Detection of Phytohormones for Early Identification of Vineyard Abiotic and Biotic Stress Factors

Rui Pedro Esteves Meirinho[†*]
Supervisors: Prof. Doctor João Pedro Estrela Rodrigues Conde[†]

Abstract

The increase in temperature due to greenhouse gases influences the development of pathogens and abiotic stresses with important consequences in agricultural production. The routine retrievals that are sent for testing to identify the infection or abiotic stress tend to be costly and time-consuming. So, there is a need to develop a point-of-use microfluidic analytical device for a fast (30 minutes), in-the-field detection. These devices are an attractive solution due to its low cost of fabrication, low volumes of reagents (5 µL) used and when allied with an immunoassay, high sensitivity can be achieved. In this dissertation I present a competitive immunoassay using a microbeads-based microfluidic biosensing device for detection ot three phytohormones that signal the presence of stresses, Jasmonic Acid (JA) and Azelaic Acid (AZA) for biotic stresses, and Abscisic Acid (ABA) for abiotic stress.
I succeeded in detecting the three acids in buffer in the following range of values: 2×10^{-2} to 9×10^{-6} mg/mL, 9×10^{-3} to 9×10^{-6} mg/mL and 2×10^{-2} to 9×10^{-5} mg/mL, for JA, AZA and ABA, respectively.
I was also able to detect a variation in signal when spiking with JA in red wine grape samples, but there is another method, such as HPLC, is necessary to validate and quantify this analyte in the real sample.
This work demonstrates that it is possible to detect these molecules in buffer and with the development of a sample treatment protocol, detect in real samples in the future.

Keywords: *Microfluidics; Agriculture; Infections; Jasmonic Acid, Azelaic Acid; Abscisic Acid*

1 Introduction

Global warming plays an important role in the majority of challenges that plants encounter, due to the rise of the average annual temperature and extreme weather conditions, grapevines are no exception. These plants are susceptible to changes in the weather. Since grapevines need a specific set of climate variables in order to develop fully. Considering this plant has a relevant role in our society due to its cultivation both as a food crop, as well as for wine production, it is important know when the grapevines are under stresses, whether these are abiotic or biotic, since the increase in global temperature is favourable to the development of pathogens [1].

Pytohormones are important agents to the regulation of growth, plant development, survival and reproduction [2]. Jasmonic Acid (JA), Azelaic Acid (AZA) and Abscisic Acid (ABA) where the phytohomones studied in this dissertation. JA is involved in plant growth development and also in its defense mechanisms against infections [3]. AZA plays an important role in a defence mechanism against infections of the planted called Systemic Acquired Resistance (SAR) by involving the gene *AZELAIC ACID INDUCED 1 (AZI1)* [4] [5]. ABA regulates the plant water balance and osmotic stress tolerance [6]. From *Feng et al.* it was possible to extrapolate an interval of concentration values for these phytohormones. To ABA the typical relevant levels were 0.1 µM to 800 µM. They also provided a range of values for another phytohormone, Salicylic Acid, that I used as baseline values for AZA and JA, which are 0.1 µM to 26 mM [7].

There are several factors that can negatively influence the growth, development and yield of the grapevine. If the plant suffers a stress, like drought or a pest attack, it leads to changes in its metabolism, in order to protect itself and adapt to the new environment. If the stress is more severe and the plant can not be restored, it can result in the plant's death. These stress factors are divided into abiotic and biotic stresses [8].

Abiotic stress is any chemical and physical change to the environment that results in a response for the plant, for instance, drought, heat, nutrient availability, changes in the salinity of the soil.

Biotic stress is any interaction between living organisms and the plant, such as insects that feed on them and diseases from virus, bacteria and fungus. [8] [9]. Pathogens can be branched into two different categories: necrotrophs, when they kill the host cells in order to feed; and biotrophs, if they feed on live cells.

Microfluidics and immunoassays were the chosen approach to address this problem. Microfluidics have several advantages: they are low-cost, use a small amount of reagents, are portable and easy to use allowing for a faster analysis and detection [10]. Immunoassays allow for a high sensitivity and specificity when used in assays.

The leading principle of an immunoassay is that binding an analyte with a specific binding molecule, in this experimental work an antibody, generates a significant signal that allows the distinction of bound analyte and free analyte [11].

Antibodies have three important attributes that play a role in immunoassays: they have an ability to bind to an ample variety of natural and man-made molecules, through the process mentioned above; they have a high specificity for the antigen to which each antibody binds; and the strength of the non-covalent bindings such as electrostatic (van der Waals) forces, hydrophobic forces, coulombic (ionic) forces or a combination of all [12] are strong enough to resist steps in the experiments, for example the wash-

*E-mail: rui.meirinho@tecnico.ulisboa.pt
†Instituto Superior Técnico, Universidade de Lisboa, Lisboa, Portugal

ing step, even though they are weaker than a covalent bound [13].

In this experimental work, the size of the molecules in study are small when compared to proteins, therefore a competitive immunoassay approach was chosen.

By choosing this approach, only one antibody is used. In a competitive immunoassay the antibody is immobilised on a solid phase, such as agarose beads. Next the analyte and labelled analyte are added at the same time, competing to bind to the antibody. In the end, the unbound material is washed away.

The value of the signal generated depends on the analyte concentration and is at its maximum when the assay is performed without the presence of analyte. By carrying out experiments with the addition of different concentrations of analyte, the values of the signal generated decrease. If a logarithmic scale is used for the x-axis, concentration on analyte, the curve resembles an "inverted" S-shape where there is a top plateau for the maximum fluorescence intensity for lower concentrations and a bottom plateau for higher concentrations of analytes,

The need for miniaturisation and microelectromechanical systems (MEMS) contributed to the rise of the Microfluidics discipline in the 90's [10]. More recently, Microfluidics led to the appearance of a new area called BioMEMS, these are used in solving complex biological problems, such as drug development [14], biosensing [15], *in vitro* organs [16], disease diagnostics [17], among others.

In BioMEMS, the polymer polydimrthylsiloxane (PDMS), whose formula is (-SI$(CH_3)_2$O)-, plays an important role in a diverse arrays of experiments, due to its characteristics. When mixed with a reticulate, curing agent in a given ratio and exposed to a temperature superior to its polymerization one, it forms an elastomer. PDMS is very useful to obtain a series of structures, such as channels to flow liquids, for different assays with a submicrometric precision. However, since it is an elastomer, the dimension of the channel should be between 5 to 500 µm, and the ratio width/height should be taken into account, since a high value for the ratio could lead to a collapse of the channel [10].

The most common methods to detect and measure phytohormones involve complex machinery and protocols, such as HPLC, and sample treatment, for example the use of liquid nitrogen [18] [19].

Grossmann et al. used a microfluidic chip platform, **Root Chip**, for root cultivation and imaging. They used *Arabidopsis* as the sample, but their device can be adapted to other species, by altering the geometry of microfluidic structure [20].

The main objective is to detect the phytohormones JA, ABA and AZA, signals of stress in plants in buffer and in real samples.

2 Materials and Methods

2.1 Reagents

Stock solutions of JA (50 mg/mL), AZA (50 mg/mL) and ABA (50 mg/mL) were prepared in Methanol. These components were purchased from Sigma-Aldrich. Anti-Azelaic Acid Polyclonal antibody, Azelaic Acid BSA-conjugated, Abscisic Acid BSA-conjugated and Anti-Abscisic Acid Polyclonal antibody were purchased from Creative Diagnostics. The blocker agent 1% Casein and phosphate buffered saline (PBS) were aquired from Thermo Fisher Scientific. The Agarose Protein A beads were bought from Cytiva.

2.2 Microfabrication

The structures are fabricated using standard soft-lithography techniques described elsewhere [14]. Briefly, two different aluminium hard masks were fabricated, for each different heights. A 200 nm aluminium (Al) layer was deposited over the clean substrate by magnetron sputtering using the Nordiko 7000 equipment (Nordiko Technical Services Ltd), followed by the deposition of a 1.5 µm thick layer of positive photoresist (PFR 7790G) coating, which is performed onto the deposited Al layer using an SVG resist coater (Silicon Valley Group Inc.). The design of the microchannel was patterned onto the photoresist through photolythography. The exposed regions of the Al layer are removed through a wet chemical etching process with aluminium etchant (Microchemicals). The hard masks was used to create SU-8 moulds, which is a negative photoresist. For the height of 20 µm, SU-8 2015 (Microchem Corp.) was spin coated onto a silicon substrate and exposed to a UV light through the appropriate hard mask followed by baking for 5 minutes at 95°C. This stack was then submerged in a solution of propylene glycol monomethyl ether acetate (PGMEA) (Sigma-Aldrich) for 2 minutes with manual orbital agitation, then rinsed with IPA (LabChem Inc.) and dried with compressed air [21]. For the second layer with 100 µm, the negative photoresist (SU-8 50, Microchem Corp.) was spin-coated on top of the silicon substrate and then baked before exposure. The stack was then exposed to UV light (UV-LED MASKING SYSTEM:UV-KUB 2, KLOÉ) followed by post-exposure bake. The silicon substrate was left to cool down and then developed with PGMEA. The silicon substrate was then hard baked at 150°C for 45 minutes and left to cool down on top of a hot plate until it reached 50°C. The finished SU-8 moulds were then used to do PDMS structures. The PDMS elastomer (Sylgard 184 PDMS, Dow Corning) was prepared by mixing PDMS and curing agent (Sylgard 184 Curing Agent, Dow Corning) in a 10:1 ratio, respectively. This mixture was then degassed for 45 minutes and then poured onto the substrate mould. This mould was then left in the oven for 90 min. Inlet and outlet holes were mechanically punched using a blunt needle (18 Gauge, Instech Laboratories, Inc.) to allow the connection of the designed chambers to the outside. In order to seal the PDMS structure, the patterned surface and a cut piece of PDMS membrane (500 µm thick) are oxidised using an oxygen plasma cleaner (Expanded Oxygen plasma cleaner PDC-002-CE, Harrick Plasma) at high power setting for 1 minute. Both oxidised surfaces are put in contact and a slight pressure is applied to promote a covalently bound structure [21].

2.3 Immunoassay

2.3.1 Protein Labelling

In order to label the conjugated phytohormone, the following protocol was used. 5 µL of $Alexa_{430}$ was added to 50 µL of the target protein, diluted in 0.1 M of sodium bicarbonate buffer, dissolved in DMSO. The mixture was left to incubate in the dark under agitation at room temperature for an hour. Using 10kDA Amicon Tube, the excess dye was washed using 500 µL of plain PBS 1x for 10 minutes at 14000 G until the permeate was no longer fluorescent.

2.3.2 Antibody Immobilisation

Agarose beads of Protein A, stored in 20% ethanol, were incubated with a chosen concentration of antibody in 19 µL on PBS plus 3 µL of beads, and then left in orbital agitation for one hour at room temperature [22].

2.4 Microfluidic methodology

The experiments were carried out in PDMS channels, as presented in Figure 1 and the general immunoassay is illustrated in Figure 3. Each experiment was carried out in one channel. Two syringes of 1 mL (Syringe 1 mL U-100 Luer-Lock) filled with 40 µL of PBS (pH 7.4), with a polyethylene tube (Instech Laboratories, Inc.) on the stub and an adapter (Instech Laboratories, Inc.) to connect the syringe to the channel through the tube, were placed in the syringe pump (New Era Pump Systems, Inc). The adapters were inserted in the $Inlet_1$ and outlet of the channel, with a gap of air between the channel and the PBS in the tube. A tip with PBS was placed in the $Inlet_2$ in order to humidify the channel through negative pressure. After the channel was humidified, the tip with PBS was carefully replaced with another with a suspension of Protein A beads functionalised with antibody. The beads were inserted into the channel at a lower flow rate, between 5 and 9 µL/min, until the channel was overfilled. Then, the beads were washed with PBS again through $Inlet_1$. A closed adapter was used to block the $Inlet_2$. The adapters from $Inlet_1$ and Outlet were carefully removed again and tips with PBS were placed in the holes. The packed structure was then stored in a container filled with DI water in the fridge overnight.

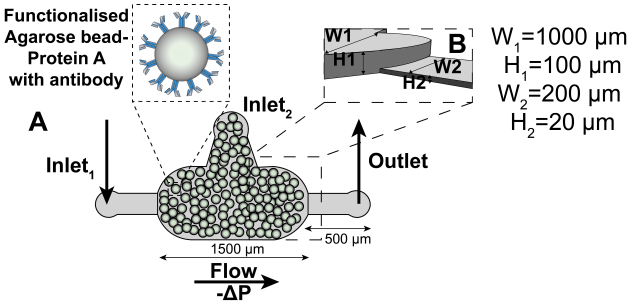

Figure 1: (A) - Microfluidic structure, overnight experiments, used on the detection of phytohormones using functionalised Agarose Protein A beads. The beads were inserted into the microchannel using $Inlet_2$. The solutions used in the assays were injected through $Inlet_1$. The mixture solution was pulled through the channel using the polyethylene tub coupled to an open metallic adapter with a syringe filled with PBS controlled by a syringe pump, applying a negative pressure at the Outlet. **(B)** - Bead trapping feature in the microchannel.

The next day, a syringe in the same conditions as mentioned before, was placed in the syringe pump. The packed structure was retrieved from the fridge and left to warm to room temperature. The metal adapter of was placed, carefully, in the Outlet of the channel and a tip with PBS placed carefully in $Inlet_1$. The beads were washed for 10 minutes with a flow of 17 µL/min, using negative pressure. The tip with PBS was carefully replaced with a tip filled with casein. This casein solution was injected with a flow of 1 µL/min for 10 minutes, and then a solution with the analytes in study was injected into the channel at a flow rate of 1 µL/min for 5 minutes. The channel was washed with plain PBS for 10 minutes at a flow rate of 5 µL/min. The fluorescence signal was measured at the end of the experiment using the fluorescence microscope, Leica DMLM using the blue filter and the digital colour camera DFC300FX, with an exposure time of 2 seconds and a gain of 1x. The blue filter used has a band pass illumination pass at wavelengths between 450 and 490 and a long pass observation above 515. The light was sourced using a mercury vapour arc lamp (100 W).

2.4.1 Sample Treatment

The grape berries were frozen at -20°C in a freezer. To macerate them, the samples were weighed and a solution of 10% Methanol in PBS was added in a ratio of 1 g of sample to 1 mL of solution. The juice obtained was filtrated and the permeate diluted to obtain a solution with 1% Methanol in PBS.

2.4.2 Image Analysis

The micrographs of conjugated phytohormones or antibody labelled with $Alexa_{430}$ were analysed using ImageJ software from National Institute of Health, USA [23]. These images are split into three channels, Red, Blue and Green. Due to the chosen fluorescent dye, only the green channel was considered. In the software, an inner and outer area of the channel were considered and measured. The final value results from the subtraction of the outer value from the inner value.

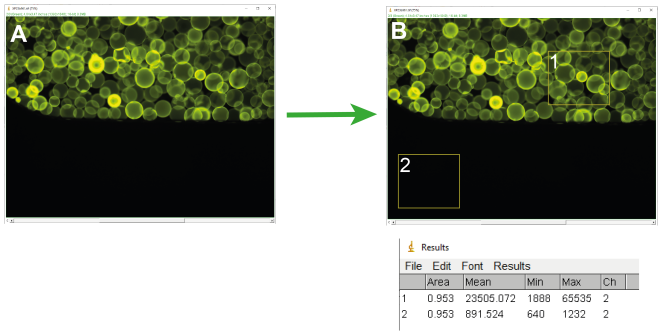

Figure 2: Image Analysis: **(A)** Source Image **(B)** The value measured form the outer area is subtracted to the value measured from the inner area.

3 Results and Discussion

The starting point for this work consisted of adapting protocols from previous and on going work performed at INESC MN [14] [22]. The competitive immunoassay was the chosen approach. This section is divided in the experiments leading to the optimal conditions in the detection of the three phytohormones, ABA, JA and AZA, in buffer and initial experiments using real samples of grapes. Throughout this section, the results obtained will be presented and explained. For an ease of language: the denomination "With Analyte" refers to the presence of a phytohormone, the competitor and the antibody in the experiment; "Without Analyte" involves the presence of competitor and antibody.

3.1 Initial Experiments

The goal with these first experiments was to understand if by immobilising the antibody I could detect the fluorescence signal, since the presence of beads increases the functional surface in microchannel. To ensure the detection

of a fluorescence signal, the concentrations of α-JA and BSA-JA-Alexa$_{430}$ were 1 mg/mL and 0.25 mg/mL, respectively. The blocking agent used here was casein 1%.

Three experiments were performed: "Without analyte", where I expected the detection of a fluorescence signal; "With Analyte", where I expected the detection of a lower value of fluorescence than the assay "Without Analyte"; and a control assay. To carry out this control, the beads that were packed into the microchannel were not functionalised with the antibody. The objective was to observe if there was a non-specific interaction between BSA-JA-Alexa$_{430}$ and Protein A. The results obtained from these experiments are in Figure 4.

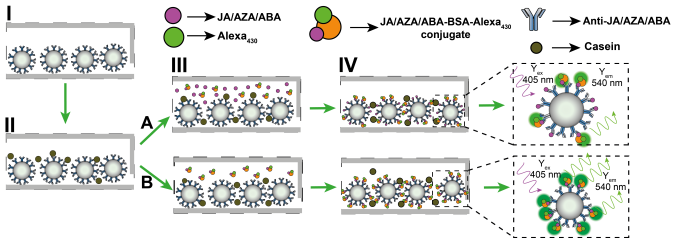

Figure 3: Schematic of the microchannel and the trapping of the beads and assay; **I** - Packing functionalised beads in microchannel; **II** - Injection of blocking using casein 1%; **III** - Injection of the conjugate plus analyte (**A** - With Analyte) or conjugate (**B** - Without Analyte) ; **IV** - Washing with plain PBS 1x and detection with a microscope.

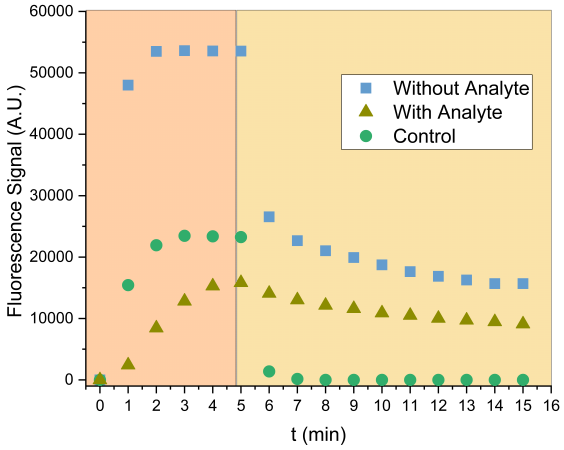

Figure 4: Graphical representation of the first experiments using Agarose Protein A beads, with fluorescence signal measured in function of time. As we can see, the fluorescence signal on the beads functionalised with 1 mg/mL of α-JA and the control increased for the first 5 minutes, and decreases when the washing step starts after the 5 minutes mark. The values for the control were zero and for the functionalised beads were over 10000 A.U.. The difference in the first 5 minutes was due to the focus of the lens. The conditions of acquisition for the Leica DMLM microscope were: exposure time 2s; gain of 1x; and objective 10x.

Figure 4 has the graphical representation of these experiments with the Fluorescence signal values (A.U.) vs time after the injection of BSA-JA-Alexa$_{430}$. The washing step corresponds to the injection of PBS 1x at the five minutes mark. The fluorescence signal values for the "Control" assay are near zero at the end of the experiment, leading me to conclude the absence of non-specific interactions. Conversely, the fluorescence signal values for the assay "Without Analyte" (1 mg/mL α-JA) decrease from around 50000 A.U. to around 15000 A.U. by the end of the experiment. . The values measured for the assay with 0.05 mg/mL of JA, "With Analyte", throughout the times of the experiment were lower than the ones obtained in the assay "Without analyte", which was expected since there is competition between JA and BSA-JA-Alexa$_{430}$. These results lead me to conclude that the binding between Protein A in the agarose beads and α-JA, and also the one between α-JA and BSA-JA-Alexa$_{430}$, were successful, but also that when the solution is injected with BSA-JA-Alexa$_{430}$ and JA there is competition between them for the binding spots in α-JA.

3.2 Different Concentrations of Antibody

After deciding to use Agarose beads with Protein A to continue the experiments, I decided to tackle the α-JA parameter. I started by packing microchannels with beads functionalised with different concentrations of α-JA, 0.5, 1, 2 and 4 mg/mL. I then flowed the blocking agent, casein 1%, followed by a solution with a known concentration of BSA-JA-Alexa$_{430}$ 0.5 mg/mL, that is the same for all the assays performed. I expected the fluorescence signal would increase with the rise of α-JA concentration. The objective with these experiments was to obtain a baseline and choose a concentration of α-JA to determine the other parameter.

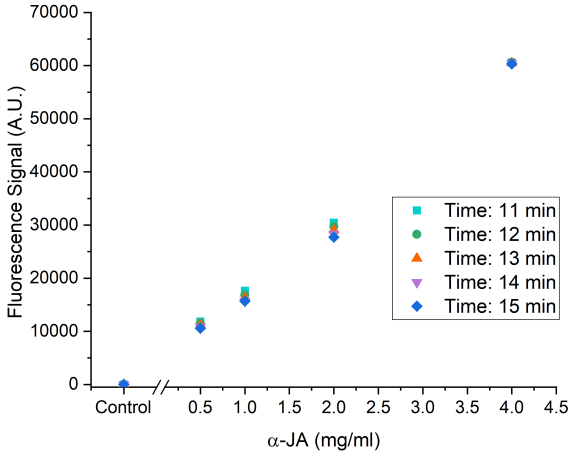

Figure 5: Graphical representation of the α-JA concentrations study using 0.5 mg/mL of BSA-JA-Alexa$_{430}$. The fluorescence signal values are for 11, 12, 13, 14 and 15 minutes time indices. 0 is the time when the solution with BSA-JA-Alexa$_{430}$ is injected into the microchannel. The conditions of acquisition for the Leica DMLM microscope were: exposure time 2s; gain of 1x; and objective 10x.

In Figure 5, I represented the values of fluorescence signal obtained for the different concentrations of α-JA at 11, 12, 13, 14 and 15 minutes. The reason for this representation was to analyse the behaviour of these values to see if they reach a saturation stage. It was expected

the fluorescence signal values would reach a plateau at higher concentrations, but as seen in Figure 5, this does not happen, meaning the saturation stage was not reached and it is possible to use higher concentrations of α-JA for the same concentration of BSA-JA-Alexa$_{430}$.

3.3 Different Concentrations of BSA-JA-Alexa$_{430}$

Once I chose a concentration of α-JA, the next step was to determine the concentration of BSA-JA-Alexa$_{430}$. In order to diminish the presence of air bubbles in the microfluidic system, I started packing the beads into the microchannels the day before, using a different structure, following the protocol described in section 2.4. I chose to vary the concentrations of BSA-JA-Alexa$_{430}$ by one order of magnitude from the stock solution. This means that the concentrations of BSA-JA-Alexa$_{430}$ that I used were 7.4 mg/mL, 7.4×10^{-1} mg/mL, 7.4×10^{-2} mg/mL and 7.4×10^{-3} mg/mL. The results from these experiments are represented in Figure 6. As seen in Figure 6 the fluorescence signal values rise with the increase of concentration of BSA-JA-Alexa$_{430}$. 0.5 mg/mL of BSA-JA-Alexa$_{430}$ was, therefore, the chosen concentration to continue with the experiments. The concentration of 1.5 mg/mL α-JA was higher than that were used in similar past works carried out at INESC MN, so for the next experiments the concentration was reduced to 0.5 mg/mL.

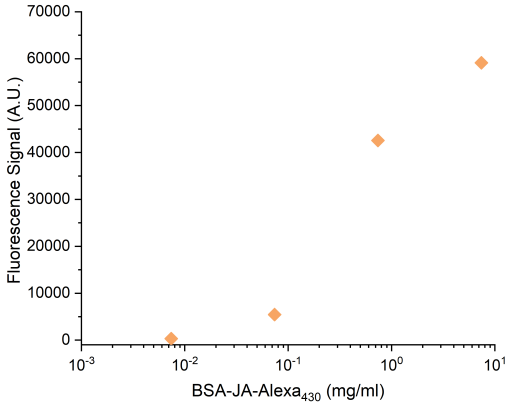

Figure 6: Graphical representation of the fluorescence signal values in function of different concentrations of BSA-JA-Alexa$_{430}$ for a concentration of 1.5 mg/mL α-JA. The conditions of acquisition for the Leica DMLM microscope were: exposure time 2s; gain of 1x; and objective 10x.

3.4 JA detection Assay

Upon deciding on the concentrations for α-JA, 0.5 mg/mL, and BSA-JA-Alexa$_{430}$, 0.5 mg/mL, the next step was the addition of different concentrations of JA to the assay. I also wanted to study the effect on fluorescence signal by increasing the time of the washing step to 35 minutes for the assay with 0 and 0.1 mg/mL of JA, and to 40 minutes for the remaining assays. The "Control" experiment was terminated at 15 minutes since its value was zero. The solutions of JA were prepared from the stock solution by diluting it in PBS 1x. The results obtained are in Figure 7 .

Figure 7 contains the fluorescence signal values over time for the different assays. In these experiments, I tried to evaluate the influence of time in the final washing step. As I concluded from the graphic, there is a gradual decrease in fluorescence signals values, and it is constant. This decrease can be due to a photobleaching phenomenon, where the fluorescent dye loses activity with the increased exposure to light. This means that the assays can end at 15 minutes after the injection of the solution with BSA-JA-Alexa$_{430}$. The lowest concentration that we were able to distinguish was 0.5 mg/mL of α-JA. As mentioned above the baseline interval concentration values for JA were between 2.10×10^{-5} mg/mL to 5.47 mg/mL. So there was a need to make further optimisations to the process, such as changing the concentrations of α-JA and BSA-JA-Alexa$_{430}$.

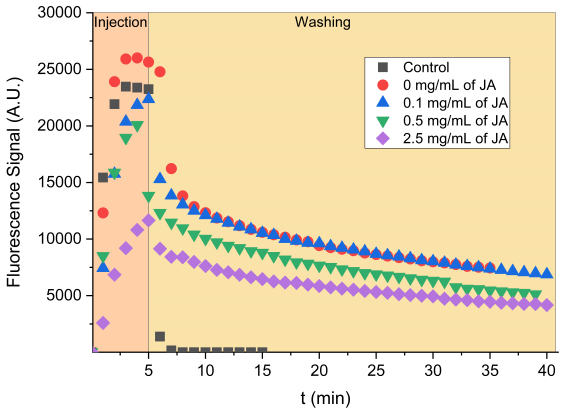

Figure 7: Graphical representation of the assays for different concentrations of the analyte JA. For these assays the following concentrations were used: 0.5 mg/mL of BSA-JA-Alexa$_{430}$ and 0.5 mg/mL of α-JA. The concentrations of JA used were: 0.1 mg/mL, 0.5 mg/mL and 2.5 mg/mL. The assay with 0 mg/mL JA is the experiment "Without Analyte". It was not possible to distinguish the signal between the assay with 0 mg/mL of JA (Without Analyte) and 0.1 mg/mL of JA. The conditions of acquisition for the Leica DMLM microscope were: exposure time 2s; gain of 1x; and objective 10x.

3.5 Study on the influence of the blocking agent

I carried out an experiment "Without Analyte" with new parameters, 0.05 mg/mL of α-JA and 0.074 mg/mL of BSA-JA-Alexa$_{430}$ and obtained a low fluorescence value, of around 300 A.U.. This led me to question whether it was possible to increase the fluorescence signal by lowering the concentration of blocking agent without increasing the non-specific interactions, following the protocol described in section 2.4.

The solution of 1% (w/v) casein was diluted to other concentrations using PBS 1x. On the graph present in Figure 8, I could see the fluorescence signal values obtained for the distinct assays with different concentrations of the blocking agent using 0.05 mg/mL α-JA and 0.074 mg/mL BSA-JA-Alexa$_{430}$. I could observe there was a significant increase in the signal when the concentration of the block-

ing agent was lowered. The assay with more relevance was 0.1% casein, since it provided a fluorescence signal more than 3 times greater than the assay using 1% casein, with no non-specific interactions. Between 0.1% and 0.05% (w/v) of casein, there was no significant change in the value obtained with antibody, but there were non-specific interactions in the 0.05% casein assay. So from then on the concentration of blocking agent used was 0.1% (w/v) casein.

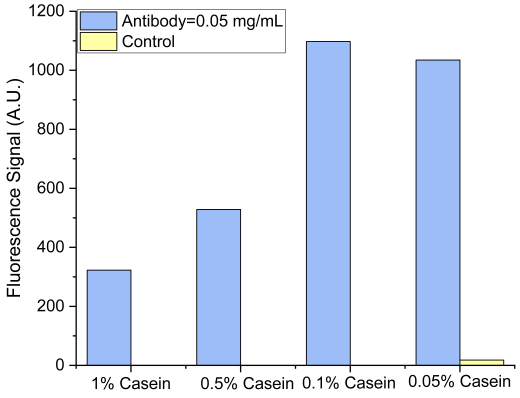

Figure 8: Graphical representation for the various casein concentration experiments. The stock solution of 1% (w/v) casein was diluted in PBS 1x to the various concentrations used in the experiments. The concentration of α-JA and BSA-JA-Alexa$_{430}$ were 0.05 mg/mL and of 0.074 mg/mL, respectively. The assays were the type "Without Analyte". The conditions of acquisition for the Leica DMLM microscope were: exposure time 2s; gain of 1x; and objective 10x.

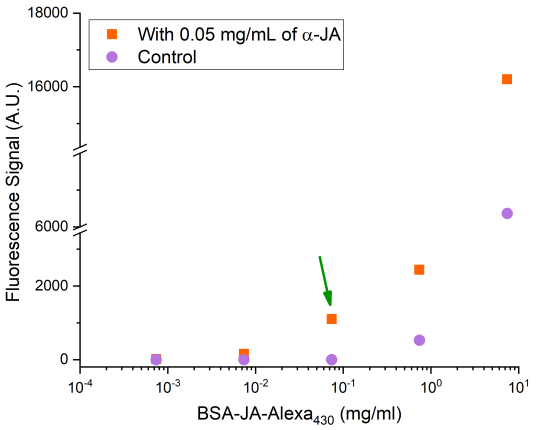

Figure 9: Graphical representation of the Fluorescence Signal values detected assays for different concentrations of BSA-JA-Alexa$_{430}$ using Casein 0.1% (w/v) as a blocking agent and controls for each experiment. The green arrow indicates the chosen concentration for the next experiments. The conditions of acquisition for the Leica DMLM microscope were: exposure time 2s; gain of 1x; and objective 10x.

3.6 Role of Methanol in the Fluorescence Signal

Considering the stock solutions of phytohormones are in 99.8% Methanol, I decided to carry out experiments "Without Analyte" where I would vary the concentration (%(v/v)) of methanol in the assay. The concentrations of methanol were: 1%, 5% and 10% (v/v). I carried out experiments for both of the sets of parameters that I had considered, to see if the variation in signal would have the same behaviour: 0.5 mg/mL of α-JA and 0.5 mg/mL of BSA-JA-Alexa$_{430}$; and 0.05 mg/mL of α-JA and 0.074 mg/mL of BSA-JA-Alexa$_{430}$. In Figure 10 its presented the results from these experiments. For both sets of parameters, I could see that with the increase of concentration of Methanol %(v/v) in the solution injected with BSA-JA-Alexa$_{430}$, the fluorescence signal values decreases. This can explain some of the results obtained in earlier experiments, since the percentage of methanol in the solutions was not a factor I took into consideration when carrying them out.

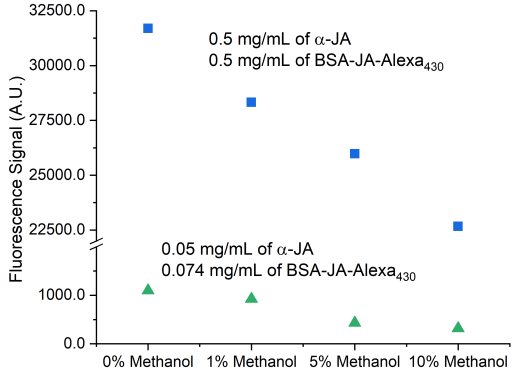

Figure 10: Graphical representation for the 2 distinct sets of assays: one with 0.5 mg/mL of α-JA and 0.5 mg/mL of BSA-JA-Alexa$_{430}$ (Blue squares); and 0.05 mg/mL of α-JA and 0.074 mg/mL of BSA-JA-Alexa$_{430}$ (Green triangles). In this experiments the concentration of methanol was varied in the following values: 0%, 1%, 5% 10% (v/v). The conditions of acquisition for the Leica DMLM microscope were: exposure time 2s; gain of 1x; and objective 10x.

3.7 Different concentrations of JA

After all the optimisations with the concentration of α-JA, BSA-JA-Alexa$_{430}$, blocking agent and methanol influence, I started to carry out experiments using the analyte JA in buffer for the 2 distinct assays: 0.5 mg/mL of α-JA and 0.5 mg/mL of BSA-JA-Alexa$_{430}$; and 0.05 mg/mL of α-JA and 0.074 mg/mL of BSA-JA-Alexa$_{430}$.

This led to the results presented in Figure 11 regarding the two different concentrations of antibody and conjugated molecule. It is possible to see the fluorescence signal values for both concentrations of antibody are very close to the assays "Without Analyte". In order to achieve a higher distinction between the concentrations of JA, I decided to change the concentration of α-JA to 0.1 mg/mL and maintained the concentration of BSA-JA-Alexa$_{430}$ equal to 0.074

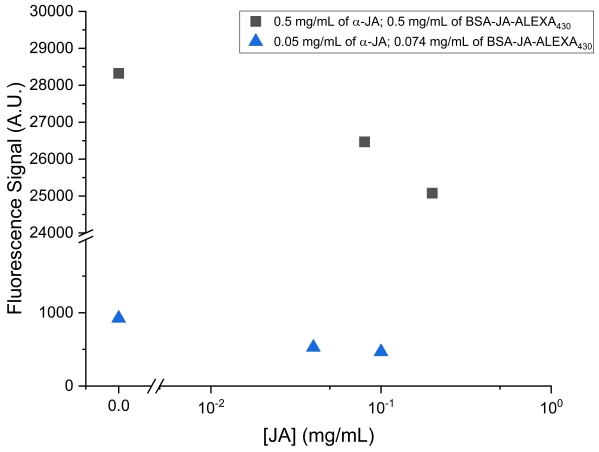

Figure 11: Graphical representation for the 2 distinct sets of assays: one with 0.5 mg/mL of α-JA and 0.5 mg/mL of BSA-JA-Alexa$_{430}$; and 0.05 mg/mL of α-JA and 0.074 mg/mL of BSA-JA-Alexa$_{430}$. It presents the fluorescence signal values in function of time for different concentrations of JA. It presents the fluorescence signal values in function of time for different concentrations of analyte. The conditions of acquisition for the Leica DMLM microscope were: exposure time 2s; gain of 1x; and objective 10x.

mg/mL. Figure 12 presents the graphical representation for these results.

There was a clear distinction of values for the different concentrations of JA in mg/mL, which were in our goal of detection, as we have seen before in the Introduction: 1×10^{-7} mol/dm^3 (2.10×10^{-5} mg/mL) to 2.6×10^{-2} mol/dm^3 (5.47 mg/mL) [7].

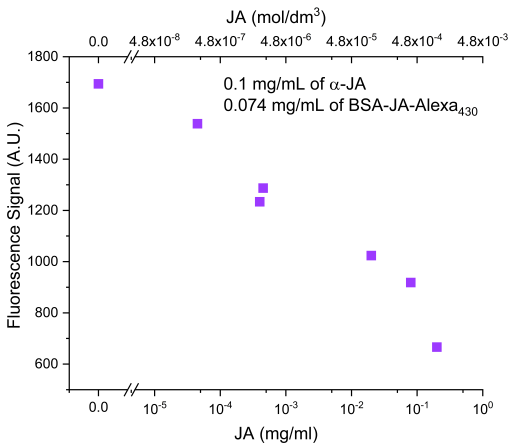

Figure 12: Graphical representation of the first detection curve for the analyte JA, with the fluorescence signal values in function of the concentration of the analyte JA. The conditions of acquisition for the Leica DMLM microscope were: exposure time 2s; gain of 1x; and objective 10x.

The solubility of JA is around 3 mg/mL. To avoid the formation of precipitates that would interfere with the detection of fluorescence signal, the concentration of analyte used was always below this value. If need be, the solution of a sample can always be diluted to be in line with the interval of detection.

3.8 Detection Curve

Using the optimizations performed for the analyte JA, I carried out similar experiments for the other two analytes, ABA and AZA. I carried out experiments to do a detection curve of the three phytohormones. The fluorescence signal values are present in Figure 14. As demonstrated above, a higher concentration of analyte leads to a decrease in the fluorescence signal values, which was what I measured. Knowing the curve would present an inverted "S" shape, with a linear area between the bottom and top plateau, I did an interpolation with the graphic points that presented a linear behaviour in the logarithmic scale for the different analytes. The detection ranges in buffer are 2×10^{-2} to 9×10^{-6} mg/mL, 9×10^{-3} to 9×10^{-6} mg/mL and 2×10^{-2} to 9×10^{-5} mg/mL, for JA, AZA and ABA, respectively.

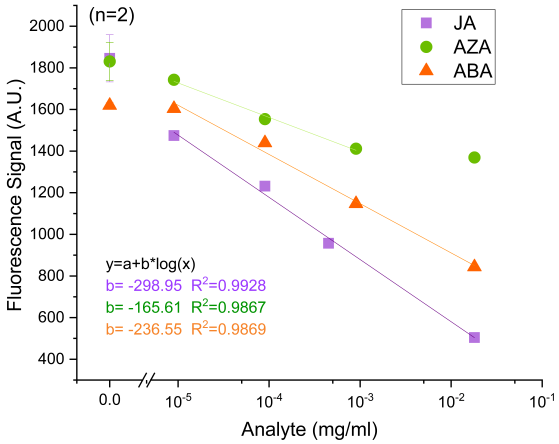

Figure 14: Graphical representation of the detection of JA, ABA and AZA in buffer with 1% methanol using the competitive immunoassay. The interpolations are present in the graphic with the equation used, slopes and R^2.The conditions of acquisition for the Leica DMLM microscope were: exposure time 2s; gain of 1x; and objective 10x.

3.9 Real Sample

One of the main objectives of this dissertation is the measurement of the different phytohormones in real samples. Due to the constriction of the concentration (% (v/v)) of methanol, we needed to take into account the preparation of the sample. I read different articles where they used a solution of 80% (v/v) methanol in PBS to macerate the samples, which was not feasible [18]. Therefore the white and red, wine and table, grape samples from different cultivars were prepared according to the protocol in section 2.4.1. The results obtained from the assays are present in Figure 13.

3.9.1 Spike Detection

In Figure 13, as exemplified with the buffer, the spike of 0.006 mg/mL of JA should decrease the value of the fluorescence signal. However this does not happen in the case of white wine grape. There is no discernable decrease since

Figure 13: Graphical representation of the fluorescence signal values obtained in buffer "Without Analyte", and the value for the presence of the analyte JA (0.006 mg/mL) and with white wine and table grapes and red wine and table grapes, with and without the spike of 0.006 mg/mL of JA. The conditions of acquisition for the Leica DMLM microscope were: exposure time 2s; gain of 1x; and objective 10x.

the fluorescence signal values measured are very similar. Regarding the white table grapes there was a decrease in fluorescence signal when adding the spike of 0.006 mg/mL of JA. In the case of red wine grapes, we can see that adding the spike of 0.006 mg/mL of JA lowered the fluorescence signal value. The red table grapes had similar fluorescence signal values, with a slight decrease when adding 0.006 mg/mL of JA.

It is important to note these results suggest the interference of the cellular matrix in the assay and also the presence of other components that may increase the fluorescence and interact with the α-JA. As I have proved above, there are also cross-contaminations between the different analytes and α-JA that will influence the detection of fluorescence signal values.

4 Conclusions and Future Work

This work demonstrated the detection of phytohormones using an immunoassay-based microfluidic device. In order to accomplish this objective, a protocol was required to be developed and optimised, in order to identify the various molecules.

The objectives set out for this dissertation were achieved since it was possible to:

- Develop a protocol to detect various concentrations of the analytes JA, AZA and ABA;
- Optimise the protocol, namely the effect of methanol's presence in the assay, maintaining it at 1% and the reduction of casein's concentration from 1% to 0.1%, raising the fluorescence signal without increasing the non-specific interactions;
- Demonstrate the viability of the protocol by obtaining curves of detection for the three phytohormones.

Another approach was tested by using positively charged beads, but the results were not satisfactory. This experiment could be further reworked using another fluorophore with a neutral charge, for instance Bodypy.

Regarding future work, this project is in its early stages, with the main goal being the detection of abiotic and biotic stresses in grapevines using a single-step device. This equipment should have a sample treatment and a detection module. The next steps should be:

- Calculation of limit of detection;
- The design of a microfluidic structure for the simultaneous detection;
- Increase the panel of phytohormones with other molecules, such as salicylic acid and resveratrol, which are known infection signals;
- Optimisation of the sample treatment protocol, to eliminate the influence of cellular matrix, for instance Aqueous Two-Phase System (ATPS) [24];
- Perform experiments in healthy and stressed samples, for both abiotic and biotic stress factors.

Acknowledgements

The work presented in this thesis was performed at INESC-MN during the period February-September 2021, under the supervision of Prof. Dr. João Pedro Estrela Rodrigues Conde.

The author would like to acknowledge Fundação para a Ciência e Tecnologia (FCT) for funding of the Research Unit INESC-MN (UID/05367/2020) through pluriannual BASE and PROGRAMATICO financing and project VineSence (PTDC/BAA-DIG/4735/2020).

References

[1] X. Venios, E. Korkas, A. Nisiotou, and G. Banilas, "Grapevine responses to heat stress and global warming," *Plants*, vol. 9, no. 12, p. 1754, 2020.

[2] C. M. Pieterse, A. Leon-Reyes, S. Van der Ent, and S. C. Van Wees, "Networking by small-molecule hormones in plant immunity," *Nature chemical biology*, vol. 5, no. 5, pp. 308–316, 2009.

[3] G. Jang, Y. Yoon, and Y. D. Choi, "Crosstalk with jasmonic acid integrates multiple responses in plant development," *International journal of molecular sciences*, vol. 21, no. 1, p. 305, 2020.

[4] J. Shah, "Plants under attack: systemic signals in defence," *Current opinion in plant biology*, vol. 12, no. 4, pp. 459–464, 2009.

[5] M. Mhlongo, F. Tugizimana, L. Piater, P. A. Steenkamp, N. Madala, and I. Dubery, "Untargeted metabolomics analysis reveals dynamic changes in azelaic acid-and salicylic acid derivatives in lps-treated nicotiana tabacum cells," *Biochemical and biophysical research communications*, vol. 482, no. 4, pp. 1498–1503, 2017.

[6] A. Shatil-Cohen, Z. Attia, and M. Moshelion, "Bundle-sheath cell regulation of xylem-mesophyll water transport via aquaporins under drought stress: a target of xylem-borne aba?" *The Plant Journal*, vol. 67, no. 1, pp. 72–80, 2011.

[7] S. Feng, C. Chen, C. Song, X. Ding, W. Wang, and L. Que, "Optical aptamer-based sensors for detecting plant hormones," *IEEE Sensors Journal*, vol. 21, no. 5, pp. 5743–5750, 2020.

[8] A. Gull, A. A. Lone, and N. U. I. Wani, "Biotic and abiotic stresses in plants," *Abiotic and biotic stress in plants*, pp. 1–19, 2019.

[9] A. Rodríguez, B. Alquézar, and L. Peña, "Fruit aromas in mature fleshy fruits as signals of readiness for predation and seed dispersal," *New Phytologist*, vol. 197, no. 1, pp. 36–48, 2013.

[10] P. Tabeling, *Introduction to microfluidics*. OUP Oxford, 2005.

[11] P. Datta, "Chapter 6 - immunoassay design," in *Accurate Results in the Clinical Laboratory (Second Edition)*, 2nd ed., A. Dasgupta and J. L. Sepulveda, Eds. Elsevier, 2019, pp. 69–73. [Online]. Available: https://www.sciencedirect.com/science/article/pii/B9780128137765000066

[12] W. Clarke, L. J. Sokoll, and A. J. Rai, "Immunoassays," in *Contemporary practice in clinical chemistry*. Elsevier, 2020, pp. 201–214.

[13] D. Wild, "Immunoassay for beginners," *The immunoassay handbook. Theory and applications of ligand binding, ELISA and related techniques. Wild DG (ed). Springer, Amsterdam*, pp. 7–10, 2013.

[14] E. J. Brás, A. M. Fortes, T. Esteves, V. Chu, P. Fernandes, and J. P. Conde, "Microfluidic device for multiplexed detection of fungal infection biomarkers in grape cultivars," *Analyst*, vol. 145, no. 24, pp. 7973–7984, 2020.

[15] I. F. Pinto, D. R. Santos, C. R. Caneira, R. R. Soares, A. M. Azevedo, V. Chu, and J. P. Conde, "Optical biosensing in microfluidics using nanoporous microbeads and amorphous silicon thin-film photodiodes: quantitative analysis of molecular recognition and signal transduction," *Journal of Micromechanics and Microengineering*, vol. 28, no. 9, p. 094004, 2018.

[16] A. Sontheimer-Phelps, B. A. Hassell, and D. E. Ingber, "Modelling cancer in microfluidic human organs-on-chips," *Nature Reviews Cancer*, vol. 19, no. 2, pp. 65–81, 2019.

[17] J. M. Campbell, J. B. Balhoff, G. M. Landwehr, S. M. Rahman, M. Vaithiyanathan, and A. T. Melvin, "Microfluidic and paper-based devices for disease detection and diagnostic research," *International journal of molecular sciences*, vol. 19, no. 9, p. 2731, 2018.

[18] S.-l. Liu, J. Wu, P. Zhang, G. Hasi, Y. Huang, J. Lu, and Y.-l. Zhang, "Response of phytohormones and correlation of sar signal pathway genes to the different resistance levels of grapevine against plasmopara viticola infection," *Plant Physiology and Biochemistry*, vol. 107, pp. 56–66, 2016.

[19] S. J. Owen, M. D. Lafond, P. Bowen, C. Bogdanoff, K. Usher, and S. R. Abrams, "Profiles of abscisic acid and its catabolites in developing merlot grape (vitis vinifera) berries," *American Journal of Enology and Viticulture*, vol. 60, no. 3, pp. 277–284, 2009.

[20] G. Grossmann, W.-J. Guo, D. W. Ehrhardt, W. B. Frommer, R. V. Sit, S. R. Quake, and M. Meier, "The rootchip: an integrated microfluidic chip for plant science," *The plant cell*, vol. 23, no. 12, pp. 4234–4240, 2011.

[21] I. Pinto, C. Caneira, R. Soares, N. Madaboosi, M. Aires-Barros, J. Conde, A. Azevedo, and V. Chu, "The application of microbeads to microfluidic systems for enhanced detection and purification of biomolecules," *Methods*, vol. 116, pp. 112–124, 2017.

[22] R. R. Soares, D. R. Santos, I. F. Pinto, A. M. Azevedo, M. R. Aires-Barros, V. Chu, and J. P. Conde, "Multiplexed microfluidic fluorescence immunoassay with photodiode array signal acquisition for sub-minute and point-of-need detection of mycotoxins," *Lab on a Chip*, vol. 18, no. 11, pp. 1569–1580, 2018.

[23] C. A. Schneider, W. S. Rasband, and K. W. Eliceiri, "Nih image to imagej: 25 years of image analysis," *Nature methods*, vol. 9, no. 7, pp. 671–675, 2012.

[24] R. Soares, P. Novo, A. Azevedo, P. Fernandes, M. Aires-Barros, V. Chu, and J. Conde, "On-chip sample preparation and analyte quantification using a microfluidic aqueous two-phase extraction coupled with an immunoassay," *Lab on a Chip*, vol. 14, no. 21, pp. 4284–4294, 2014.