast suspension is placed in the measuring cell

ITC was performed on protoplast suspension that was placed in the measuring cell. The protoplast suspension was prepared as described above in 4.2.4. The degassed conservation solution containing 1 µL/mL of DMSO and 10 µM of surfactin was placed in the measuring cell, while the degassed protoplast suspension was transferred in the measuring cell by using a glass syringe and held under agitation during ITC measurement. The same ITC measurement was performed with the conservation solution containing 1 µL/mL of DMSO without surfactin instead of 1 µL/mL of DMSO and 10 µM of surfactin.

The second graph on **figure 4.10** represents the raw data obtained when protoplasts were in contact with surfactin. The first graph on **figure 4.10** is the control, without surfactin, therefore there is no interactions between protoplasts and surfactin. The signal obtained for protoplasts with surfactin is approximately ten times higher than for the control. Therefore, it can be concluded that there was an interaction between the protoplasts and surfactin.

The total lipid surface area of tomato root protoplasts was calculated.

The protoplast concentration was about 280 protoplasts per µL and the volume of the measuring cell is 1500 µL. Therefore, the total number of protoplasts in the measuring cell during ITC measurement is 420 000. Assuming that the average diameter of tomato root protoplasts is 50 µm and by neglecting the thickness of the bilayer, the total surface area of tomato root protoplasts exposed during ITC measurement is approximately 0.0066 m^2, which is a hundred times higher than the surface area of protoplasts when they were placed in the injection syringe (i.e. 0.0000788 m^2) but also a hundred times lower than the total surface area of liposomes in a concentration of 5 mM (i.e. 0.602 m^2). Therefore, ITC measurement with protoplasts in the measuring cell should be performed again with protoplast concentration a hundred times higher.

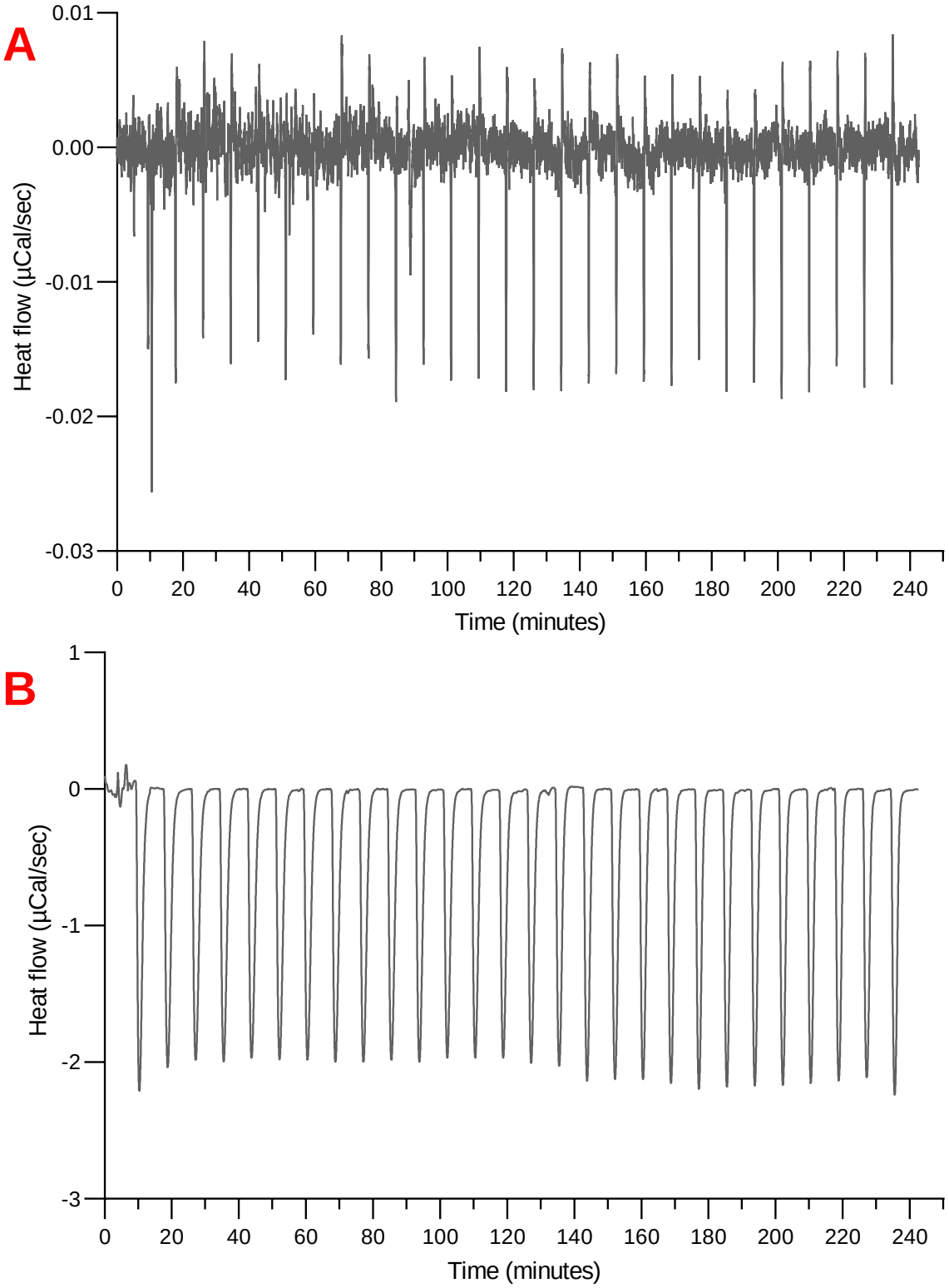

Figure 4.10: **ITC raw data of ITC measurement performed on protoplast suspension, made from 7-day-old roots incubated for 17 hours without agitation, placed in the measuring cell.** (A) ITC raw data where the conservation solution containing 1 µL/mL of DMSO is placed in the injection syringe and the protoplast suspension is placed in the measuring cell, with an average concentration of 278.3 protoplasts/µL (B) ITC raw data where the conservation solution containing 1 µL/mL of DMSO and 10 µM of surfactin is placed in the injection syringe and the protoplast suspension is placed in the measuring cell, with an approximate concentration of 280 protoplasts/µL.

4.3 Flow cytometry and data analysis of tomato root protoplasts

Flow cytometry is a fast technique used to analyze cells and other particles in a solution (Zhou et al., 2019). Flow cytometry can be used to count protoplasts in the solution and also to make a viability test on protoplasts, since flow cytometry can detect fluorescence. The advantages of this technique are its rapidity, sensitivity and multiparameter detection (Zhou et al., 2019; Badaró Costa et al., 2018).

In flow cytometry, each particle goes through a laser and two main parameters are measured. First, the cell size is measured with the Forward Scatter Channel (FSC). As the cells go through the light, the light is scattered forward and is collected by the FSC. Similarly, the cell granularity and complexity are measured with the Side Scatter Channel (SSC), where cells scattered the light perpendicularly to the laser beam. Both parameters help to define a cell population. In addition, the green fluorescence is measured with the forward light 1 (FL1) channel (Goetz et al., 2018).

The protoplast suspension was prepared as described above in 4.2.3. Then, the protoplast suspension was diluted 32 times so the absorbance of the solution is between 0.1 and 0.2 and the number of events measured with the flow cytometer is between 800 and 1200 events per second. This dilution is carried out so the protoplasts and particles are sufficiently separated to pass one by one in front of the laser and so they do not clog the device. Flow cytometry was performed on this protoplast suspension.

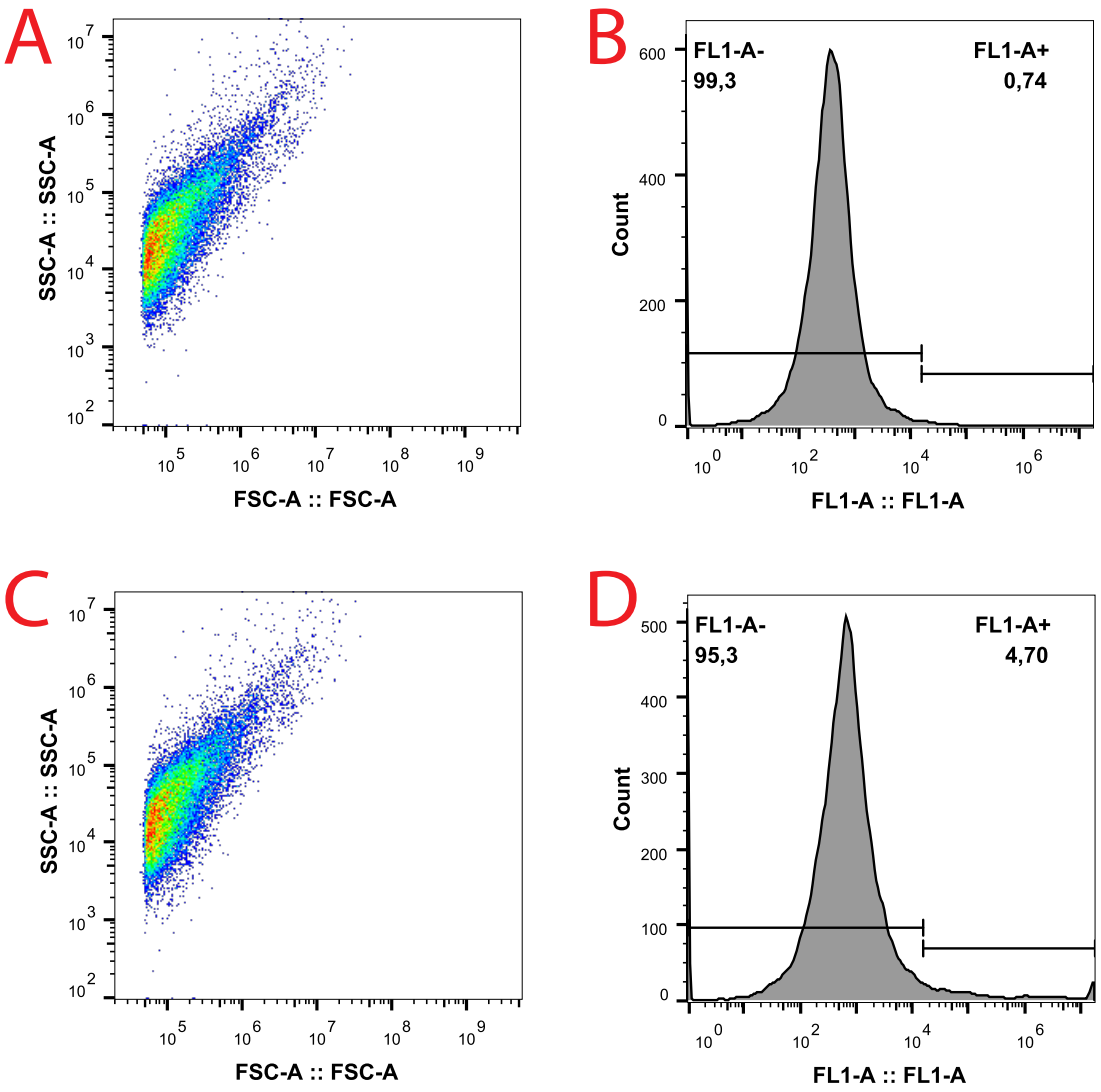

Figure 4.11: **Tomato root protoplast analysis by flow cytometry.** (A) FSC-SSC plot of unstained tomato root protoplats (B) Fluorescence intensity plot of unstained tomato root protoplasts (C) FSC-SSC plot of tomato root protoplats stained with FDA (D) Fluorescence intensity plot of tomato root protoplasts stained with FDA

The different elements of the protoplast suspension are presented on **figure 4.11 A and C** according to their size and granularity and on **figure 4.11 B and D** according to their number and their intensity of fluorescence. It can be observed that the amount of fluorescent events increases from 0.74 % to 4.70 % when protoplast suspension is stained with FDA. However, before performing flow cytometry measurement, the number of protoplasts was counted on a Bürker chamber with a microscope. It was observed that the protoplast suspension contained a large number of debris. The initial protoplast suspension had a concentration of 280 protoplasts/µL and has been diluted 32 times. Therefore, the protoplast suspension analyzed with the flow cytometer has in theory a concentration of approximately 9 protoplasts/µL. Flow cytometry was performed on 20 000 events, or approximately 23 µL of protoplast suspension. Therefore technically, only about 210 protoplasts were analyzed out of 20 000 events, meaning that the suspension contains a lot of debris. It can be seen on **figure 4.11** that most of the population does not emit fluorescence, which is probably due to the high number of debris present in the suspension.

4.4 Measurement of ROS production in tomato root protoplasts in presence of surfactin

It was demonstrated that when surfactin is in contact with root tissues, it can induce systemic resistance in the host plant. Surfactin also stimulates early defense-related events, such as the production of ROS (Debois et al., 2015; Henry et al., 2011; Cawoy et al., 2014). In addition, Henry et al. (2011) has clearly demonstrated that surfactin induced the accumulation of intracellular ROS in tomato roots. Therefore, it is important to verify that surfactin can interact not only with tomato roots, but also with tomato root protoplasts, by measuring its production of ROS when they are in contact with surfactin.

7-day-old tomato roots were incubated for 17 hours without agitation. The solution was filtrated, centrifuged at 700 rcf for 6 minutes and washed on time with the saline washing solution and two times with the conservation solution. After each wash, the solution was centrifuged and the supernatant was discarded. Finally, protoplasts were resuspended in the conservation solution. The protoplast suspension contained approximately 280 protoplasts/µL.

The protoplast suspension was incubated in the dark for 10 minutes with 50 µL/mL of a solution composed of the conservation solution containing 1 µL/mL of a solution of DMSO containing 100 µM of DCFDA. Then, in four wells of a 96-well plate, 100 µL of this solution was mixed with 100 µL of the conservation solution containing 30 µL/mL of a solution of ethanol containing 10 mM of surfactin. In addition, in four other wells of the same plate, controls were prepared similarly with 100 µL of the protoplast suspension and 100 µL of the conservation solution containing 30 µL/mL of ethanol without surfactin. Therefore, in each well, protoplast concentration is approximately 1.3×10^5 protoplasts/mL.

Every 2 minutes the probe fluorescence emission was measured in 5 positions in all wells. The average of these 5 values was calculated for each well every 2 minutes. Data from two wells, including

one containing surfactin and one control that do not contain surfactin, were aberrant and therefore were excluded. Then, the average fluorescence emission for the three repetitions was calculated for each measure. Therefore, means of the fluorescence emission for the control and surfactin solutions were obtained every 2 minutes.

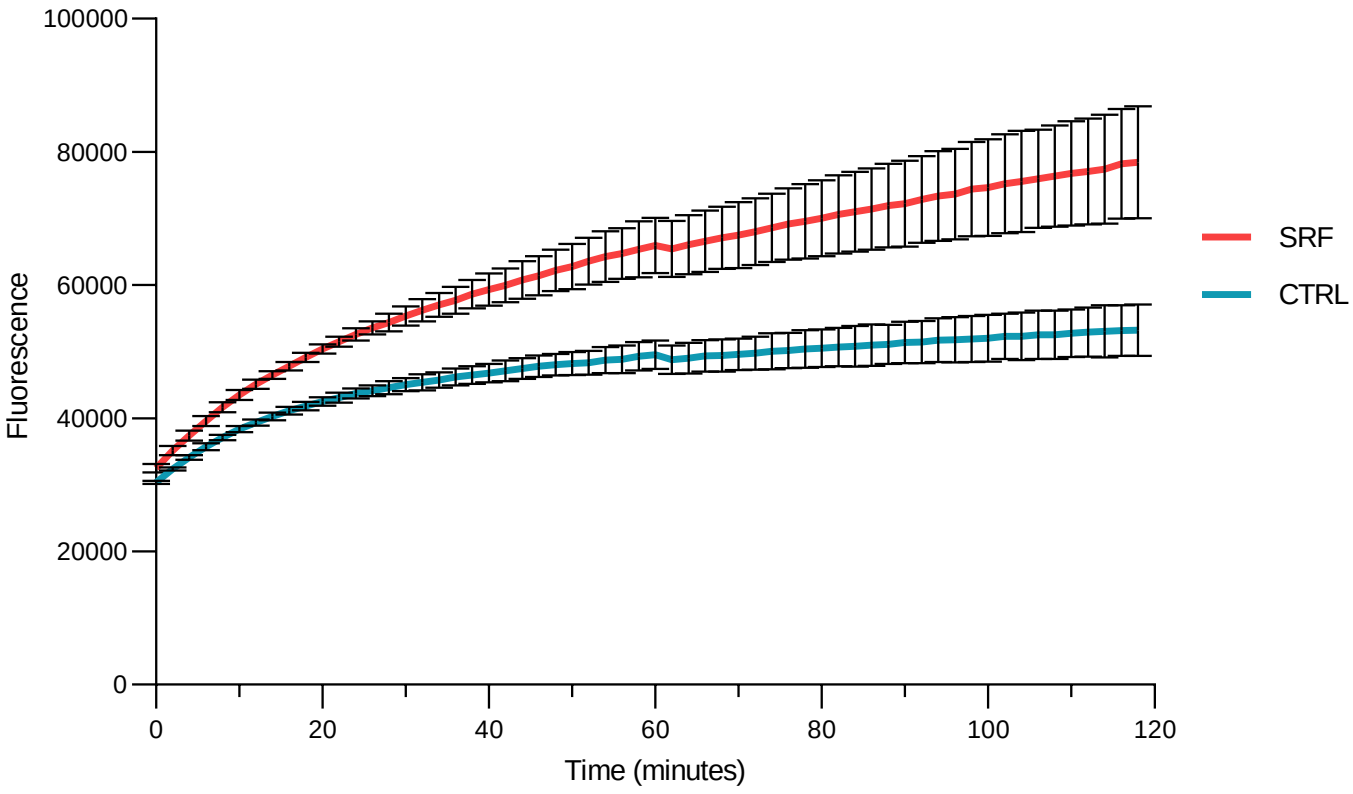

Figure 4.12: **Measurement of ROS production in tomato root protoplasts in presence of surfactin.** The blue line represents the emission of fluorescence by DCFDA in tomato root protoplasts in presence of surfactin. The grey line represents the emission of fluorescence by DCFDA in tomato root protoplasts in absence of surfactin.

On **figure 4.12**, it can be seen that the emission of fluorescence is always higher for the sample containing surfactin than for the control sample that does not contain surfactin. It can therefore be concluded that surfactin has a significant effect on the emission of fluorescence by DCFDA in protoplasts. It can be supposed that the presence of surfactin induces the production of ROS by protoplasts, which means that surfactin can induce a signal in protoplasts.

Chapter 5

DISCUSSION AND TECHNICAL PERSPECTIVES

Protoplasts are models that can provide useful information on biological mechanisms such as defense response in plants (Boudsocq et al., 2010; Pecher et al., 2014). They are living cells that are delimited by a PM but no longer have a cell wall, which is a complex matrix of polysaccharides, including cellulose, hemicellulose and pectin (Ochoa-Villarreal et al., 2012). The plant PM is mainly composed of lipids, including glycerolipids, sphingolipids and sterols (Furt et al., 2011), and proteins.

5.1 Optimization of tomato root protoplast isolation

In order to optimize the isolation of tomato root protoplast, the effect of several factors on protoplast production yield was tested. Those factors are the parameters of centrifugation, the age of tomato roots, the time of incubation with enzymes and the agitation during incubation with enzymes. Therefore, protoplast isolation was performed with different treatments, varying according to the different factors. For each treatment, the protoplast production yield was calculated. Each factor has been shown to have a significant influence on protoplast production yield (**Figure 4.1**, **Figure 4.3**, **Table 4.2** and **Table 4.3**).

First, the centrifugation time and speed have a strong influence on the protoplast production yield. As Sangra et al. (2019) and Jia et al. (2016) already demonstrated on leaf and mesophyll protoplasts, too low speed and time of centrifugation could not allow the complete formation of the pellet and lots of protoplasts remained in the supernatant, causing a low protoplast production yield. In addition, too high speed and time of centrifugation cause the rupture of the membrane of a part of the protoplasts, resulting in a low protoplast production yield and percentage of viability. Therefore, optimal centrifugation parameters can be defined as the lowest duo of speed and time of centrifugation enabling the formation of a firm pellet. This is the reason why in this study, after several experiments, the centrifugation parameters selected are 600 rcf and 6 minutes.

Secondly, the time of incubation with enzymes has also an influence on the protoplast production yield. Here, two different times of incubation were tested, including 3h30 and 17 hours of incubation. It was shown that an incubation of 17 hours provided a larger quantity of protoplasts than an incubation

of 3h30. As Sangra et al. (2019) already suggested for leaf protoplasts and Zhou et al. (2019) and Jia et al. (2016) for mesophyll protoplasts, too low incubation time could not induce the total digestion of tissues, while too long incubation could damage protoplast membranes since all tissues will be digested. Here, it was shown that the protoplast production yield for roots incubated for 3h30 is too low and an incubation of 17 hours was selected.

In addition, it was shown that the agitation during incubation also had an influence on the protoplast production yield only when roots were incubated for 3h30 in the enzymatic solution. As said above, a too short incubation with enzymes will not provide the complete digestion of tissues. Agitation during incubation facilitates contact between enzymes and tissues (Sangra et al., 2019). As tissues are not totally digested with an incubation of 3h30, the protoplast production yield increases when the solution is agitated during incubation because it increases contact between enzymes and tissues. For an incubation of 17 hours, there was no significant difference when the solution was agitated or not. However, there was a slight decrease of protoplast production yield and percentage of viability when roots were agitated during incubation. As mentioned by Sangra et al. (2019); Jia et al. (2016), when protoplasts, after tissue digestion, are exposed to the enzymes for too long, enzymes can become toxic. As all tissues will be digested and protoplasts will be exposed to enzymes, protoplast membranes will be digested, which will decrease the protoplast production yield. Therefore, the solution was not agitated during the incubation of 17 hours in order to decrease the toxicity of enzymes.

Finally, it was demonstrated that the age of roots has an influence on the protoplast production yield. Globally, 9-day-old roots provide a higher protoplast production yield than 7-day-old roots. Lin et al. (2016) previously demonstrated that younger tissues are less resistant to enzymes because their cell walls contained less cellulose than older tissues. And a too long incubation with enzymes could be harmful, causing the degradation of some protoplasts. Sangra et al. (2019) previously demonstrated on leaf protoplasts that the age of plant material has an influence on protoplast production yield. Similarly, too young leaves provided a low protoplast production yield and percentage of viability. On the contrary, too old plant materials are more developed and became more resistant to enzymes. Therefore, a too short exposure time with enzymes might not release digest all cell walls. As 7-day-old roots provided a large quantity of viable protoplasts with optimal time of incubation and agitation, they were preferred over 9-day-old roots because they can be obtained in two days less.

Optimized parameters for protoplast isolation are presented in **table 5.1**.

Table 5.1: Summary table of the optimized parameters for tomato root protoplast isolation

Protoplast isolation parameters	
Age of roots	7 days
Time of incubation with enzymes	17 hours
Agitation during incubation with enzymes	0 rpm
Centrifugation parameters	600 rcf; 6 minutes
Washing steps	Once with the saline washing solution and once with the conservation solution

However, a more rigorous statistical analysis should be undertaken to confirm the results. First, to get a more complete statistical analysis, protoplast suspension should be prepared from 9-day-old roots incubated for 17 hours with and without agitation. Therefore, the three-factor ANOVA and ANOVA on the interaction between the age of roots and the time of incubation could be performed. In addition to obtain more robust results, at least three protoplast suspensions from different tomato root sources should be prepared for each treatment. And for each protoplast suspension, protoplast production yield should also be counted at least three times.

In addition to the four factors tested to optimize protoplast isolation from tomato roots, other factors must be tested. First, the concentration of mannitol in the enzymatic solution could be optimized. As Sangra et al. (2019) demonstrated on leaf protoplasts and Zhou et al. (2019) and Jia et al. (2016) on mesophyll protoplasts, if the concentration of mannitol is too low, the solution is hypotonic and protoplasts can burst but if the concentration of mannitol is too high, the solution is hypertonic and protoplasts can fuse and die. It is therefore important to find the optimal concentration of mannitol so that protoplasts are not under osmotic stress. Secondly, the type and concentration of enzymes in the enzymatic solution could be optimized. Sangra et al. (2019), Jia et al. (2016) and Zhou et al. (2019) all tested different enzyme types and concentrations in order to select the most efficient combination. However, isolation conditions are strongly dependent on the plant species and even between the different tissues of the same plant, there can be differences in the isolation conditions (Zhou et al., 2019). This is why all isolation factors should be optimized on tomato roots in our case.

Moreover, percentages of viability obtained for each treatment are not totally reliable because FDA dye only makes viable protoplasts fluorescent, while dead protoplasts are not. However, some protoplasts exploded and they were not counted in the total number of protoplasts. Therefore, the total number of protoplasts and the number of dead protoplasts are underestimated. Huang et al. (1986) described an efficient double staining method where FDA is used as a dye for viable protoplasts and propidium iodide (PrI) is used as a dye for dead protoplasts. When protoplasts are double-stained with PrI-FDA, viable protoplasts are fluorescing in green and dead protoplasts and cell debris present a yellow-orange color. So, it would be interesting to test this double staining method to compare percentages of viability that were obtained.

5.1.1 Optimization of the purification of the protoplast suspension

Before performing any experiment on protoplasts, it is important to have a protoplast suspension containing as few debris as possible. Specifically for ITC, each impurity could induce a small heat flow and therefore biasing the heat flow due to an interaction between protoplasts and surfactin. Similarly, it is important that the composition of the solution containing the protoplasts and that the composition of the solution containing the surfactant are as similar as possible. A difference in composition of those two solutions could also induce a heat flow, which is not due to an interaction between protoplasts and surfactin. The protoplasts should ideally be washed two times with the saline washing solution to decrease the number of debris and so the composition of the solution is similar to the one containing the surfactant. It was shown that the protoplast concentration decreased a lot when protoplasts were washed two times rather than one and does not significantly decrease when

protoplasts were washed three times rather than two (**Figure 4.4**). It can be supposed that after each wash, if the pellet is too small, a small quantity of the pellet, containing the protoplasts, is lost and the protoplast production yield decreases. Therefore, only two washes could avoid wasting a too large number of protoplasts.

It is important to note that the number of roots used to measure the protoplast production yield for protoplasts that were washed once is more than three times bigger than the number of roots used to measure the protoplast production yield for protoplasts that were washed two and three times. This could be implicated in the big difference in protoplast production yield and future experiments should be performed with a larger quantity of roots. As shown in 4.2.3., when the protoplast isolation was performed on a large number of root material, the number of washes does not seem to have an effect on the protoplast production yield.

5.2 Optimization of isothermal titration calorimetry measurement on protoplasts

First, it is important to note that for each experiment tested, statistical analysis was performed on the means of three counts on the same protoplast suspension. Therefore, results obtained are not totally reliable but they are useful to get a first idea. To have more reliable results the experiments should be repeated at least three times and for each repetition, protoplast production yield or protoplast concentration should be calculated also at least three times.

5.2.1 Evaluation of tomato root protoplasts behavior during isothermal titration calorimetry preparation and measurement when protoplast suspension is placed in the injection syringe

It was decided that ITC measurement will be first performed with protoplast suspension in the injection syringe. In this design commonly used with liposome models (Lebecque et al., 2019), the experiment allows determining the binding constant in only one measurement and hence, characterizing the affinity of a surfactant for a membrane.

Therefore, before proceeding to ITC measurement on protoplasts, the behavior of protoplasts during ITC preparation steps and after ITC measurement must be evaluated. It was shown that there was no significant decrease of protoplast concentration between protoplasts before ITC measurement, degassed protoplasts and protoplasts that went through the injection syringe (**Figure 4.4** and **Table 4.5**). However, the generation of the protoplasts used for this experiment presented an unexpectedly low protoplast production yield. A low initial protoplast concentration and a big standard error can explain why the differences in protoplast concentration after each step of ITC preparation were not significant. Even if it was not statistically significant, a decreasing trend in the concentration of protoplasts during ITC preparation steps was observed. The experiment should be performed again and the number of repetitions of counting should be increased, to see if there is a significant difference of protoplast concentrations. In addition, the experiment should be performed again with a larger quantity of protoplasts in the injection syringe during ITC measurement. As the protoplast

suspension was diluted in the solution placed in the measuring cell, the final concentration in the measuring cell was too diluted and the counting on Bürker chamber was not reliable, providing a protoplast concentration with a really high standard error.

Isothermal titration calorimetry measurement of the interaction between protoplasts and surfactin when protoplast suspension is placed in the injection syringe

When the protoplasts are placed in the syringe and the surfactin in the measuring cell, no interaction was observed when protoplasts are injected in the cell (**figure 4.6**). This could be due to the low concentration of protoplasts in the syringe. In order to verify this hypothesis, the total lipid surface area of protoplasts injected during ITC was calculated and compared with the total lipid surface area of successful ITC experiments. The lipid surface area exposed by protoplasts was very low and it is probably the reason why no interaction between protoplasts and surfactin was detected.

5.2.2 Evaluation of *Arabidopsis* leaf protoplasts behavior during isothermal titration calorimetry preparation and measurement when protoplast suspension is placed in the injection syringe

As leaf protoplast production is easier and provides a larger quantity of protoplasts, an ITC measurement was performed with leaf protopalsts within the injection syringe. The higher concentration of protoplasts in this case led to an obstruction of the needle of the syringe because of their decantation.

In addition, the behavior of leaf protoplasts after 5 hours in the saline washing solution was investigated. It was shown that there is a significant decrease of the protoplast concentration when the protoplast suspension that went through the syringe was kept for 5 hours at 4°C (**Figure 4.7** and **Table 4.6**). In addition, it can be observed that the percentage of viability of protoplasts after 5 hours decreases for the degassed suspension and for the suspension that went through the syringe. It could be supposed that the conservation of protoplasts in the saline washing solution is not adequate and that ITC should be performed on protoplasts conserved in a solution with mannitol that helps to maintain osmotic pressure within the protoplasts. However, it is important to mention that leaf protoplasts are more fragile than root protoplasts.

5.2.3 Evaluation of tomato root protoplasts behavior during isothermal titration calorimetry preparation and measurement when protoplast suspension is placed in the measuring cell

As said above, when the protoplast suspension is placed in the injection syringe, the final protoplast concentration in the measuring cell is very low. In addition, highly concentrate protoplast suspension is likely to clog the injection syringe. And finally, it was supposed that protoplasts will be better conserved in the conservation solution, containing mannitol, rather than in the saline washing solution during the ITC measurement. All these limitations led to performing a second approach with ITC where a highly concentrated root protoplast suspension, where the protoplasts were suspended in the conservation solution, and which was placed in the measuring cell, while the solution containing surfactin was placed in the injection syringe.

First, the behavior of protoplasts during ITC preparation steps and measurement was investigated. It was shown that the protoplast concentration significantly decreased after degassing and after going through the glass syringe used to fill the measuring cell (**Figure 4.8** and **Table 4.7**). However, it was shown that the protoplast concentration before and after ITC measurement was statistically equal, by taking into account the dilution factor due to the addition of the solution from the injection syringe. It can be concluded that ITC measurement can be performed on root protoplasts because the experiment does not cause the protoplast death. The number of viable protoplasts during ITC was constant and data obtained were not skewed.

Isothermal titration calorimetry measurement of the interaction between protoplasts and surfactin when protoplast suspension is placed in the measuring cell

When the protoplasts are placed in the measuring cell and the surfactin in the injection syringe, a clear interaction between protoplasts and surfactin was observed (**Figure 4.10**). The peaks of the experiment performed with surfactin were ten times higher than when there was no surfactin. It can be concluded that ITC measurement with protoplast suspension in the measuring cell seems very promising. Finally, the total lipid surface area of protoplasts in this experiment was one hundred times higher than the one in the experiment when protoplasts were in the injection syringe but it was still one hundred times lower than with the one of an experiment with liposomes. The same ITC measurement can be performed with a more concentrated protoplast suspension to observe the impact of the protoplast concentration on the measured heat flow.

In conclusion, to perform ITC measurement on protoplasts it is necessary to use a sufficiently concentrated protoplast suspension placed in the measuring cell and to work with protoplasts resuspended in a solution containing mannitol, for a good conservation. In addition, ITC measurement on protoplast suspension in the injection syringe could not be performed because, with an appropriate protoplast concentration, the suspension will be prone to clog the needle of the injection syringe.

5.3 Flow cytometry and data analysis of tomato root protoplasts

In order to calculate more accurately and reliably the protoplast production yield and percentage of viability, flow cytometry can be used. Counts using a Bürker chamber are not the most accurate because only a small volume of suspension is analyzed and because it depends on the operator competences while it is not the case with flow cytometry. In addition, flow cytometry is much less time consuming and is therefore more suitable for routine counting. However, it is essential to calibrate flow cytometry with an observation of protoplast suspension under a microscope. With flow cytometry it can be difficult to differentiate the protoplast population from the debris population and it is therefore important to have an idea of the quantity of protoplasts in order to interpret flow cytometry results. Once flow cytometry calibration is made, it is a good routine method.

Flow cytometry was performed on 23 µL of a protoplast suspension containing 280 protoplasts per µL, which was counted on a Bürker chamber, and diluted 32 times. Therefore, approximately 210 protoplasts were analyzed by flow cytometry, which represents less than 1 % of protoplasts among

all the particles present in the solution. An increase of fluorescence can be observed when protoplast suspension was stained with FDA (**Figure 4.11**). It includes the fluorescence emitted by protoplasts but also by some debris. The number of debris in the protoplast suspension was too high to accurately calculate the protoplast production yield and the percentage of viability. The protoplast suspension should be better purified. As described by Fontes et al. (2010), Hughes et al. (1978) and Gamborg and Phillips (1995), protoplasts could be purified by discontinuous gradient centrifugation. With this technique, solutions, containing various concentrations of carbohydrates and salts, are prepared. Protoplasts are transferred in a mix of two solutions, which is centrifuged and protoplasts are recovered at the interface between the two solutions, while debris remain in the solutions. However, Ferrer et al. (1985) demonstrated that filtration was an efficient purification method. The protoplast suspension is firstly filtrated with a large-pore filter in order to remove large fragments while protoplasts are in the solution. Then, the suspension is filtrated a second time with a small-pore filter and small debris remain in solution while protoplasts are trapped in the filter. Finally, the filter is placed in solution in order to liberate stuck protoplasts. However, both purification methods should be tested and the most efficient one should be selected.

Successful flow cytometry was previously performed on leaf and mesophyll protoplasts (Badaró Costa et al., 2018; Zhou et al., 2019), while it did not work on root protoplasts. The range of size for root protoplasts is larger than for leaf protoplasts (Mazarei et al., 2008) and it could justify the success of flow cytometry measurement on leaf and mesophyll protoplasts. It means that it is harder to find root protoplast population on a FSC-SSC dot plot than for leaf protoplast populations.

5.4 Measurement of ROS production in tomato root protoplasts in presence of surfactin

The production of ROS by root protoplasts was evaluated in presence and in absence of surfactin. It was shown that root protoplasts in presence of surfactin produced more ROS than root protoplasts in absence of surfactin (**Figure 4.12**). It can be deduced that surfactin can interact with protoplasts and induce a signal, leading to the production of ROS, such as it was observed in tobacco and tomato roots (Henry et al., 2011; Cawoy et al., 2014), suggesting that protoplasts and plants respond similarly to surfactin. To conclude, as protoplasts and intact cells respond similarly to surfactin, protoplasts are therefore relevant models for the study of biological mechanisms.

Chapter 6

CONCLUSION AND PERSPECTIVES

In order to understand the molecular mechanism of the perception process of surfactin, an elicitor produced by several *Bacillus subtilis* strains (Eeman et al., 2006; Henry et al., 2011; Cawoy et al., 2014; Deleu et al., 2003), membrane models are required. Biomimetic models, such as liposomes, can be used even if they are not able to reproduce the complexity of the plant PM. Therefore, protoplasts are required to confirm results obtained with liposomes because they better represent the complexity and composition of the plant PM.

The optimization of the isolation of tomato root protoplasts has shown that protoplasts from 7-day-old roots that were incubated for 17 hours without agitation provide adequate protoplast production yield and percentage of viability. However, the production of root protoplasts is trickier than the production of leaf protoplasts, as the protoplast production yield is proportionally lower for roots (Pindel, 2007), and requires more optimization steps. A part of this optimization has been achieved but other parameters, such as the concentration of mannitol and the concentration and type of enzymes in the enzymatic solution, must still be optimized (Sangra et al., 2019; Jia et al., 2016; Zhou et al., 2019). In addition, the purification of protoplasts must also be optimized in order to avoid the presence of debris and to allow the study of the protoplasts with specific techniques like the isothermal titration calorimetry and the flow cytometry.

Then, ITC measurements were performed on protoplast suspension, firstly placed in the injection syringe and secondly placed in the measuring cell. When the sufficiently concentrated root protoplast suspension was placed in the measuring cell and surfactin in the injection syringe, an interaction was observed. In addition, it was shown that protoplasts do not die or explode during ITC measurement.

In this study, the optimization of root protoplast isolation protocol and the optimization of ITC measurement on protoplasts was performed on tomato root protoplasts. The final purpose is to perform ITC measurement on *Arabidopsis thaliana* root protoplasts to evaluate their interaction with surfactin. This is the most frequently used plant model. Its main advantages are that it has a rapid life cycle, it is easy to grow, it is low-cost, it has one of the simplest genomes known for a higher plant and it is easy to genetically transform, providing a large number of mutant possibilities (Johnson

and Bouchez, 2007; Koornneef and Meinke, 2010; Diaz, 2019). Tomato plants were used for a first optimization because they provide a larger quantity of biological material for the same amount of time. So, the next step will be to adapt the method using *Arabidopsis* roots.

After the optimization of protoplast isolation and ITC preparation steps on protoplasts, the molecular interaction between surfactin and the plant PM was investigated. First, the induction of a signal in root protoplasts by surfactin was verified. It was shown that surfactin induces the production of ROS by root protoplasts, suggesting that surfactin is perceived by the protoplasts and is able to trigger the same early events of the plant defense mechanism in protoplasts than in intact cells.

ITC measurements performed on root protoplasts with surfactin have shown that surfactin is able to interact with the protoplasts, as it was already observed on liposomes with a composition mimicking the plant PM. The profile of the heat flow is not yet optimal to determine the thermodynamic parameters of the binding. Some further optimization like the purification of the protoplasts, the choice of their initial concentration in the measuring cell and, the concentration of surfactin in the syringe are still required.

Nevertheless, the ITC measurement on protoplasts is very promising. Results will make possible to fill the gap between the ones obtained in biophysics on the liposomes, a very simplified cell model, and the ones carried out in biology on real cells, tissues or the whole plant. The use of protoplasts from roots muted in a specific lipid or protein could lead to a better understanding of biological mechanisms like the elicitor perception by the plants. In the case of surfactin, it could confirm that some specific lipids of the plant PM play a main role in its perception by the plant cell and may influence the activity of mechanosensitive channels by regulating the plasma membrane tension.

Chapter 7

APPENDIX

Table 7.1: P-values of Tukey's test made on average protoplast concentrations of protoplasts from 7-day-old roots obtained after 17 hours of incubation, without agitation, to evaluate the influence of washing on protoplast production yield. P-values : NS (Not significative) $> 0.05; * \leqslant 0.05; ** \leqslant 0.01; * * * \leqslant 0.001; * * ** \leqslant 0.0001$.

	1 wash	2 washes
2 washes	0.0000362	-
3 washes	0.0000174	0.2907028

Table 7.2: P-values of Tukey's test made on average protoplast concentrations of protoplasts before and after ITC measurements where protoplast suspension was placed in the injection syringe. P-values : NS (Not significative) $> 0.05; * \leqslant 0.05; ** \leqslant 0.01; * * * \leqslant 0.001; * * ** \leqslant 0.0001$.

	Protoplasts before ITC	Degassed protoplasts
Degassed protoplasts	0.2085037	-
Protoplasts after going through the syringe	0.1246413	0.9147755

Table 7.3: P-values of Tukey's test made on average protoplast concentrations obtained for degassed protoplasts and protoplasts that went through the injection syringe before and after 5 hours on *Arabidopsis* leaf protoplasts obtained after 17 hours of incubation, without agitation. P-values : NS (Not significative)$> 0.05; * \leqslant 0.05; ** \leqslant 0.01; * * * \leqslant 0.001; * * ** \leqslant 0.0001$.

	Freshly degassed protoplasts	Degassed protoplasts after 5 hours	Fresh protoplasts after going through the syringe
Degassed protoplasts after 5 hours	0.1675610	-	-
Fresh protoplasts after going through the syringe	0.9503786	0.3336918	-
Protoplasts after going through the syringe after 5 hours	0.0021403	0.0427745	0.0039604

Table 7.4: P-values of Tukey's test made on average protoplast concentrations of protoplasts before and after ITC measurements where protoplast suspension was placed in the measuring cell. P-values : NS (Not significative) $> 0.05; * \leqslant 0.05; ** \leqslant 0.01; * * * \leqslant 0.001; * * ** \leqslant 0.0001$.

	Protoplasts before ITC	Degassed protoplasts	Protoplasts after going through the glass syringe
Degassed protoplasts	0.0197512	-	-
Protoplasts after going through the glass syringe	0.0001390	0.0081097	-
Protoplasts after ITC in the measuring cell	0.0001666	0.0106167	0.9965015

Bibliography

Alagumuthu, M., Dahiya, D., and Singh Nigam, P. (2019). Phospholipid—the dynamic structure between living and non-living world; a much obligatory supramolecule for present and future. *AIMS Molecular Science*, 6(1):1–19.

Aoyagi, H. (2011). Application of plant protoplasts for the production of useful metabolites. *Biochemical Engineering Journal*, 56(1-2):1–8.

Asai, T., Tena, G., Plotnikova, J., Willmann, M. R., Chiu, W.-L., Gomez-Gomez, L., Boller, T., Ausubel, F. M., and Sheen, J. (2002). MAP kinase signalling cascade in Arabidopsis innate immunity. *Nature*, 415:977–983.

Bach, M., Schnitzler, J. P., and Seitz, H. U. (1993). Elicitor-induced changes in Ca2+ influx, K+ efflux, and 4-hydroxybenzoic acid synthesis in protoplasts of Daucus carota L. *Plant Physiology*, 103(2):407–412.

Badaró Costa, N. L., Carvalho, C. R., and Clarindo, W. R. (2018). Improved procedures to assess plant protoplast viability: Evidencing cytological and genomic damage. *Cytologia*, 83(4):397–405.

Balasubramanian, V., Vashisht, D., Cletus, J., and Sakthivel, N. (2012). Plant β-1,3-glucanases: Their biological functions and transgenic expression against phytopathogenic fungi. *Biotechnology Letters*, 34(11):1983–1990.

Bengochea, T. and Dodds, J. H. (1986). *Plant Protoplasts: A Biotechnological Tool for Plant Improvement*. Springer Netherlands, 1 edition.

Bhojwani, S. and Razdan, M. (1996). Plant Tissue Culture: Theory and Practice, a Revised Edition. *Modern Applications of Plant Biotechnology in Pharmaceutical Sciences*, pages 1–779.

Boudsocq, M., Barbier-Brygoo, H., and Laurière, C. (2004). Identification of nine sucrose nonfermenting 1-related protein kinases 2 activated by hyperosmotic and saline stresses in Arabidopsis thaliana. *Journal of Biological Chemistry*, 279(40):41758–41766.

Boudsocq, M., Willmann, M. R., McCormack, M., Lee, H., Shan, L., He, P., Bush, J., Cheng, S.-H., and Sheen, J. (2010). Differential innate immune signalling via Ca2+ sensor protein kinases. *Nature*, 464(7287):418–422.

Browse, J., Somerville, C. R., and Slack, C. R. (1988). Changes in lipid composition during protoplast isolation. *Plant Science*, 56(1):15–20.

Buchanan, B. B., Gruissem, W., and Jones, R. L. (2015). *Biochemistry & molecular biology of plants*, volume 3. John Wiley & Sons, 2 edition.

Cacas, J. L., Buré, C., Grosjean, K., Gerbeau-Pissot, P., Lherminier, J., Rombouts, Y., Maes, E., Bossard, C., Gronnier, J., Furt, F., Fouillen, L., Germain, V., Bayer, E., Cluzet, S., Robert, F., Schmitter, J. M., Deleu, M., Lins, L., Simon-Plas, F., and Mongrand, S. (2016). Revisiting plant plasma membrane lipids in tobacco: A focus on sphingolipids. *Plant Physiology*, 170(1):367–384.

Cacas, J. L., Furt, F., Le Guédard, M., Schmitter, J. M., Buré, C., Gerbeau-Pissot, P., Moreau, P., Bessoule, J. J., Simon-Plas, F., and Mongrand, S. (2012). Lipids of plant membrane rafts. *Progress in Lipid Research*, 51(3):272–299.

Casares, D., Escribá, P. V., and Rosselló, C. A. (2019). Membrane lipid composition: Effect on membrane and organelle structure, function and compartmentalization and therapeutic avenues. *International Journal of Molecular Sciences*, 20(9).

Cawoy, H., Mariutto, M., Henry, G., Fisher, C., Vasilyeva, N., Thonart, P., Dommes, J., and Ongena, M. (2014). Plant Defense Stimulation by Natural Isolates of Bacillus Depends on Efficient Surfactin Production. *Molecular Plant-Microbe Interactions*, 27(2):87–100.

Chen, M. and B. Cahoon, E. (2009). *Plant Sphingolipids: Structure, Synthesis and Function*, volume 30.

Cheng, Y. and Bélanger, R. R. (2000). Protoplast preparation and regeneration from spores of the biocontrol fungus Pseudozyma flocculosa. *FEMS Microbiology Letters*, 190(2000):287–291.

Davey, M. R., Anthony, P., Power, J. B., and Lowe, K. C. (2004). Protoplast Applications in Biotechnology. *Encyclopedia of crop and plant science*.

Davey, M. R., Anthony, P., Power, J. B., and Lowe, K. C. (2005). Plant protoplasts: Status and biotechnological perspectives. *Biotechnology Advances*, 23(2):131–171.

Debois, D., Fernandez, O., Franzil, L., Jourdan, E., de Brogniez, A., Willems, L., Clément, C., Dorey, S., De Pauw, E., and Ongena, M. (2015). Plant polysaccharides initiate underground crosstalk with bacilli by inducing synthesis of the immunogenic lipopeptide surfactin. *Environmental Microbiology Reports*, 7(3):570–582.

Deleu, M., Bouffioux, O., Razafindralambo, H., Paquot, M., Hbid, C., Thonart, P., Jacques, P., and Brasseur, R. (2003). Interaction of surfactin with membranes: A computational approach. *Langmuir*, 19(8):3377–3385.

Deleu, M., Crowet, J. M., Nasir, M. N., and Lins, L. (2014). Complementary biophysical tools to investigate lipid specificity in the interaction between bioactive molecules and the plasma membrane: A review. *Biochimica et Biophysica Acta - Biomembranes*, 1838(12):3171–3190.

Deleu, M., Lorent, J., Lins, L., Brasseur, R., Braun, N., El Kirat, K., Nylander, T., Dufrêne, Y. F., and Mingeot-Leclercq, M. P. (2013). Effects of surfactin on membrane models displaying lipid phase separation. *Biochimica et Biophysica Acta - Biomembranes*, 1828(2):801–815.

Deshpande, S. and Dekker, C. (2018). On-chip microfluidic production of cell-sized liposomes. *Nature Protocols*, 13(5):856–874.

Diaz, M. (2019). Arabidopsis Thaliana: From Weed to Model Organism. *Current Protocols in Essential Laboratory Techniques*, 19(1):1–14.

Doktorova, M., Heberle, F. A., Eicher, B., Standaert, R. F., Katsaras, J., London, E., Pabst, G., and Marquardt, D. (2018). Preparation of asymmetric phospholipid vesicles for use as cell membrane models. *Nature Protocols*, 13(9):2086–2101.

Donato, P., Dugo, P., and Mondello, L. (2013). Separation of Lipids. In *Liquid Chromatography: Applications*, pages 203–248. Elsevier.

Eeman, M., Berquand, A., Dufrêne, Y. F., Paquot, M., Dufour, S., and Deleu, M. (2006). Penetration of surfactin into phospholipid monolayers: Nanoscale interfacial organization. *Langmuir*, 22(26):11337–11345.

Eeman, M. and Deleu, M. (2010). From biological membranes to biomimetic model membranes. *Biotechnology, Agronomy and Society and Environment*, 14(4):719–736.

Evrard, A., Bargmann, B. O., Birnbaum, K. D., Tester, M., Baumann, U., and Johnson, A. A. (2012). Fluorescence-Activated Cell Sorting for Analysis of Cell Type-Specific Responses to Salinity Stress in Arabidopsis and Rice. *Methods in molecular biology*, 913:265–276.

Ferhan, A. R., Yoon, B. K., Park, S., Sut, T. N., Chin, H., Park, J. H., Jackman, J. A., and Cho, N. J. (2019). Solvent-assisted preparation of supported lipid bilayers. *Nature Protocols*, 14(7):2091–2118.

Ferrer, A., Altabella, T., Arró, M., and Boronat, A. (2017). Emerging roles for conjugated sterols in plants. *Progress in Lipid Research*, 67(2017):27–37.

Ferrer, S., Ramón, D., Salom, J., Vicente, E., and Uruburu, F. (1985). Protoplasts from Podospora anserina: Isolation, purification, and transformation. *Current Microbiology*, 12(5):301–306.

Fontes, N., Silva, R., Vignault, C., Lecourieux, F., Gerás, H., and Delrot, S. (2010). Purification and functional characterization of protoplasts and intact vacuoles from grape cells. *BMC Research Notes*, 3:1–7.

Fowke, L. C. and Constabel, F. (1989). *Plant Protoplasts*. CRC Press.

Fuchs, I., Stölzle, S., Ivashikina, N., and Hedrich, R. (2005). Rice K+ uptake channel OsAKT1 is sensitive to salt stress. *Planta*, 221(2):212–221.

Fujimoto, T. and Parmryd, I. (2017). Interleaflet coupling, pinning, and leaflet asymmetry-major players in plasma membrane nanodomain formation. *Frontiers in Cell and Developmental Biology*, 4(JAN):1–12.

Furt, F., Simon-Plas, F., and Mongrand, S. (2011). Lipids of the Plant Plasma Membrane. *The Plant Plasma Membrane*, (November 2010):353–377.

Gamborg, O. L. and Phillips, G. C. (1995). *Plant Cell, Tissue and Organ Culture: Fundamental Methods*. Springer-Verlag Berlin Heidelberg, 1 edition.

Goetz, C., Hammerbeck, C., and Bonnevier, J. (2018). *Flow Cytometry Basics for the Non-Expert*. Springer International Publishing, 1st ed. edition.

Gong, B. Q., Guo, J., Zhang, N., Yao, X., Wang, H. B., and Li, J. F. (2019). Cross-Microbial Protection via Priming a Conserved Immune Co-Receptor through Juxtamembrane Phosphorylation in Plants. *Cell Host and Microbe*, 26(6):810–822.

Grimm, J. B., Heckman, L. M., and Lavis, L. D. (2013). The chemistry of small-molecule fluorogenic probes. In *Progress in Molecular Biology and Translational Science*, volume 113, pages 1–34. Elsevier Inc., 1 edition.

Grosjean, K., Der, C., Robert, F., Thomas, D., Mongrand, S., Simon-Plas, F., and Gerbeau-Pissot, P. (2018). Interactions between lipids and proteins are critical for organization of plasma membrane-ordered domains in tobacco BY-2 cells. *Journal of Experimental Botany*, 69(15):3545–3557.

Grosjean, K., Mongrand, S., Beney, L., Simon-Plas, F., and Gerbeau-Pissot, P. (2015). Differential effect of plant lipids on membrane organization specificities of phytosphingolipids and phytosterols. *Journal of Biological Chemistry*, 290(9):5810–5825.

Hardy, G. J., Nayak, R., Alam, S. M., Shapter, J. G., Heinrich, F., and Zauscher, S. (2012). Biomimetic supported lipid bilayers with high cholesterol content formed by α-helical peptide-induced vesicle fusion. *Journal of Materials Chemistry A*, 22(37):19506–19513.

Hardy, G. J., Nayak, R., and Zauscher, S. (2013). Model cell membranes: Techniques to form complex biomimetic supported lipid bilayers via vesicle fusion. *Current Opinion in Colloid & Interface Science*, 18(5):448–458.

Hartmann, M. A. (1998). Plant sterols and the membrane environment. *Trends in Plant Science*, 3(5):170–175.

Henry, G., Deleu, M., Jourdan, E., Thonart, P., and Ongena, M. (2011). The bacterial lipopeptide surfactin targets the lipid fraction of the plant plasma membrane to trigger immune-related defence responses. *Cellular Microbiology*, 13(11):1824–1837.

Huang, C.-N., Cornejo, M. J., Bush, D. S., and Jones, R. L. (1986). Estimating viability of plant protoplasts using double and single staining. *Protoplasma*, 135:80–87.

Hughes, B. G., White, F. G., and Smith, M. A. (1978). Purification of Plant Protoplasts by Discontinuous Gradient Centrifugation. *Biochemie und Physiologie der Pflanzen*, 172(3):223–231.

Isalomboto Nkanga, C., Murhimalika Bapolisi, A., Ikemefuna Okafor, N., and Werner Maçedo Krause, R. (2019). General Perception of Liposomes: Formation, Manufacturing and Applications. In *Liposomes - Advances and Perspectives*.

Israelachvili, J. N., Mitchell, D. J., and Ninham, B. W. (1975). Theory of self-assembly of hydrocarbon amphiphiles into micelles and bilayers. *Journal of the Chemical Society, Faraday Transactions 2: Molecular and Chemical Physics*, 72:1525–1568.

Jelenska, J., Davern, S. M., Standaert, R. F., Mirzadeh, S., and Greenberg, J. T. (2017). Flagellin peptide flg22 gains access to long-distance trafficking in Arabidopsis via its receptor, FLS2. *Journal of Experimental Botany*, 68(7):1769–1783.

Jia, N., Zhu, Y., and Xie, F. (2018). An efficient protocol for model legume root protoplast isolation and transformation. *Frontiers in Plant Science*, 9(June):1–7.

Jia, X., Zhang, X., Qu, J., and Han, R. (2016). Optimization Conditions of Wheat Mesophyll Protoplast Isolation. *Agricultural Sciences*, 07(12):850–858.

Jiang, Z., Zhou, X., Tao, M., Yuan, F., Liu, L., Wu, F., Wu, X., Xiang, Y., Niu, Y., Liu, F., Li, C., Ye, R., Byeon, B., Xue, Y., Zhao, H., Wang, H. N., Crawford, B. M., Johnson, D. M., Hu, C., Pei, C., Zhou, W., Swift, G. B., Zhang, H., Vo-Dinh, T., Hu, Z., Siedow, J. N., and Pei, Z. M. (2019). Plant cell-surface GIPC sphingolipids sense salt to trigger Ca2+ influx. *Nature*, 572:341–346.

Johnson, X. and Bouchez, D. (2007). The model species, Arabidopsis thaliana. In *Plant Models in Genomics*, number January 2007, pages 385–396.

Jouanneau, J. P., Lapous, D., and Guern, J. (1991). In plant protoplasts, the spontaneous expression of defense reactions and the responsiveness to exogenous elicitors are under auxin control. *Plant Physiology*, 96(2):459–466.

Jouhet, J. (2013). Importance of the hexagonal lipid phase in biological membrane organization. *Frontiers in Plant Science*, 4:1–5.

Kilic, A. and Kok, F. N. (2016). Biomimetic lipid bilayers on solid surfaces: Models for biological interactions. *Surface Innovations*, 4(3):141–157.

Kök, F. N., Yildiz, A. A., and Inci, F. (2019). *Biomimetic Lipid Membranes: Fundamentals, Applications, and Commercialization*. Springer International Publishing, 1 edition.

Koornneef, M. and Meinke, D. (2010). The development of Arabidopsis as a model plant. *the Plant Journal*, 61(6):909–921.

Kurusu, T., Kuchitsu, K., Nakano, M., Nakayama, Y., and Iida, H. (2013). Plant mechanosensing and Ca2+ transport. *Trends in Plant Science*, 18(4):227–233.

Lebecque, S., Lins, L., Dayan, F. E., Fauconnier, M. L., and Deleu, M. (2019). Interactions between natural herbicides and lipid bilayers mimicking the plant plasma membrane. *Frontiers in Plant Science*, 10(March):1–11.

Ledoux, Q., Van Cutsem, P., Mark, I. E., and Veys, P. (2014). Specific localization and measurement of hydrogen peroxide in Arabidopsis thaliana cell suspensions and protoplasts elicited by COS-OGA. *Plant Signaling & Behavior*, 9(5):e28824 1–6.

Li, J., He, Q., and Yan, X. (2011). *Molecular Assembly of Biomimetic Systems*. Wiley-VCH, 1 edition.

Lin, F., Manisseri, C., Fagerström, A., Peck, M. L., Vega-Sánchez, M. E., Williams, B., Chiniquy, D. M., Saha, P., Pattathil, S., Conlin, B., Zhu, L., Hahn, M. G., Willats, W. G., Scheller, H. V., Ronald, P. C., and Bartley, L. E. (2016). Cell wall composition and candidate biosynthesis gene expression during rice development. *Plant and Cell Physiology*, 57(10):2058–2075.

Liu, S., Liu, H., Lin, M., Xu, F., and Lu, T. J. (2017). Intracellular Microfluid Transportation in Fast Growing Pollen Tubes. In *Modeling of Microscale Transport in Biological Processes*, pages 155–169.

Lu, L., Schertzer, J. W., and Chiarot, P. R. (2015). Continuous microfluidic fabrication of synthetic asymmetric vesicles. *Lab on a Chip*, 15(17):3591–3599.

Maget-Dana, R. and Ptak, M. (1995). Interactions of surfactin with membrane models. *Biophysical Journal*, 68(5):1937–1943.

Maintz, J., Cavdar, M., Tamborski, J., Kwaaitaal, M., Huisman, R., Meesters, C., Kombrink, E., and Panstruga, R. (2014). Comparative Analysis of MAMP-induced Calcium Influx in Arabidopsis Seedlings and Protoplasts. *Plant and Cell Physiology*, 55(10):1813–1825.

Mamode Cassim, A., Gouguet, P., Gronnier, J., Laurent, N., Germain, V., Grison, M., Boutté, Y., Gerbeau-Pissot, P., Simon-Plas, F., and Mongrand, S. (2019). Plant lipids: Key players of plasma membrane organization and function. *Progress in Lipid Research*, 73:1–27.

Martinez, J. C., Murciano-Calles, J., Cobos, E. S., Iglesias-Bexiga, M., Luque, I., and Ruiz-Sanz, J. (2013). Isothermal Titration Calorimetry: Thermodynamic Analysis of the Binding Thermograms of Molecular Recognition Events by Using Equilibrium Models. In *Applications of Calorimetry in a Wide Context - Differential Scanning Calorimetry, Isothermal Titration Calorimetry and Microcalorimetry*.

Mastuti, R. and Rosyidah, M. (2018). In vitro enzymatic isolation of protoplasts from tissues of the medicinal plant Physalis angulata L. *AIP Conference Proceedings*, 020002(2018):1–6.

Mazarei, M., Al-Ahmad, H., Rudis, M. R., and Stewart, C. N. (2008). Protoplast isolation and transient gene expression in switchgrass, Panicum virgatum L. *Biotechnology Journal*, 3(3):354–359.

Mennicke, U. and Salditt, T. (2002). Preparation of solid-supported lipid bilayers by spin-coating. *Langmuir*, 18(21):8172–8177.

Michaelson, L. V., Napier, J. A., Molino, D., and Faure, J. D. (2016). Plant sphingolipids: Their importance in cellular organization and adaption. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*, 1861(9):1329–1335.

Mongrand, S., Stanislas, T., Bayer, E. M., Lherminier, J., and Simon-Plas, F. (2010). Membrane rafts in plant cells. *Trends in Plant Science*, 15(12):656–663.

Moreau, R. A., Nyström, L., Whitaker, B. D., Winkler-Moser, J. K., Baer, D. J., Gebauer, S. K., and Hicks, K. B. (2018). Phytosterols and their derivatives: Structural diversity, distribution, metabolism, analysis, and health-promoting uses. *Progress in Lipid Research*, 70(2018):35–61.

Newell, C. A. (2000). Plant Transformation Technologies. *Molecular Biotechnology*, 16:53–65.

Ochoa-Villarreal, M., Aispuro-Hernndez, E., Vargas-Arispuro, I., and Ngel, M. (2012). Plant Cell Wall Polymers: Function, Structure and Biological Activity of Their Derivatives. In *Polymerization*, pages 63–86.

Pecher, P., Eschen-Lippold, L., Herklotz, S., Kuhle, K., Naumann, K., Bethke, G., Uhrig, J., Weyhe, M., Scheel, D., and Lee, J. (2014). The Arabidopsis thaliana mitogen-activated protein kinases MPK3 and MPK6 target a subclass of 'VQ-motif'-containing proteins to regulate immune responses. *New Phytologist*, 203(2):592–606.

Pereno, V., Carugo, D., Bau, L., Sezgin, E., Bernardino De La Serna, J., Eggeling, C., and Stride, E. (2017). Electroformation of Giant Unilamellar Vesicles on Stainless Steel Electrodes. *ACS Omega*, 2(3):994–1002.

Pilet, P. E. (1985). *The Physiological Properties of Plant Protoplasts*. Springer-Verlag Berlin Heidelberg.

Pindel, A. (2007). Optimization of isolation conditions of Cymbidium protoplasts. *Folia Horticulturae*, 19(2):79–88.

Razafindralambo, H., Dufour, S., Paquot, M., and Deleu, M. (2009). Thermodynamic studies of the binding interactions of surfactin analogues to lipid vesicles Application of isothermal titration calorimetry. *Journal of Thermal Analysis and Calorimetry*, 95(3):817–821.

Sangra, A., Shahin, L., and Dhir, S. K. (2019). Optimization of Isolation and Culture of Protoplasts in Alfalfa (Medicago sativa) Cultivar Regen-SY. *American Journal of Plant Sciences*, 10(07):1206–1219.

Santas, J., Codony, R., and Rafecas, M. (2013). Phytosterols: Beneficial Effects. In *Natural Products: Phytochemistry*, pages 3437–3464.

Sheen, J. (2001). Signal transduction in maize and Arabidopsis mesophyll protoplasts. *Plant Physiology*, 127(4):1466–1475.

Sheikhpour, M., Barani, L., and Kasaeian, A. (2017). Biomimetics in drug delivery systems: A critical review. *Journal of Controlled Release*, 253(2017):97–109.

Shen, H.-H. H., Thomas, R. K., Penfold, J., and Fragneto, G. (2010). Destruction and Solubilization of Supported Phospholipid Bilayers on Silica by the Biosurfactant Surfactin. *Langmuir*, 26(10):7334–7342.

Simon-Plas, F., Perraki, A., Bayer, E., Gerbeau-Pissot, P., and Mongrand, S. (2011). An update on plant membrane rafts. *Current Opinion in Plant Biology*, 14(6):642–649.

Simons, K. and Sampaio, J. L. (2011). Membrane Organization and Lipid Rafts. *Cold Spring Harb Perspect Biol.*, 3(10):a004697 1–17.

Song, C., Zhang, S., and Huang, H. (2015). Choosing a suitable method for the identification of replication origins in microbial genomes. *Frontiers in Microbiology*, 6(1049):1–18.

Wu, F., Chi, Y., Jiang, Z., Xu, Y., Xie, L., Huang, F., Wan, D., Ni, J., Yuan, F., Wu, X., Zhang, Y., Wang, L., Ye, R., Byeon, B., Wang, W., Zhang, S., Sima, M., Chen, S., Zhu, M., Pei, J., Johnson, D. M., Zhu, S., Cao, X., Pei, C., Zai, Z., Liu, Y., Liu, T., Swift, G. B., Zhang, W., Yu, M., Hu, Z., Siedow, J. N., Chen, X., and Pei, Z. M. (2020). Hydrogen peroxide sensor HPCA1 is an LRR receptor kinase in Arabidopsis. *Nature*, 578(7796):577–581.

Yu, M., Cui, Y., Zhang, X., Li, R., and Lin, J. (2020). Organization and dynamics of functional plant membrane microdomains. *Cellular and Molecular Life Sciences*, 77(2):275–287.

Zhou, Q., Jiang, Z., Li, Y., Zhang, T., Zhu, H., Zhao, F., and Zhao, Z. (2019). Mesophyll protoplast isolation technique and flow cytometry analysis of ancient platycladus orientalis (Cupressaceae). *Turkish Journal of Agriculture and Forestry*, 43(3):275–287.

Zorzoli, R., Raúl, G., Gustavo, P., Rodríguez, R., and Picardi, L. A. (2007). Advances in Biotechnology : Tomato as a Plant Model System. *Functional Plant Science and Biotechnology*, 1(1):146–159.