José Eduardo Nunes Marques

Assays of *In vitro* germination of kiwi pollen

Master thesis on Plant Biodiversity and Biotechnology,
Supervised by Professor Dr. Jorge Manuel Pataca Leal Canhoto
Department of Life Sciences, Faculty of Sciences and Technology, University of Coimbra

Coimbra, June 2018

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Thesis submitted to the University of Coimbra as a requirement to obtain the title of Master in Plant Biodiversity and Biotechnology, supervised by Professor Doctor Jorge Manuel Pataca Leal Canhoto from the Department of Life Sciences, Faculty of Science and Technology, University of Coimbra.

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Images on the cover. Top left and bottom corners, *Actinidia deliciosa* female flowers with its main pollinator *Apis mellifera.* Top right corner, *Actinidia deliciosa* developing fruit. Bottom right corner, *Actinidia deliciosa* male flowers in different stages of development.

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Abbreviations

2,4-D - 2,4-Dichlorophenoxyacetic acid

APK – Associação Portuguesa de kiwicultura

BAP - 6-Benzylaminopurine

FAO – Food and Agriculture Organization

FAA- Formol Acetic Alcohol

GA_3 - Gibberellic acid

IBA - Indole-3-butyric acid

PGRs – Plant growth regulators

PSA - *Pseudomonas syringae* pv *actinidiae*

Abstract

Actinidia deliciosa presents a distinctive flavor and appearance that made this cultivar become the most exported of all kiwi species. At the moment, Portugal occupies the 13th position on the world ranking of production of kiwi. *Actinidia deliciosa* commerce in Portugal faces some fluctuations. PSA bacteria, the inexistence of genotypes fitted to our climate and soil, and the unreliable natural pollination are some of the factors leading to the unstable production of kiwi.

Better pollination and germination of pollen grains result in a higher of seed that are directly related to fruit weight. Therefore, pollination is one of the factors that have strong impact on productivity of a vine, and the producers are now investing in artificial pollination in order to make the yields more reliable.

Many researchers have described the importance of the Effective Pollination Period and how pollination can affect the production. With this in mind, this research focused on determining when pollen was more fit to pollinate and germinate, and to develop a pollen extender formula that could boost the production.

Germination assays performed demonstrated that pollen collected from early branches, from flowers on earlier stages of their development, present higher germination ratios (91%) and therefore have more capability to pollinate. The pollen also showed to be able to germinate and grow when female flowers were pollinated *in vitro*.

Whereas fresh pollen may have higher germination ratios, properly stored pollen can maintain a high capability of germination (83%) and may even present better results when applied combined with hormones and a thickening agent.

In order to optimize the artificial pollination, a new pollen extender formula was developed. The pollen extender containing 2,4-D + IBA (both at 1mg/L), carrageenan 0.5% (w/v) and 2 M sucrose gave interesting results increasing germination rates by 10% (92%) and showing a pollen tube length of around 1.4 mm.

These results are promising and further tests on the field are necessary to verify whether this extender can be successfully applied.

Keywords: *Actinidia deliciosa*, Artificial pollination, carrageenan, in vitro, Pollen extender

Resumo

Actinidia deliciosa apresenta um paladar e aparência distintivo que tornaram este cultivar no mais exportado de todas as espécies de kiwi.

Nos dias de hoje, Portugal ocupa a décima terceira posição no ranking mundial de produção de kiwi. O comércio de *Actinidia deliciosa,* em Portugal, sofreu bastantes flutuações. A bactéria do PSA, a inexistência de genótipos apropriados para o nosso clima e solo, e a inconstante polinização natural são alguns dos fatores que tornam a produção de kiwi instável. Uma melhor polinização e germinação dos grãos de pólen resulta em frutos com maior número de sementes que irá afetar diretamente o calibre do fruto. Consequentemente, a polinização é um dos fatores que tem maior impacto na produção da vinha e, por isso, os produtores começaram a investir na polinização artificial de modo a tornar as colheitas mais uniformes.

Muitos investigadores descreveram a importância do Período Eficaz de Polinização e como a polinização afeta diretamente a produção. Neste contexto, esta tese focou-se na determinação de quando o pólen está mais apto para polinizar e germinar, e em desenvolver um novo pólen *extender* capaz de aumentar a produção.

Ensaios de germinação revelaram que o pólen recolhido de ramos jovens e de flores em estados mais precoces no seu desenvolvimento apresentam taxas de germinação mais elevadas (91%) e, portanto, estão mais eficazes na polinização. O pólen mostrou ser igualmente capaz de desenvolver o tubo polínico no pistilo de flores femininas quando a polinização ocorreu *in vitro*.

Apesar do pólen fresco ter taxas de germinação mais elevadas, pólen apropriadamente armazenado consegue manter uma alta capacidade germinativa (83%) e pode até apresentar resultados mais elevados quando aplicado juntamente com hormonas e um agente espessante. De forma a otimizar a polinização artificial, desenvolveu-se um novo pólen *extender* composto por 2,4-D + IBA (1mg/L cada), carragenanas 0,5% e sacarose 2M, que mostrou resultados muito interessantes, tendo-se obtido um aumento na taxa de germinação de cerca de 10% (92%) e um aumento no alongamento do tubo polínico que chegou aos 1,4 mm.

Estes resultados são bastante promissores. Futuros testes em campo poderão são necessários para se este novo pólen *extender* pode ser aplicado com sucesso na polinização artificial desta espécie.

Palavras-chave: *Actinidia deliciosa*, Carragenanas, Polinização, Polinização artificial, Pollen *extender*

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1. Introduction

Kiwifruit *Actinidia deliciosa* (A.Chev.) C.F. Liang & A.R.Ferguson is a dioecious fruiting vine indigenous to South-East Asia. The *Actinidia* culture regarding commercial purpose, mainly focused on the Hayward cultivar, started in New Zealand during the second quarter of the 20th century (Goodwin *et al*., 2013). The research funded by the New Zealand's government and private companied, to enhance the production, led to the name of the fruit commonly called Kiwi due to the name of the bird national icon. It was introduced in Portugal in 1973, more specifically in a small orchard in the city of Vila Nova de Gaia, district of Porto (Martino, 2006). In the first years of the 80's decade, with the arrival of the French Agricultural Expert, Bernard Blanc, who provided the necessary knowledge, the first small orchards with commercial purposes and with high production potential were created (Martino, 2006).

The biochemical properties of the fruit, with a high content of vitamin C content (126mg/100g of fresh fruit weight) (Huang *et al*., 1997), made it popular among consumers as a healthy fruit. Moreover, it shows a new and rich fruitiness reminiscent of an exotic fruit, as seen in figure 1.

Figure 1. Fruits of the cultivar Hayward. **A.** Inside aspect of kiwifruit – green with a great number of seeds. **B.** Outside aspect of kiwifruit

These characteristics gave kiwi a great relevance at the social point of view and, soon, new kiwi orchards were being created all over the Portuguese territory for commercial purpose.

The creation of cultures in inadequate climatic zones and the lack of knowledge about this species culture lead to the 1993, kiwis market crisis marked by the heavy drop of the kiwi production and market value (Fig. 2, Martino, 2006).

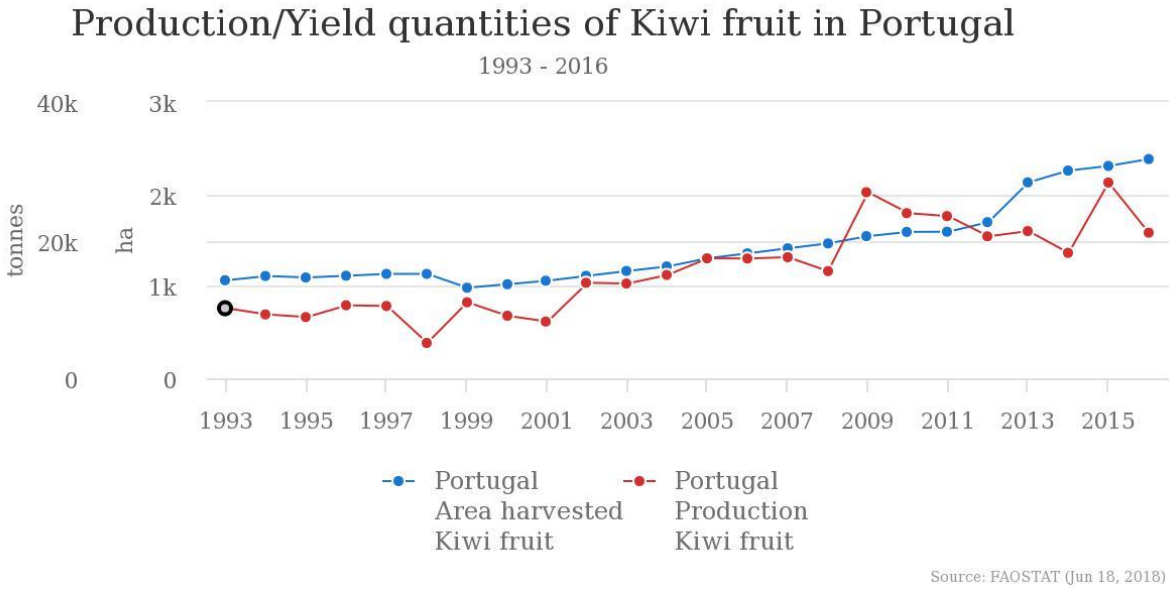

Figure 2. Evolution of kiwi production in Portugal from 1993 to 2016. From: FAOSTAT, 2018.

The years after the market crash remained at a low production rate until 2000 when the production began to rise again. The consistent research done on this species conditions in order to improve cultures explains how this culture was able to recover its expansion until today. Despite the problems this culture faced, the kiwi culture is improving and getting a more important role in the Portuguese economy. By analyzing the data from FAO (Food and Agriculture Organization of the United Nations) we can understand the difficulties this culture endured through the years. It is easily seen that the culture does not have a regular growth, having gains and losses amongst the years. From 2000 to 2009 the production registered an almost impaired growth, and 2009 recorded the highest yield (Fig. 2).

From 2009 to today, the culture had suffered losses in its yield. The main factor to this decrease in the production is a bacterial disease caused by *Pseudomonas syringae pv. actinidiae*, most commonly called PSA. This pathogen can easily multiply and quickly spread to new areas (Vanneste *et al*., 2013). This bacterial outbreak can devastate entire cultures and it is especially hard to control due to the limited number of tools and products available (Vanneste *et al*., 2013). In 2013, 3 years after the discovery of this disease, in New Zealand, the biggest exporter, around 1400 orchards (52% of New Zealand's total production) have been infected with the PSA bacteria (Vanneste *et al*., 2013). Besides this disease, unfavorable weather during the pollination season, or disturbances affecting the natural pollinators, can negatively impact the production (Antunes, 2008).

With the goal to make kiwi production more reliable in Portugal the research project I9K, a partnership between the Instituto Pedro Nunes, and research institutions as the Centre for Functional Ecology of the University of Coimbra, producers and other stakeholders,

started this program to implement strategies that can improve the sustainability of the culture. This program is focused in two main problems, one is to develop an easier and faster ways to identify the PSA bacteria on cultures and creating an effective treatment for it, and the other is to understand pollination and pollen behavior in the field, thus contributing to increase fruit production.

With the support of the scientific research being done around this species, the kiwi culture has the potential to grow even more in the internal Portuguese market and could become a good exportation resource. In 1987, Pyke conducted a research that lead to the conclusion that fruit weight was correlated with seed number, indicating that high rates of pollination are important to produce bigger fruits since an inadequate quantity or quality of pollen can reduce plant reproductive success (seed quantity or quality) (Ashman *et al*., 2004). These conclusions draw a new path for the investigation, which would turn its focus to finding a better understanding on pollination: when it was done, how it was done and how it could be enhanced.

For pollination to be successful it has to happen during a specific period that is called Effective Pollination Period (EPP) (Sanzol & Herrero, 2001). This concept was developed to assess flower receptivity. It is defined as the number of days during which pollination is effective in producing a fruit and it is determined by the longevity of the ovules minus the time lag between pollination and fertilization (Ortega *et al.,* 2004). The EPP has a key role for fruit formation and development and its understanding is crucial to increase productivity (Gonzalez & Coque,1995). EPP is influenced by 3 main events, namely, stigmatic receptivity, pollen tube kinetics, and ovule longevity (Sanzol & Herrero, 2001). Stigmatic receptivity is the ability of the stigma to allow pollen germination (Sanzol & Herrero, 2001), and it is related to the changes resulting from the maturation of the stigma. The stigmatic receptivity reaches its peak upon stigmatic papilla degeneration which produces secretions considered to be related to the recognition and hydration processes (Sanzol & Herrero, 2001). As stigmatic maturation proceeds, the degeneration of the stigma cell wall occurs, which is concomitant with the end of the stigmatic receptivity (Sanzol & Herrero, 2001).

Pollen tube kinetics is the relationship between pollen tube growth and pistil. At the beginning of the germination, there are starch reserves along all the style tract (Hopping, 1979). Pollen tube growth is coincident with the disappearing of the starch reserves and appearance of another secretion, mostly carbohydrates, near the obturator (Herrero *et al.,* 1996). Research carried out to investigate this kinetics showed that the secretion did not disappear in unpollinated flowers while in pollinated flowers the secretion disappeared concomitantly with the growth of the pollen tube (Hopping, 1979). Alongside that, it has been shown that pollen tube growth is not uniform along the style and changes in speed are

significant along the different barriers that pollen tube has to overcome (Sanzol & Herrero, 2001).

The last event limiting the EPP is the longevity of the ovules. Abnormalities in ovule formation and development are common (Tonutti *et al.,* 1991) and they may happen during ovule formation or even after fertilization (Tonutti *et al.,* 1991). Even when the ovule seems to be formed correctly, its short lifespan is often a limiting factor for fertilization that can be slowed by abiotic or endogenous factors (Postweiler *et al.,* 1985).

In 2013, a study was conducted to better understand the EPP in *Actinidia* species. On this test, different flowers were hand pollinated from days one to six after its opening. The results showed that the seed number significantly decreased when the flower's age was four days old or higher and the highest number of seeds was registered at day 2 of pollination (Goodwin *et al*., 2013). Having this in mind, it's really important to notice that the anther dehiscence is not always in perfect coordination with the female flower EPP. Therefore, it's important that the pollen is adequately transported to the stigma during its prime. Another option to synchronize pollination with EPP is to collect male pollen and apply it on the female flower when the flowering stage is more appropriate (Razeto *et al.,* 2005). Results showed that pollen may be stored for long periods of time. If the storage is from 1 to 32 weeks, a temperature of -18 ºC can guarantee successful germination rates of 80 to 90% (Bomben *et al*.,1999). On the other hand, If pollen is to be stored for more than a year then temperatures of -80 ºC must be used to guarantee germination rates of more than 60% for 3 years (Bomben *et al*.,1999).

The fruit set in kiwifruit is mostly affected by its own flower biology. Being a dioecious species, whose female flower's pollen is unable to germinate and to carry gametes for fertilization, it is very important that the male flower's pollen is properly transported (Costa,1993).

The increasing of the pollination effectiveness transport starts with the organization of the orchards. The orchards are mostly trained on a pergola trellis, although some orchards can still be trained in T-bars, with the proportion of 1:6 male/female distribution (Ferguson *et al*., 1999). In kiwi, main pollinator agents are wind and honey bees (Intoppa & Piazza, 1990) although it has been reported that some small insects might also aid pollination (Palmer-Jones & Clinch, 1974).

Figure 3. *Actinidia deliciosa var. Hayward* trained on a pergola trellis from the region of Vila do Conde.

In order to evaluate the relevance of the anemophilous pollination in this species experiments carried out involved enclosing orchards or vines in cages that excluded the possibility of being accessed by insects. In those cages, the pollination was exclusively made by wind. The results showed that wind was inadequate for high production levels and most of the fruit set was small and did not have the necessary weight to be marketable (Costa *et al*., 1993). These results clearly showed that insects have a central role on kiwi pollination and further fruit production (Gonzalez *et al.,* 1998). Honey-bees (*Apis mellifera*) are the main pollinators in kiwifruit (Vaissiere *et al*., 1996). Although this species may face some problems that impair their pollination efficiency

As it is well known, kiwi shows a short flowering period, which for itself is already a threat for the fruit production and can difficult bee pollination. Moreover, in the Northern hemisphere, the flowering period occurs often during heavy rain conditions, making difficult the activity of the insects (Vaissiere *et al.,* 1991).

The orchard arrangement also affects bee pollination, as the distance from the male to the female flower can diminish the amount of pollen that the female flower receive. A study carried out by Goodwin *et al*. (1999) showed that the number of seeds decreased 2.3% per meter of distance between male and female plants. Besides, different hives also have unpredictable foraging efficiency, and it can be even more magnified by the kiwifruit incapacity to produce nectar. In experiments it was shown that honey-bees never attempted

to lick moisture from the base of the petals or stigma; pollen transfer is only done by the bee's movements from male to female flowers (Goodwin *et al*., 2013).

As mentioned before the female flowers also produce pollen. Although it is unable to germinate, it is used as an attraction to the honeybees, since they are incapable to determine the nutritional value of the pollen they collect (Free, 1993). Previous studies, in which both male and female flowers were presented on a tray, indicated a preference of the insects to pistillate flowers, leading to the conclusion that pistillate flowers pollen display a high level of attractiveness (Goodwin *et al.,* 2013). This preference recorded on honey-bees in Hayward orchards can be a problem to the production, since it may affect the movement of honey-bees between male and female flowers and pollination (Goodwin *et al.,* 2013).

Another factor that has been proven to have great relevance to fruit weight is the number of visits to the female flower during the pollination. In 2013, Goodwin *et al*. designed an experiment to identify the relevance of honey-bees on kiwifruit pollination. To determinate, the effect of honeybee's visits, female flowers were marked and enclosed on pollen proof bags while being video recorded to register the number of visits and the time each visit took place (Goodwin *et al.,* 2013). Results confirmed that the number of seeds is strongly related to the number of bee visits. Flowers that received a single bee visit did not develop fruits or produced only a small fruit with an average of 51 seeds (Goodwin *et al.,* 2013). On single visit cases, the flowers on which bee's visit took longer periods were the ones able to set a fruit, the single bee visits recorded and able to produce a fruit had an average time of 38.8 s (Goodwin *et al.,* 2013). The number of seeds per fruit increased, and therefore it's weight, increased up to five visits, concluding that the yield quality is strongly affected by an effective pollination (Goodwin *et al.,* 2013).

Natural pollination is affected by multiple factors. Non-coincident flowering period on male and female flowers, weather conditions at the flowering period, bee's foraging efficiency and orchard arrangement, can affect the pollination and therefore affect the quality of the fruit. Since honey-bees and wind cannot guarantee an effective and reliable pollination, only hand pollination can assure a maximal fruit size in each year (Costa *et al*., 1993). Hand pollination can be performed by rubbing a recently open staminate flower over the pistillate flower for a couple seconds, depending on the cultivar (Razeto *et al*., 2005). According to studies described by Sale in 1985 and Kulczewski in 1988, each male flower can pollinate about five female flowers, which will originate a larger fruit with a better overall quality than those set by bee pollination (Razeto *et al*., 2005). However, hand pollination is not without problems since it requires a great effort - around 100 hours per man are necessary to hand pollinate 1 ha of a mature kiwifruit orchard (Costa *et al*., 1993). Nowadays more effective methods are available such as kiwi pollinator machines (mechanical pollination) that allows pollen gathering and dispersal in spite of their high costs (Razeto *et al*., 2005). In this type of

pollination, a small duster provided with a mixture of pollen and the pollen extender lycopodium powder (1:1) is used. This type of pollination requires fewer hand work but provided somehow lower results than those obtained by hand-pollination (Razeto *et al*., 2005). The mixture dispersion to the air might present a reason why the mechanical pollination was not as efficient as hand-pollination.

In Japan, where artificial pollination is essential to produce a kiwifruit able to be marketable, a new strategy was created. Pollen was suspended in a mix of thickening agents and sugars, to decrease the expenses of the hives feed, and to provide a medium in which pollen is not dispersed thru the air while pollinating (Yano *et al*., 2007). Studies aimed to identify new thickening agents to produce liquid pollen extender were made. With the idea to produce a liquid pollen extender that could be relatively cheap, new thickening agents were tested. Between all the different thickening agents tested, agar, guar gam, xanthan gum and carrageenan displayed the best results (Yano *et al.,* 2007). Among these thickening agents, carrageenan was the one which aroused more interest. Carrageenan are linear sulfated polysaccharides that are extracted from red edible seaweeds (Imeson *et al.,* 1997). Carrageenan have a wide array of functional properties which include thickening, gelling and stabilization (Prajapati *et al.,* 2016). They are used in various products such as pharmaceuticals, food, cosmetics, printing and textile formulation (Campo *et al*., 2009). Its hydrocolloids disperse in water to give a thickening or viscosity producing effect being this the prime reason for their overall use (Saha *et al*., 2010). For that reason, carrageenan might present itself as a good alternative as a thickening agent for new pollen extender formula.

Plant hormones play a key role in developmental processes such as fruit and seed maturation (Mohammad *et al.*, 2014). They are a group of naturally occurring, organic substances which influence physiological processes at low concentrations (Davies, 2010). Plant hormones are a unique set of compounds, with unique metabolism and properties that may be of interest for this study. Scientists have described hormones that play important roles in the fruit set. Although, some studies showed that exogenous hormones, when applied to the fruit itself did not produce the expected results (Hopping, 1979). Tests with the combination of hormones, done in kiwifruit, showed that both seed dry weight and fruit weight were not affected by any treatment (Hopping, 1979).

With the aim of making the kiwifruit culture more profitable, this study revolved around understanding the floral biology of kiwifruit and producing a new liquid pollen extender

Pollen collected from flowers in different stages was tested in order to establish a correlation between flowering stage and germination ratios. Since it had been proven to work, in coffee (Terzi *et al*., 1995) and in bananas (Alvard *et al.,* 1993), pollen germination was tested on a liquid medium, and its germination ratio was compared with the solid medium ratio.

The main goals of this research were to characterize pollen germination *in vitro* and to identify how different compounds can affect pollen germination. These *in vitro* studies are crucial to develop new strategies that can be applied for artificial pollination and to increase the efficiency of fertilization and fruit formation. Thus, we have tested the role of different growth regulators as well as different thickening agents on pollen germination. Assays of pollen germination following conservation were also carried out to verify which approaches are more interesting for pollen conservation and further germination.

2. Material and Methods

2.1. Plant material

Pollen was collected during the pollination period of 2015 from vines, trained in a pergola trellis, growing on a region near Cantanhede. Pollen was stored -20 ºC to guarantee its viability. During 2017, a new set pollen was collected to compare germination rates between stored pollen and “fresh” pollen. To do so, the anthers were isolated and placed in a Petri dish covered with aluminum foil and were gently stirred to promote pollen release from the anthers (Fig. 4).

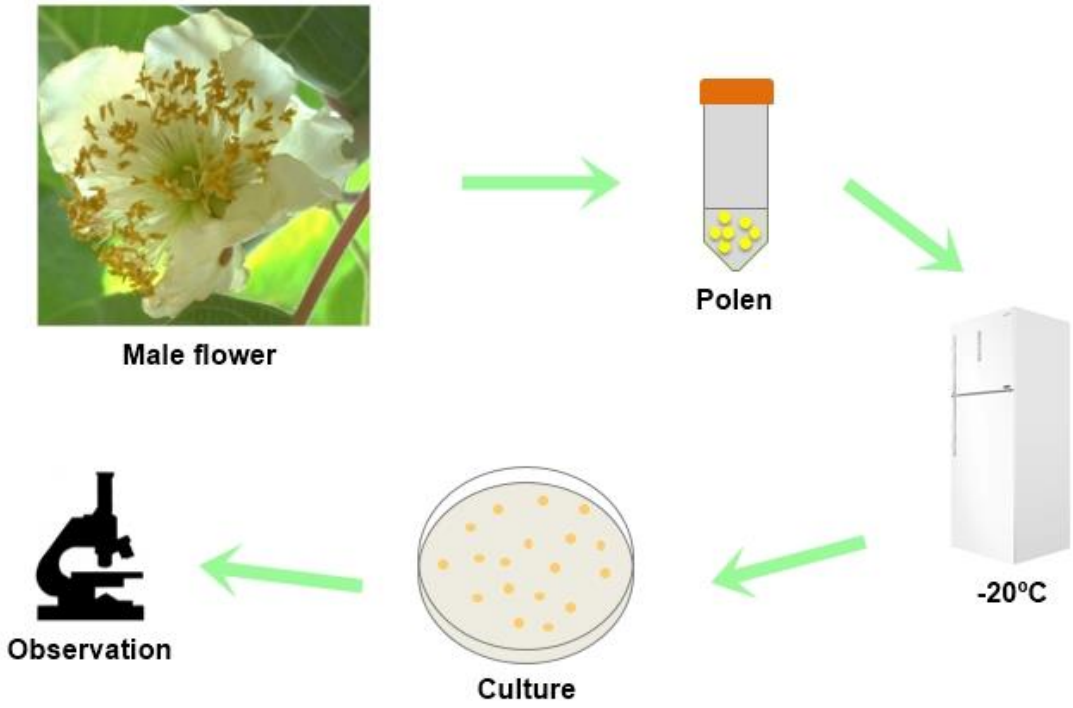

Figure 4: Representation of the process used to evaluate the germination ratio of stored pollen.

2.2. Methods

Both pollen viability and the efficiency of the treatments applied were tested by evaluating germination rates. To do so, pollen was set to germinate in different culture mediums and the percentage of germination evaluated after 24h. From 24h to 48h no statistically significant differences were found in several of the tests carried out, therefore, its good practice to assume differences are only significant during the first 24h of germination.

To count pollen germination, squares of 0.5x 05 cm were cut from the medium, placed on a microscope slide and stained with 1% acetocarmine. When counting the pollen, we observed how many grains germinated in a group of 100 grains. That way, we could easily obtain the percentage of germination. To assume that a pollen grain had germinated, the length of the pollen tube had to be longer than the diameter of the pollen grain. Three replicas were made per preparation in order to ensure the reliability of the results.

2.2.1. Pollen germination in stored and fresh pollen.

Pollen had already been collected from this same vine, at the pollination period of May 2015 and stored at -20 ºC.

The purpose of this test was to compare the efficiency of germination between stored pollen and fresh pollen, while also testing whether female pollen was able to germinate in *in vitro* condition. Flowers were sorted according to their flowering stage and their sex.

Flower buds that were closed or only partially open were classified as stage 1 flowers, as seen in figure 5A, whereas flower buds almost completely open or completely open were determined as stage 2 flowers, as seen in figure 5B. Pollen was collected from flowers from both stages and sex and set to germinate separately. During the development of this project, quality of the pollen was also tested according to the age of the branch. Early branches were separated from older branches and flowers were collected individually. Pollen was set to germinate, and its ratios were compared.

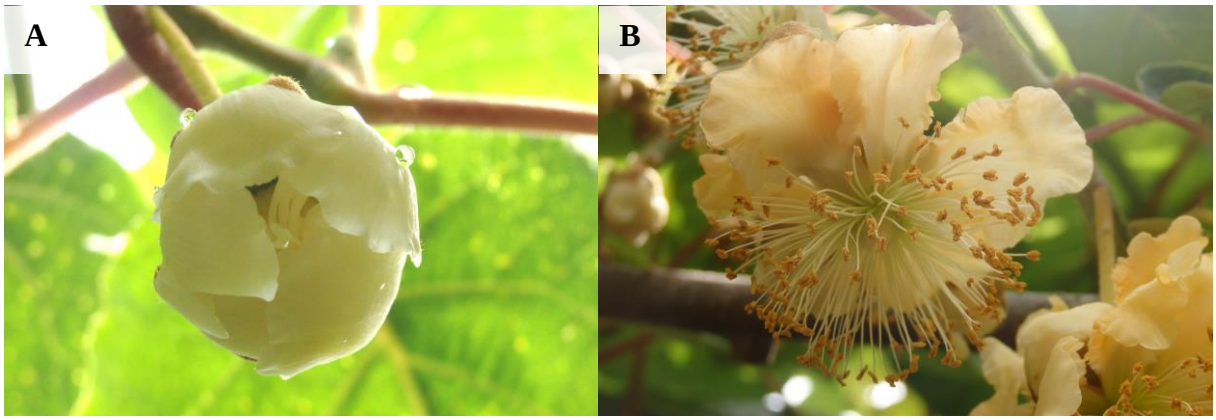

Figure 5. Kiwi flowers in stage 1 (A) and stage 2 (B) of its development.

To initiate the cultures, 3 mg of pollen from each category was placed in a Petri dish, with the aid of a brush, in a pre-tested culture medium which assures a good efficiency of germination in pollen of different species, including kiwi (Morgado, 2015). The composition of this culture medium is described in table 1.

Table 1. Components of the culture medium used to test pollen viability.

Compound	Chemical Formula	Quantity
Calcium Chloride	$CaCl_2$	15 mg/L
Potassium Nitrate	KNO_3	10 mg/L
Boric Acid	H_3BO_3	5 mg/L
Sucrose	$C_{12}H_{22}O_{11}$	6 %
Agar		6 g/L
Distilled Water	H_20	

Before distribution in Petri dishes (6 cm diameter) the pH of the medium was adjusted to 5.8 using KOH or HCl. Cultures were kept at 25 ºC under a photoperiod of 12h light and 12h dark per day. Pollen germination ratio was counted 24h after culture initiation.

2.2.2. Pollen germination in the pistil.

The experiment was carried out during June 2017 with pollen collected from the pollination period of May of that same year. Male flowers were collected and separated again by bud stage. Female flowers that were identified to be on its prime for receiving pollen were collected and their petals were removed in order to remain only the female reproductive organs. Pollen was collected and applied in the stigma of these flowers (Fig. 6) that were cultivated in Petri dishes containing a simple medium used to avoid flower dehydration and rapid senescence (Table 2). After 24 h of pollination styles were fixed in a FAA solution containing 90% ethanol at 70º, 5% of formol and 5% of acetic acid for a period of 24h.

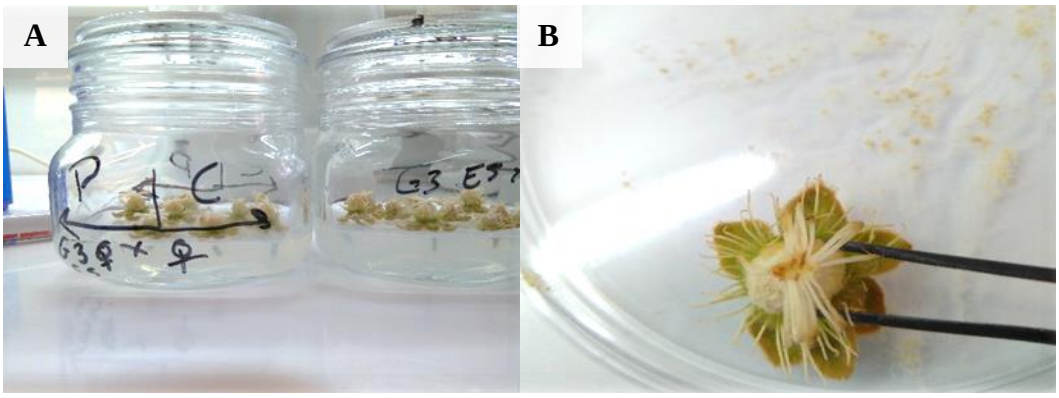

Figure 6. *in vitro* artificial pollination method preformed in *Actinidia deliciosa*. **A.** Pollinated flowers placed on the pollination medium separated according to which pollen they were pollinated with. **B.** Female flower being pollinated with the aid of a tweezer, holding the female flower while a brush gently drops the pollen.

Table 2. Medium used for artificial pollination of female flowers in vitro.

Compound	Chemical formula	Quantity
Ascorbic Acid	$C_6H_8O_6$	100 mg/L
Distilled Water	H_2O	200 ml
Agar		5 g/L

After those 24 hours, the styles were washed in running water to remove excess of FAA and then immersed in a solution of sodium hydroxide (NaOH) for 8h to soft the tissues. Following washing for three times in water the samples were dyed with aniline blue (0,1M) to visualize the pollen tubes in the style. Observations were carried out in a fluorescence microscope (Leika DM4000B) using a wave length of 365nm.

2.2.3. Pollen germination in liquid medium.

Besides jellified media, pollen germination was also tested in liquid medium. Germination in liquid medium is an uncommon method to germinate pollen, that has not yet been properly studied, but could possibly be a new efficient method to carry out *in vitro* experiments. The problem associated with this method comes from the osmotic pressure that the pollen grain cells endure during emersion in a solution.

To minimize this problem, pollen needs to be rehydrated before being placed in the solution. For that purpose, pollen was placed on a Petri dish which was floating in water

inside a plastic bag with a wide mouth. This method created an atmosphere rich in water that would rehydrate the pollen to prevent an osmotic shock that would affect the viability.

Following rehydration, pollen was tested in two different solutions. One solution (Table 3) has been used by researches working with kiwi in New Zealand (Séverine, personal communication).

Table 3: Components of liquid medium solution.

Compound	Chemical formula	Quantity
Boric Acid	H_3BO_3	0.05 g
Sucrose	$C_{12}H_{22}O_{11}$	50 g
Distilled Water	H_2O	500 ml

The other solution was an attempt to create a more nutrient-rich solution that would improve the germination ratio. This solution had of sucrose, boric acid, calcium chloride, potassium nitrate and distilled water as shown in table 4.

Table 4: Components of nutrient rich liquid medium solution.

Compound	Chemical Formula	Quantity
Calcium Chloride	$CaCl_2$	15 mg/L
Potassium Nitrate	KNO_3	10 mg/L
Boric Acid	H_3BO_3	5 mg/L
Sucrose	$C_{12}H_{22}O_{11}$	6 %
Distilled Water	H_20	

For each treatment, 3 mg of pollen were placed in 4 ml of solution. To compare the pollen grain germination rate, a drop of the solution was placed on a microscope slip and stained with acetocarmine after 24 h of germination. Slides were observed in a microscope and the percentage of grains germinated determined

2.2.4. Effect of plant growth regulators on pollen germination.

To evaluate the effect of plant growth regulators (PGRs) on pollen germination tests several compounds were tested. Hormones known to have the more impact on the plant development were selected, according to scientific research. The ideal concentration to produce effect was also selected by previous literature (Hopping ,1975 & Iwahori *et al.,* 1988).

PGRs were applied individually and combined in groups of 2 and applied as shown in table 5. The basal medium was the same indicated in section 2.1 and prepared in the same way.

Table 5. PGRs tested on the germination of kiwi pollen.

Treatment	Culture medium	Hormone(s)	Quantity
A	Pollen germination medium	2,4-D	2 mg/L
B	Pollen germination medium	IBA	2 mg/L
C	Pollen germination medium	BAP	2 mg/L
D	Pollen germination medium	GA_3	2 mg/L
AB	Pollen germination medium	2,4-D + IBA	1 mg/L each
AC	Pollen germination medium	2,4-D + BAP	1 mg/L each
AD	Pollen germination medium	2,4-D + GA_3	1 mg/L each
BC	Pollen germination medium	IBA + BAP	1 mg/L each
BD	Pollen germination medium	IBA + GA_3	1 mg/L each
CD	Pollen germination medium	BAP + GA_3	1 mg/L each

2.2.5. Effect of thickening agents on pollen germination

Thickening agent is a very important component of the artificial pollination medium. This component adds thickness to the pollen solution avoiding it to spread through the wind and resulting in a more efficient pollination. The thickening agent must be able to add consistency to the pollen solution without making inaccessible the nutrients of the medium.

To assure that the thickening agent would not negatively impact pollen germination, pollen was mixed with different types and concentrations of thickening agents, as shown in table 6, and sucrose 2M.

Table 6. Thickening agents applied to the pollen.

Thickening agent	Quantity
Bactoagar	0.1%
Pectin	1.0%
Starch	0.5%
Gelrite	0.5%
Cellulose	0.5%
Cellulose	1.0%
Nanocellulose	1.0%
Nanocellulose	5.0%
Carrageenan	0.25%
Carrageenan	0.5%
Carrageenan	1.0%
Carrageenan	1.5%
Carrageenan	2.0%

Pollen was mixed with the thickening agents and sucrose, and the mixture was applied in a culture medium for germination. Culture medium was composed of sucrose, agar, boric acid, calcium chloride, potassium nitrate and distilled water as shown in table 1. Pollen was counted 24h after it was set to germinate, and results were compared to assure the effectiveness of the thickening agent.

2.2.6. Pollen germination using a pollen extender.

After selecting the most efficient thickening agent and hormones, tests were carried out to evaluate a new pollen extender. To do so pollen was mixed with the thickening agent and the hormones and set to germinate in a culture medium composed of sucrose, agar, boric acid, calcium chloride, potassium nitrate and distilled water as shown in table 1.

A solution of distilled water, sucrose, carrageenan and hormones was made according to the table 7.

Table 7. Composition of the pollen extender.

Compound	Quantity
Carrageenan	0,5%
2,4-D + IBA (Hormones)	1mg/L each
Sucrose	2M
Pollen	3 mg per 5ml of solution
Distilled water	

Pollen extender was then applied with a pipette and with a sprayer. Pollen germination was counted 24h after it was set to germinate, and the results were compared with the germination ratio of experiments 2.1 (Pollen Viability comparison. Viability of female flowers' pollen), 2.4 (The effect of plant growth regulators on pollen germination) and 2.5 (The effect of thickening agents of the culture medium on the pollen germination).

2.2.7. Measurement of pollen tube growth.

To assure that the pollen tube reached the ovule during the EPP, tests were carried out to measure the speed of growth of the pollen tube per hour on a 24h period. The objective behind this experiment was to determine whether pollen extender, promotes pollen tube growth, a parameter that could be important for the effectiveness of artificial pollination.

Pollen was mixed with the pollen extender and applied in a culture medium composed of sucrose, agar, boric acid, calcium chloride, potassium nitrate and distilled water as shown in table 1.

2.2.8. Viability of pollen extender.

To assure viability of pollen extender, the content made was stored in a fridge at temperatures of -80 ºC for 31 days and then tested in a culture medium composed of sucrose, agar, boric acid, calcium chloride, potassium nitrate and distilled water as shown in table 1.

The content was left to unfreeze for 2h at 25 ºC before being used. Pollen was counted 24 h after it was set to germinate. The germination ratio was compared with the ones from the experiment 6.

2.2.9. Statistical analysis.

All the results obtained, pollen germination rate and length of pollen tube, were statistically analyzed using both STATISTICA 7 and Microsoft Excel software. The data were gathered in table and graphics and then mathematically tested by using the One Way ANOVA parametric test for results with a normal distribution and Tukey's range-test to compare the differences between treatments.

3. Results

3.1. Pollen germination in stored and fresh pollen.

It was decided to use a period of 24h in all germination tests, in order to grant a better consistency of the results. Fresh pollen presented the highest germination ratios (91% germination ratio in early stages). It was recorded differences between germination ratios of pollen collected from early branches or old branches and between pollen collected from flowers in early stages or flowers in older stages.

Stored pollen presented reliable germination ratios according to the period it was stored, only loosing 6% of its germination capability compared to fresh pollen from early stages of plant development.

Pollen collected from early branches (one-year old branches that are not completely lignified) showed higher germination ratios when compared to older branches (Fig. 7) and these differences were statistically significant (p= 0.000140), when collected from flowers at earlier stages of development.

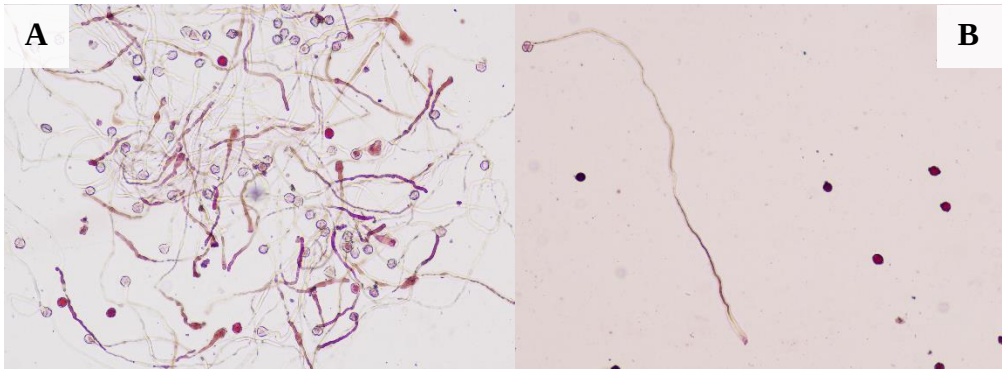

Figure 7. Pollen germination. Observations (x20) were made after pollen staining with acetocarmine and culture on a jellified medium**. A.** Germination of pollen collected from flowers at stage 2 of development of early branches. **B.** Germination of pollen collected from flowers in stage 2 of development of old branches.

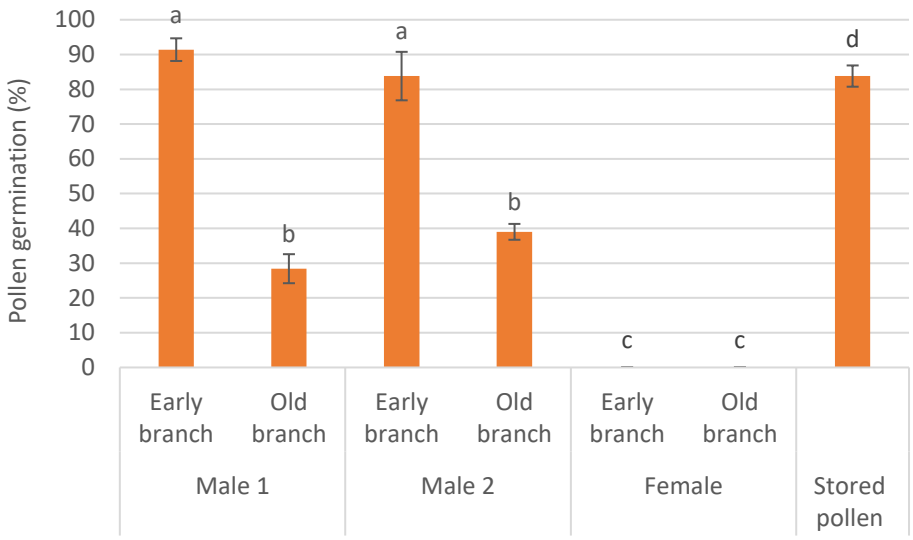

Figure 8. Effect of the origin of the flowers (old versus young branches) on pollen germination on flowers on stage I of their development. Pollen from female flowers and stored pollen were also tested. Each value represents the mean ± SD of seven independent experiments. Treatments indicated with different letters have been shown to have statistically significant differences using Tukey's range test.

Flowers on stage 1 collected from early branches, on both replicas, have germination ratios above 80%, meanwhile flowers on the same stage but collected from older branches presented germination ratios lower than 40%, as we can see in figure 8.

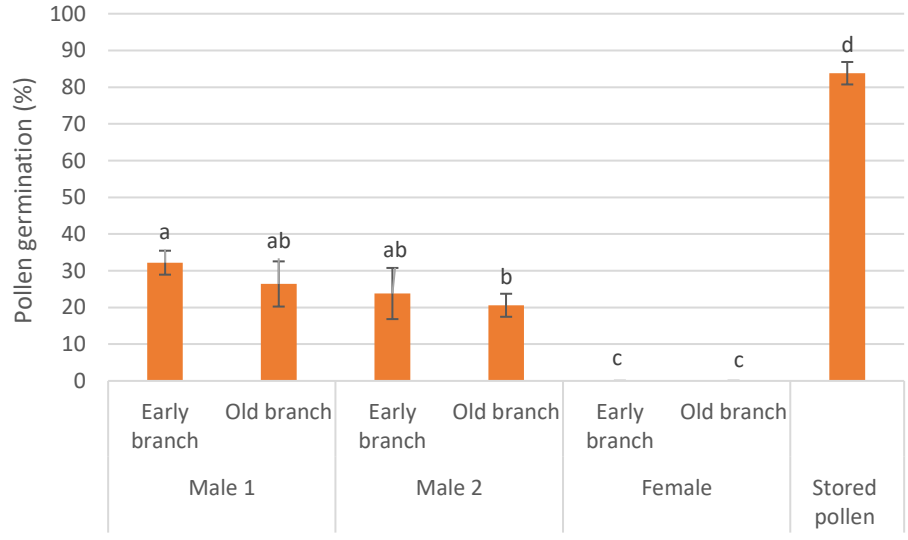

Figure 9. Effect of the origin of the flowers (young versus old branches) on pollen germination on flowers on stage II of their development. Pollen from female flowers and stored pollen were also tested. Each value represents the mean ± SD of seven independent experiments. Treatments indicated with

different letters have been shown to have statistically significant differences using Tukey's range test.

Flowers on stage 2 collected from early branches, present germination ratios as high as 32% while flowers on the same stage but collected from older branches presented germination ratios lower than 28%, as we can see in figure 9.

As a rule, pollen collected from flowers on stage 1 displayed better germinations when collected from early branches than from older branches (Fig. 8). Pollen collected from flowers on stage 2 does not show statistically significant differences on their germination capability when collected from early or old branches (Fig. 9).

The feature more relevant for the success of germination was the flowering stage at the moment that pollen was collected. Flowers acquire different conformations during the flowering bud development as shown in figure 10. Flowering occurs concomitantly with pollen development resulting in a more efficient pollination.

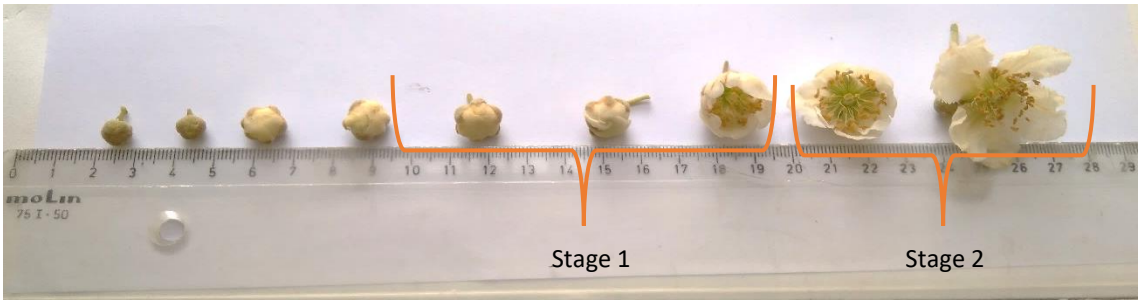

Figure 10. Flowers of *A. deliciosa* at different stages of development. The stages 1 and 2 referred in the text are indicated in the figure by the respective numbers.

During the early stages of pollen development, flowers have their petals pointing to the middle, granting a closed structure in which pollen is capable to complete its development.

As pollen development progresses, petals start pointing out, and present a "bell-like" shape, which will partially allow, both pollinators and wind, to remove pollen to initiate pollination. During this research, we called this stage of the development stage 1.

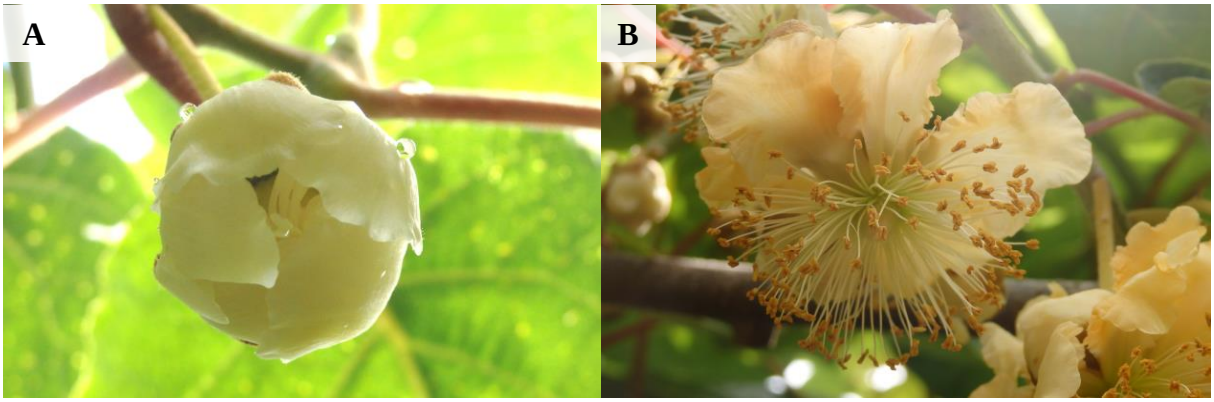

Figure 11. Male flowers in different stages of development. **A.** Male flower in stage 1. **B.** Male flower on stage 2.

As pollination period reaches its conclusion, petals progressively distance themselves further from each other, exposing even more the anthers, and by consequence pollen, to the pollination agents. Regarding this conformation, we named it stage 2 (Fig. 11).

Pollen collected from flowers in stage 1 of their development, when collected from early branches, showed stunning values of germination ratios (91% and 83%), as we can see in figure 12 and 13, when compared to the much lower germination ratios from pollen collected also from early branches but from flowers on stage 2 of their development (32% and 23%), (Fig 12 & 13).

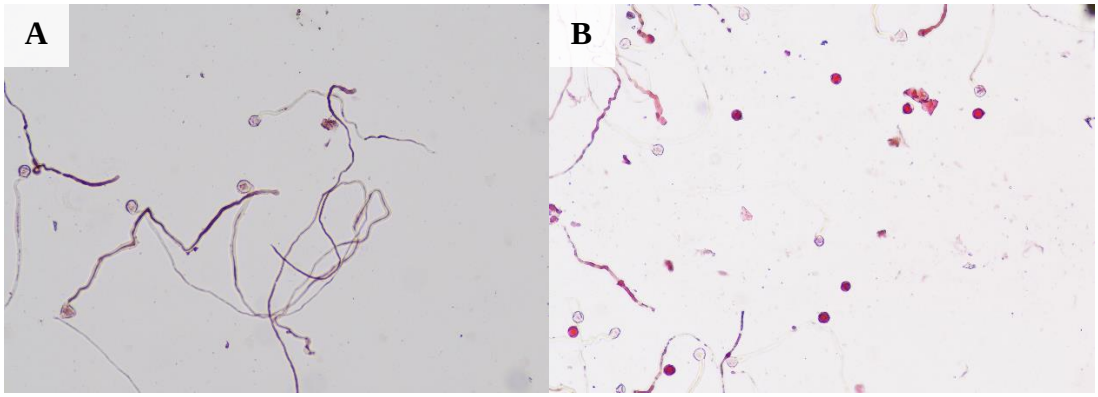

Figure 12. Pollen germination. Observations (x20) were made after pollen staining with acetocarmine and culture on a jellified medium. **A.** Germination of pollen collected from early branches from flowers on stage 1 of their development. **B.** Germination of pollen collected from early branches from flowers on stage 2 of their development.

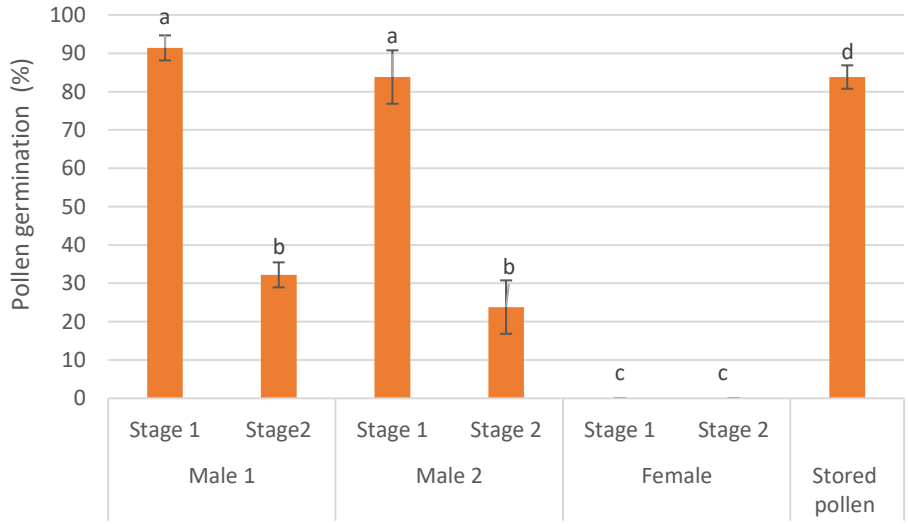

Figure 13. Effect of the origin of the pollen (younger flower development versus later flower development) on pollen germination on pollen collected from early branches. Pollen from female flowers and stored pollen were also tested. Each value represents the mean ± SD of seven independent experiments.

Treatments indicated with different letters have been shown to have statistically significant differences using Tukey's range test.

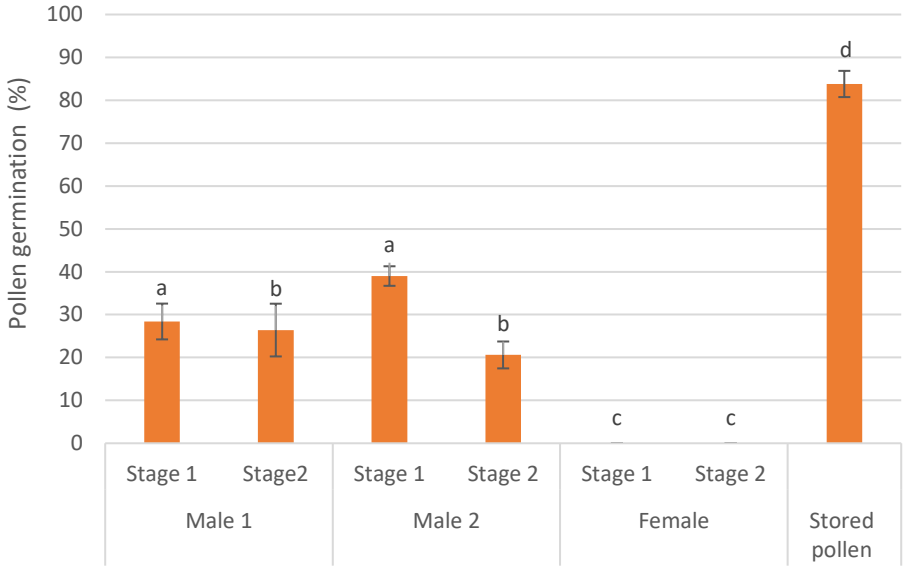

Figure 14. Effect of the origin of the pollen (younger flower development versus later flower development) on pollen germination on pollen collected from old branches. Pollen from female flowers and stored pollen were also tested. Each value represents the mean ± SD of seven independent experiments. Treatments indicated with different letters have been shown to have statistically significant differences using Tukey's range test.

After comparing the germination ratios from both flowering stages, we were able to conclude that pollen decreases its capability to germinate with time. Younger stages demonstrated higher germination ratios than those of older stages.

Pollen shows a higher germination ratio when its collected from early branches from flowers on stage 1 of their development (Fig. 14).

This experiment also showed that female pollen is not viable. Female pollen is not capable of germinating in culture medium, *in vitro*. Results revealed that pollen collected from younger stages can maintain its germination capability if it is properly stored, as shown in figure 16. Stored pollen exhibited germination rates of 83%, only dropping 8% compared to the higher values obtained with fresh pollen. (Fig. 8,9,13 &14).

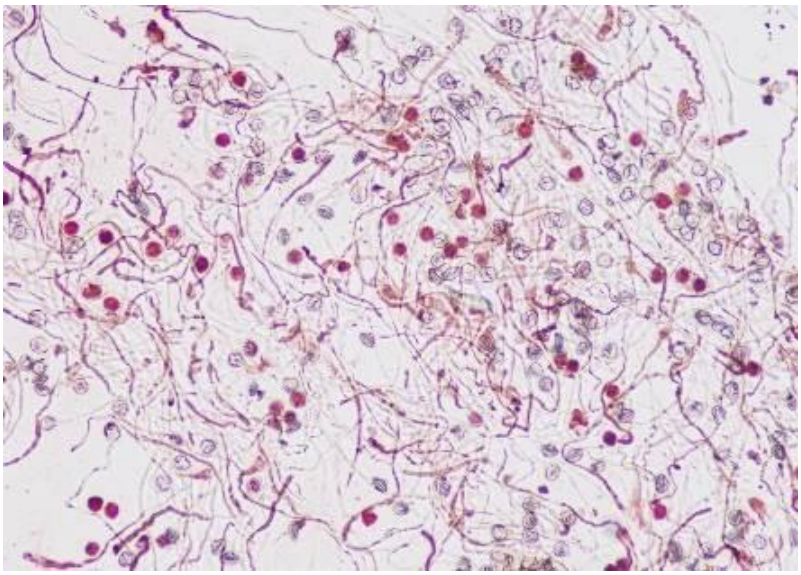

Figure 15. Stored Pollen germination. Observations (x20) were made after pollen staining with acetocarmine and culture on a jellified medium.

3.2. Pollen germination in the pistil.

On this experiment, pollen was artificially applied into female flowers and the germination was evaluated by fluorescence microscopy.

Stored pollen, fresh male pollen and fresh female pollen capability to pollinate were tested. The results showed that pollen from female flowers could not germinate (Fig. 16).

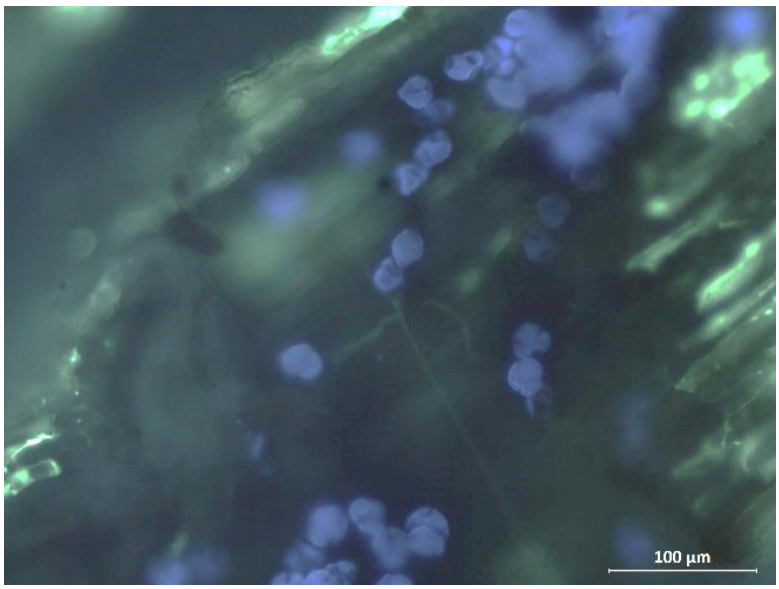

Figure 16. Pollen grains (turquoise blue) that did not germinate in the stigma of the flower. Observations (x40) were made in a fluorescence microscope using an ultraviolet filter of 365nm.

Both stored and fresh pollen from male flowers were viable and successfully pollinated the female flowers (Fig.17). On both figures the fluorescent green tubes, are the pollen tubes being formed from the “blue” pollen grains.

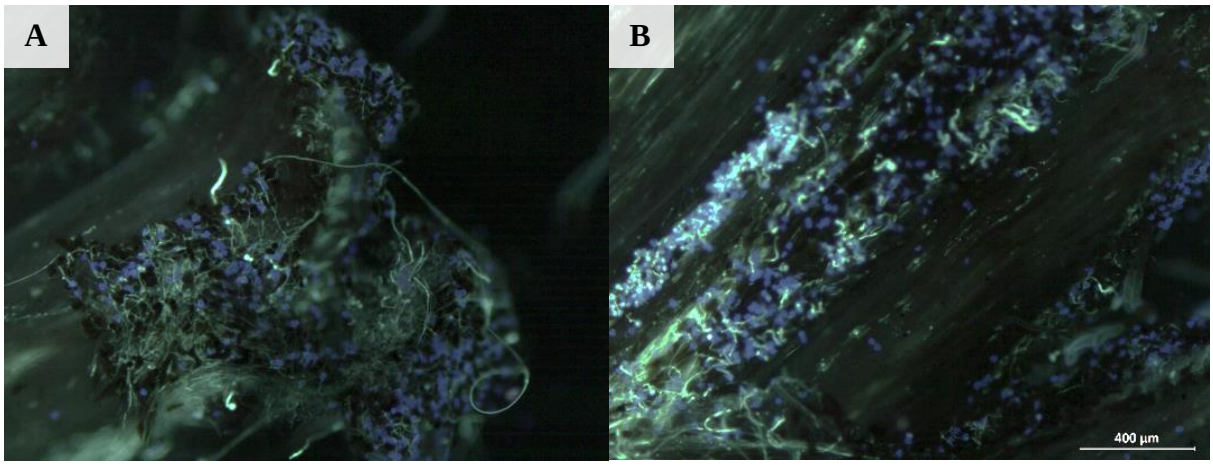

Figure 17. Pollen grains (turquoise blue) that germinate in the stigma of the flower, being able to develop pollen tube (fluorescent green). Observations (40x) of stored pollen (A) and fresh pollen (B) were made in a fluorescence microscope using an ultraviolet filter of 365nm.

3.3. Pollen germination in liquid medium.

In this experiment, stored pollen was used to evaluate pollen capability to germinate in liquid medium.

Results showed that there are statistically significant differences between establishing pollen in a solid culture medium or in a liquid culture medium ($p=0.00102$). Pollen germination ratio was manifestly higher in solid culture medium with a percentage of 81%, while in liquid medium the percentage was never higher than 63%, (Fig. 18).

The different solutions used to germinate pollen also showed differences. The “Séverine” solution displayed ratios of 42% while Nutrient rich solution was able to reach 63% of pollen germination. These results demonstrated statistically significant differences ($p=0.00159$), (Fig. 18).

These results showed that pollen is able to germinate in liquid medium, but its rates are lower than in solid culture medium. Therefore, solid culture medium presents itself as a better solution to preform germination assays.

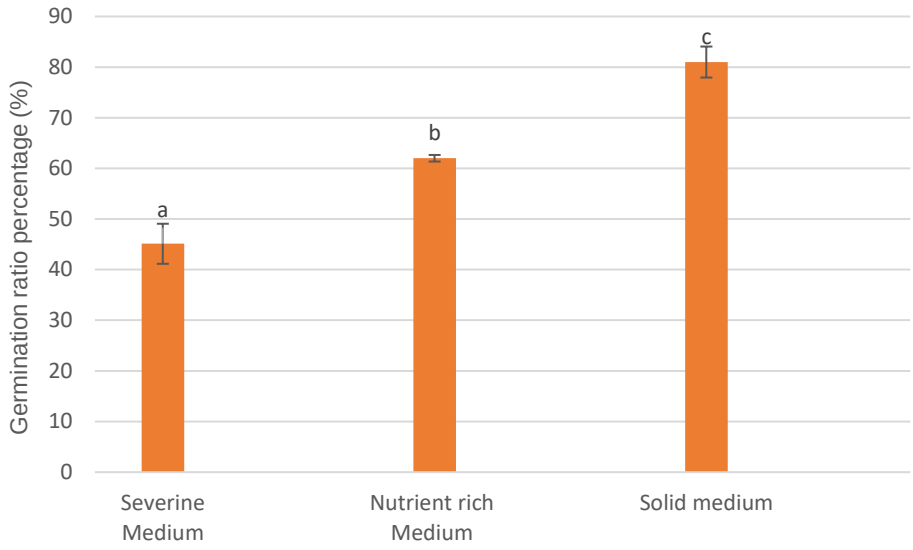

Figure 18. Effect of the germination medium on pollen germination. Each value represents the mean ± SD of three independent experiments. Treatments indicated with different letters have been shown to have statistically significant differences using Tukey's range test.

3.4. Effect of plant growth regulators on pollen germination.

During this experiment, culture medium was treated with hormones individually or in combinations of two, and the results were compared to identify which treatment could possibly enhance pollen germination.

Hormones were added to the culture medium where the pollen was then applied, as done in all previous experiments. Four different hormones were selected (2,4-D, BAP, IBA and GA_3) and applied individually or in combinations of two, resulting in 10 different treatments.

This experiment had the main goal of identifying the treatment that had greater impact on pollen germination in order to use it in the development of a new pollen extender formula.

To determine which treatment presented a more desired effect, both pollen germination ratios and the length of pollen tube were analyzed.

Comparing the germination ratios between all ten treatments, we were able to separate 6 treatments (BAP, GA_3, 2,4-D+ BAP, 2,4-D+IBA, 2,4-D+GA_3 and IBA+GA_3) with more effect, presenting germination ratios higher than 81% (value of germination ratio without any treatment), (Fig. 19).

Also, we could identify 2 treatments that drastically decreased the germination ratios to percentages under 50% (lower than those of liquid medium), being those 2,4-D and BAP +GA_3 (Fig. 19).

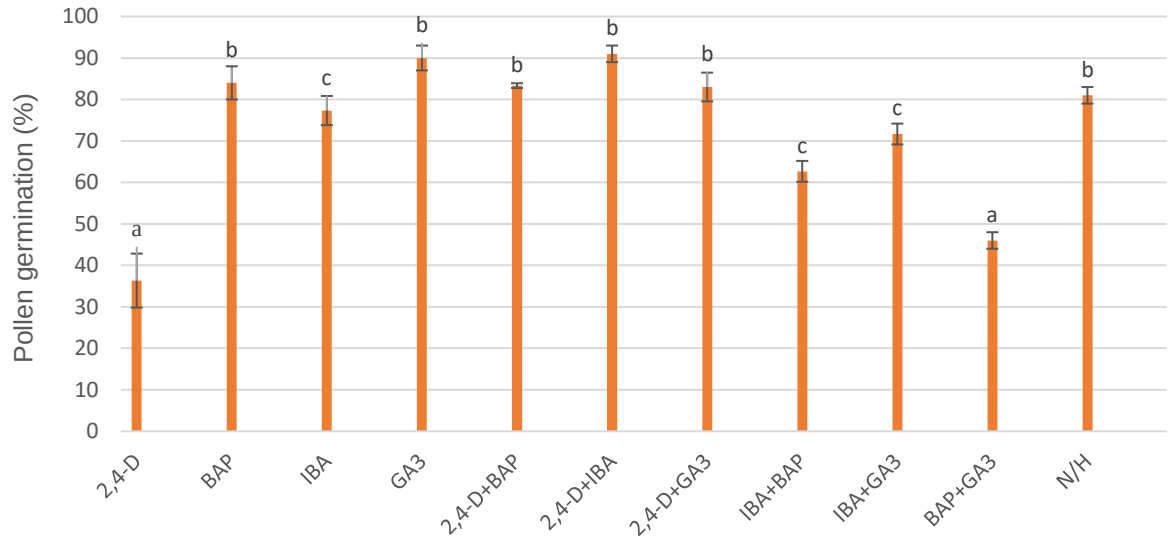

Figure 19. Effect of the different hormonal treatments on the germination ratios of *Actinidia* deliciosa stored pollen. Pollen without hormones was also tested. Each value represents the mean ± SD of eleven independent experiments. Treatments indicated with different letters have been shown to have statistically significant differences using Tukey's range test.

Focusing more on the treatments with capability to enhance pollen germination we can visualize that two treatments are also capable of reaching germinations ratios higher than 90%, which is 10% higher than the normal germination ratio for *in vitro* trials.

Accordingly, pollen germination does not depend solely on its capability to germinate, needing also an adequate development of the growth of the pollen tube in order to reach the ovule. Therefore, the length of pollen tube was also of great importance to this study.

Analyzing the length of pollen tubes, we observe that two treatments show statistically significant differences when comparing to all other treatments.

Both Treatments with 2,4-D+IBA and BAP+GA_3 showed pollen tubes much longer than the average pollen tube (without treatment). Their size was superior than 1,4mm (Fig. 20)

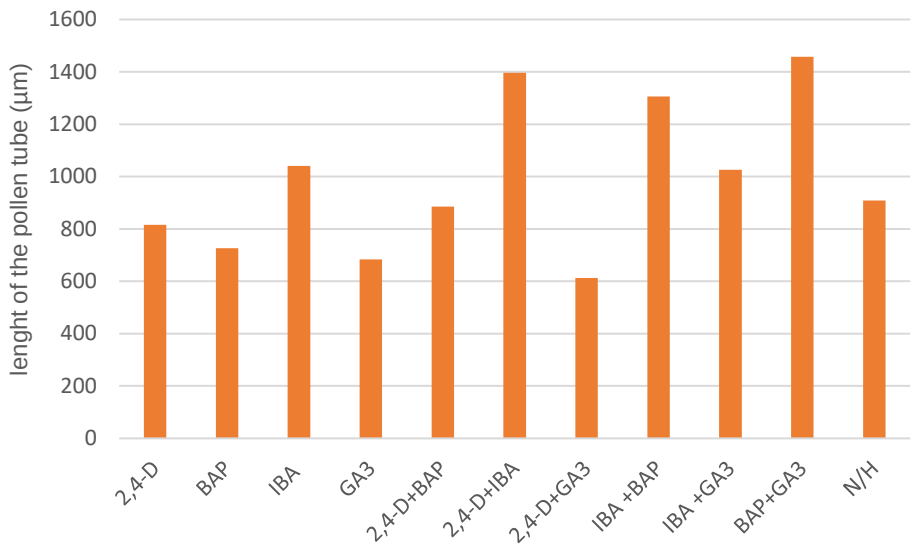

Figure 20. Effect of the different hormonal treatments on pollen tube length of *Actinidia deliciosa* stored pollen. Pollen without hormones was also tested. Each value represents the mean of eleven independent experiments.

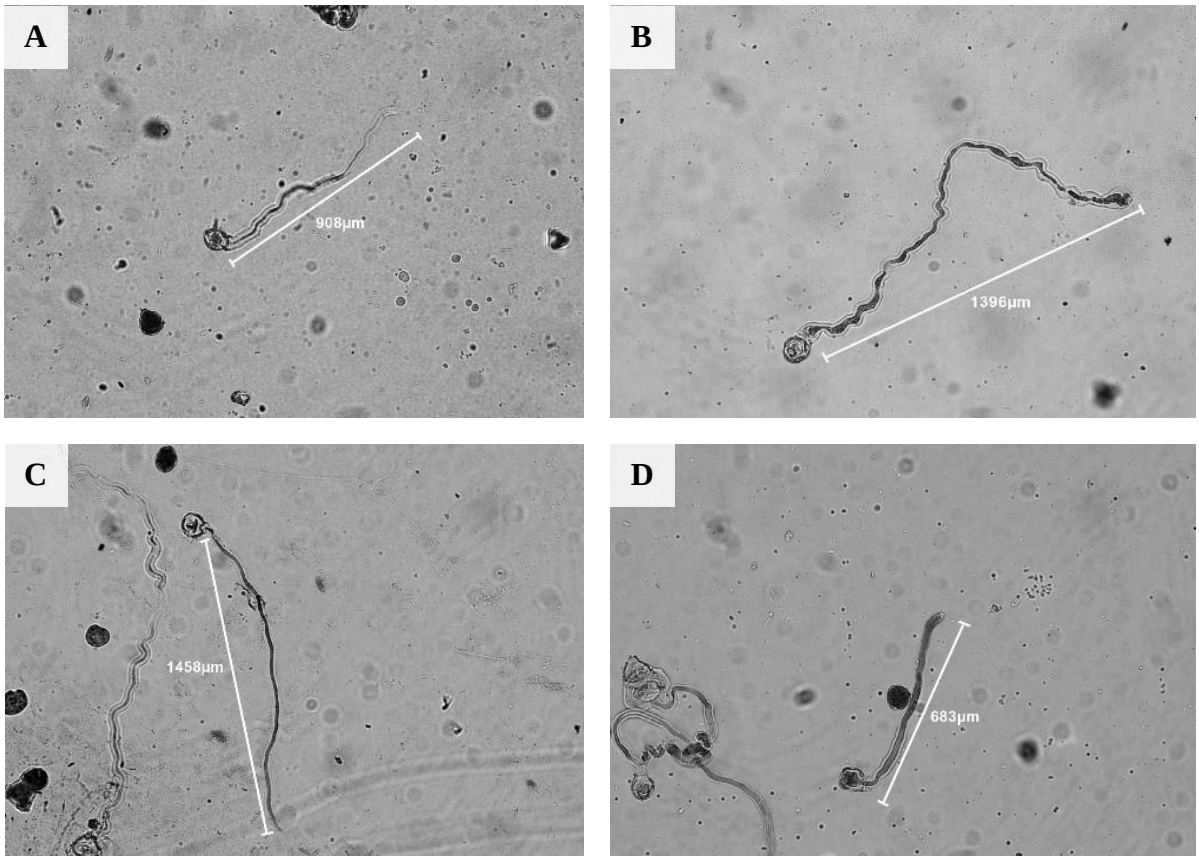

Figure 21. Pollen tube length of stored pollen. **A.** Pollen without any hormonal application. **B.** Pollen with hormonal application of 2,4-D+IBA. **C.** Pollen with hormonal application of BAP+GA_3. **D.** Pollen with hormonal application of GA_3. Observations (x20) were made after pollen staining with acetocarmine and culture on a jellified medium.

These results demonstrated that the treatment with 2,4-D+IBA was the most effective (Fig. 20 & 21A).

By analyzing the germination ratios of the 2,4-D+IBA treatment and comparing it to the germination ratio of no hormones, Tukey's test demonstrated the existence of statistically significant differences (p= 0.000179).

Since it not only increased germination ratio but also enhanced pollen tube growth to almost double of its normal size, this experiment lead to the choice of the combination of 2,4-D+IBA as the establishment of the new pollen extender formula.

3.5. Effect of thickening agents on pollen germination.

In this experiment, pollen was mixed with a thickening agent before being added to the culture medium. Pollen was mixed with 13 different treatments and pollen germination ratios were compared.

When cellulose was used as thickening agent, the pollen got stuck in a solution excessively thick that did not allow it to use the nutrient in the medium and therefore did not permit the germination. Carrageenan and Starch presented good germination ratios (approximately 80%) (Fig 23), being extremely close to the normal germination ratios (ratios without any thickening agent).

Since carrageenan is a natural product extracted from algae, this research focused more on this option. To do so, a gradient of carrageenan was made to recognize which percentage provided the best consistency.

According to the results, the percentage of 0,5% confers the best consistency and therefore this was the value selected to use on the process of conceiving a new pollen extender formula as we can see in figure 22 and 23.

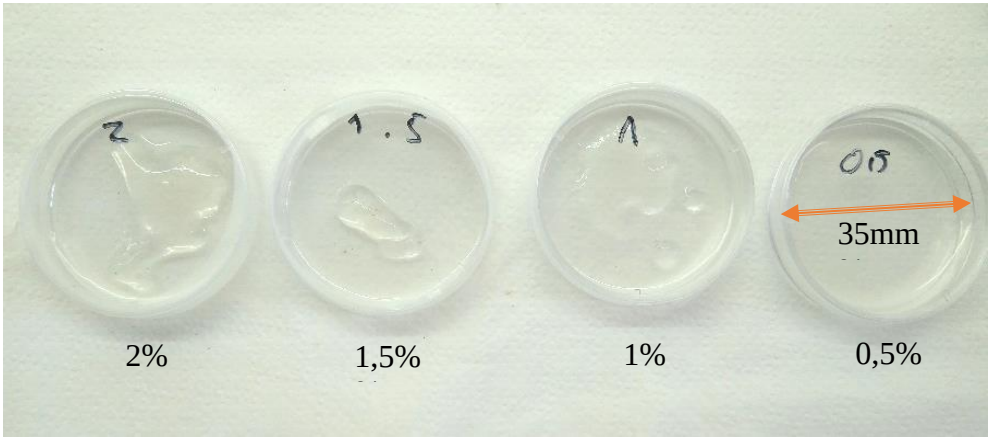

Figure 22. Photograph of the different consistencies of the thickening agent, according to the percentage of carrageenan added. Gradient decreased 0,5% starting from 2% and until 0,5%. From Left to right petri dish 2%, 1,5%, 1% and 0,5%.

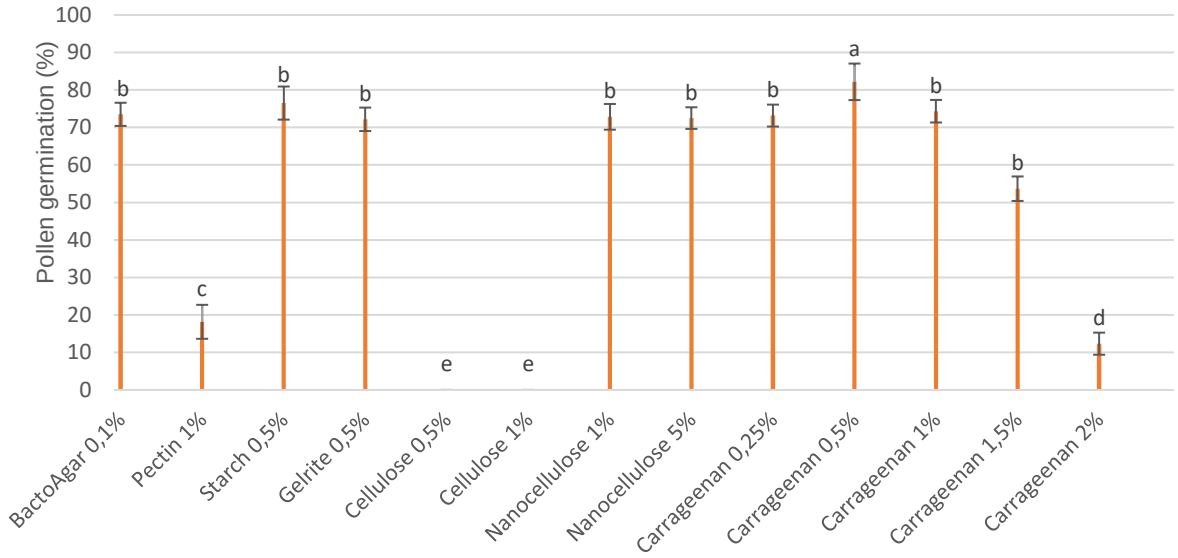

Figure 23. Effect of the different thickening agents on the germination ratios of *Actinidia* deliciosa stored pollen. Each value represents the mean ± SD of thirteen independent experiments. Treatments indicated with different letters have been shown to have statistically significant differences using Tukey's range test.

3.6. Pollen extender.

In this experiment a new pollen extender formula was tested.

Results shown that applying pollen with the new pollen extender formula has a positive effect on the pollen germination ratios.

Pollen extender formula germination ratio presented no statistically significant differences in comparison with the experiments with hormones, meaning that the thickening agent did not much effect on these results (p= 0.681609), (Fig. 24).

Tukey's range test revealed that there are statistically significant differences between pollen extender and no treatment, meaning that pollen extender highly impacted germination ratios, improving them from 82% to 93% (p=0.000683), (Fig. 24).

These results were very promising, and this new formula can greatly impact the kiwi culture.

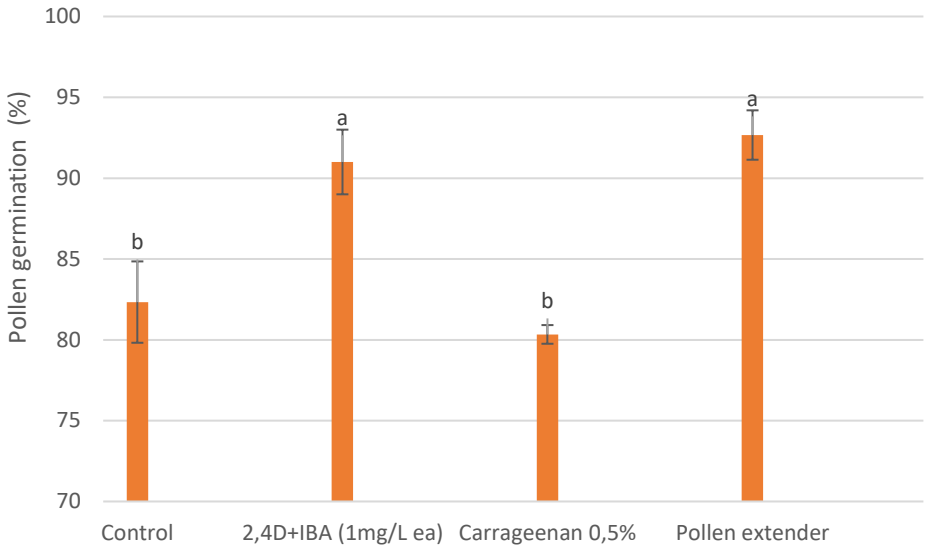

Figure 24. Effect of the treatments on the germination ratios of *Actinidia* deliciosa stored pollen. Pollen without any treatment was also tested. Each value represents the mean ± SD of four experiments. Treatments indicated with different letters have been shown to have statistically significant differences using Tukey's range test.

3.7. Gradient of growth of pollen tube.

During this try out, pollen tube length was measured every hour for a period of 24h, after being germinated with the new pollen extender formula, as described in table 7. This test is very important to assure that the pollen tube grows during the Effective Pollination Period culminating in a more efficient pollination.

Results showed that pollen tube had grown during all 24h until reaching a maximum length of 1,3mm, (Fig. 25). Also, this result proved that the new pollen extender improves growing rates of the pollen tube, which makes the pollination more effective.

Pollen tube growing in the control, without pollen extender, reached an average length of 0,9mm, which is a value much lower when compared to the 1,3mm that the pollen extender formula was able to obtain.

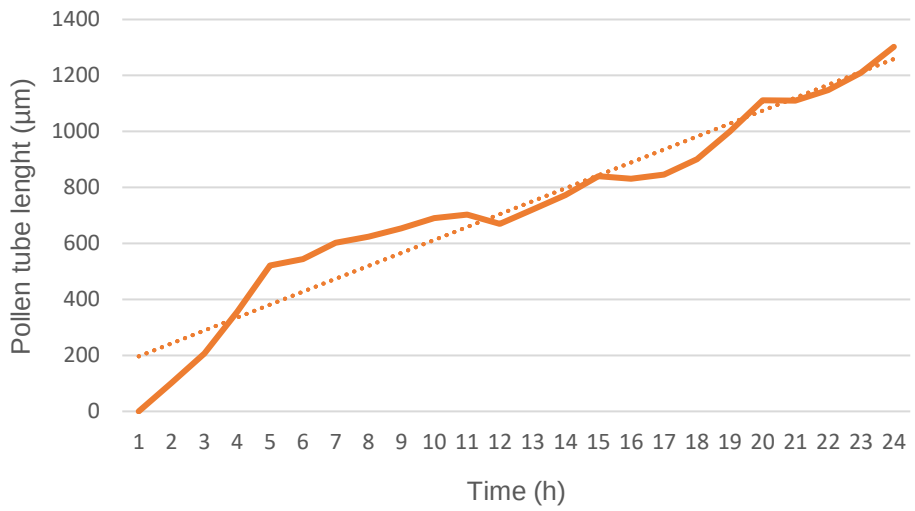

Figure 25. Effect of the pollen extender on the length of the pollen tube growth of *Actinidia deliciosa*.

3.8. Viability of pollen extender.

This experiment tested the capability of the pollen extender to be stored for a period of time.

Pollen extender was stored for 31 days before being tested. Germination ratios of the stored pollen extender (92%) did not show any statistically significant differences when compared to fresh pollen extender (p= 0.981937), proving that storing pollen extender wo not negatively impact its effect, (Fig. 26).

Stored pollen extender formula was also compared to germinating pollen without any pollen extender. Results shown statistically significant differences between these two methods, affirming that stored pollen extender still positively had an impact in the pollen germination ratios (p=0.003886) (Fig. 26).

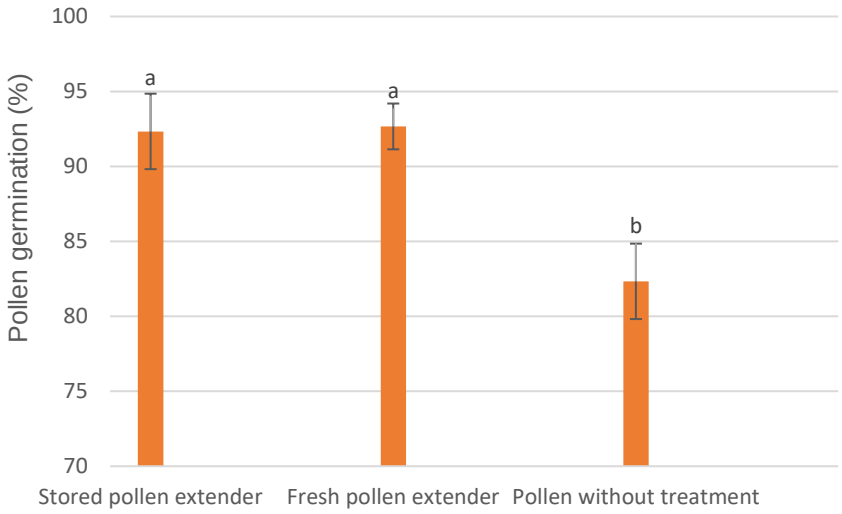

Figure 26. Effect of the treatments on the germination ratios of Actinidia deliciosa stored pollen. Pollen without any treatment was also tested. Each value represents the mean ± SD of three experiments. Treatments indicated with different letters have been shown to have statistically significant differences using Tukey's range test.

4. Discussion

In order to assess potential of kiwi pollen to germinate, pollen was collected and cultured in medium containing sucrose 6%, agar 6 g/L, boric acid 5 mg/L, calcium chloride 15 mg/l, and potassium nitrate 10 mg/l, and distilled water. This medium was previously tested and verified to successfully induce pollen germination in kiwi and other species. Pollen collected from male flowers was collected from a vine in Cantanhede. The assays showed that stored pollen was able to germinate at reasonable high rates. The rational basis for this experiment relies on previous work (Bomben *et al.*, 1999), which state that pollen stored at -80 ºC can keep a germination rate higher than 60% for more than three years. Although it may be argued that fresh pollen may still have better performance, the lack of an uneven amount may cause that a portion of female flowers can often receive an inadequate supply of pollen. Because of that, techniques on pollen storage and enhancement of artificial pollination have great emphasis.

Germination assays were carried out to identify the differences of pollen capability to germinate at different times of the anthesis. To do so, 4 different moments of development were defined: 1) young branches and flowers on the first stages of development, 2) young branches and flowers on final stages of development, 3) Old branches and flowers on the first stages of development, and 4) Old branches and flowers on the final stages of development. The purpose of this analysis was to evaluate if pollen had the same germinative potential in all stages of the plant cycle or if it's potential would either increase or decrease with time. On female plants, it is known that younger branches are the most productive ones, thus being called productive structures. Productive structures, regarding this project, refer to one-year-old branches where floral induction had already happened and that on the second year will produce fruits (Antunes, 2008). With that information in mind, we hypothesized that regarding male plants, younger branches would also have the best performance regarding pollen quality. Results showed that branches stages affect the pollen germinative potential. Pollen collected from earlier branches demonstrated higher germination ratios when compared to pollen collected from older branches. While pollen collected from younger branches was able to reach 91% on germination rates, older branches could not obtain percentages higher than 36%.

Since branch development affects pollen quality, it was obvious the necessity to study the impact of the flowering stage on pollen quality. In 1979, Hopping's research lead to the discovery that 50% of pollen produced by male flowers is released from the anther before petals are completely open. It is also known that pollen dispersion occurs continuously for 1 or 2 days after the complete opening of petals and it ends in coordination with the

senescence of the petals. It was idealized that, since most of the pollen is released in the earlier stages of flowering development, the flowering stage could influence pollen germination potential. To confirm these experiments carried out evaluated pollen germination on pollen collected from flowers in two different stages of their development. Results pointed out to an enormous difference between the germination ratios of both flowering stages. While flowers on the first stages of their development reached germination ratios of 91%, flowers on the last stages of their development reached the germination plateau at 32%.

These results confirmed the hypothesis and further research on this subject may extensively improve artificial pollination. Effective Pollination Period is a concept that has been studied for a long period of time, although, it focuses mainly on the organs directly related to producing fruits (Sanzol, 2011). An extended research on pollen produced by male flowers on *Actinidia* can lead to the development of a method to identify pollen developmental stages, which could be of very important on plantations that perform previous pollen produced by male flowers acquisition in order to carry out artificial pollination.

The germination potential of pollen produced by female flowers was also addressed in this thesis. Previous studies have recorded the unviability of this pollen. In 1967, Schroeder & Fletcher affirmed that Hayward pistillate flowers pollen is not viable and, in 2004, an investigation carried out by Coimbra et al. showed that the male sterility on female flowers of *Actinidia deliciosa* occurs after the microspores are released from the tetrads. In female flowers, microspores undergo programmed cell death (PCD), resulting on the condensation and shrinkage of the cytoplasm, changes of the plasma membrane and nuclear envelope and condensation of chromatin (Coimbra *et al*., 2004). During this thesis, pollen produced from female flowers was set to germinate under the same conditions than pollen obtained from male flowers. The results obtained coincide with the previous literature. None of the pollen retrieved from female flowers placed on the culture medium was able to germinate (Costa, 1993). This evidence introduces some questions about pollen produced by female flowers function and necessity. Although pistillate flowers pollen has little or no nutritional value for insects that collect it (Jay & Jay, 1993), it is still attractive to the pollinators, who, in case of honey bees, ca not identify its lack of nutritional value (Goodwin & Steven, 1993). Further experiments, excising the anthers on female flowers, can lead to the discovery of the real purpose of this sterile pollen.

The ability of pollen to germinate in the pistil was also evaluated using fresh pollen from male flowers, fresh pollen from female flowers and stored pollen. As expected due to the results of the previous experiment, pollen from flowers was unable to germinate, while both fresh pollen from male flowers and stored pollen were able to germinate and grow in the style.

During pollen germination along the style tract, the pollen tube growth occurs concomitantly with the consumption of a secretion abundant on the style, (Gonzalez *et al.,* 1996). This secretion is depleted when the pollen tube reaches the ovule. The precise composition of this secretion or its function it is not completely known, although its relation with pollen tube growth is clear (Gonzalez *et al.,* 1996).

The style tract is initially covered by starch reserves, as they are being consumed, are being replaced for the new secretion. Experiment that can extract this new secretion and perform a detailed analysis of its composition may lead to a new and better understanding of the pollen-pistil interaction. This information will lead to a better understanding of the mechanism used by the style tract to induce pollen tube growth which may consequently be the motor of development of a new pollen extender formula, specialized on the pollen tube needs during its growth.

In the meantime, a side investigation was performed to investigate the capability of *Actinidia* pollen to germinate *in vitro* in a liquid medium. It was presumed that the problem regarding liquid medium germinations revolved around the osmotic difference that the pollen membranes are submitted to. A solution to minimize this problem is to perform the rehydration of the pollen previous its deposition on the solution.

The Séverine solution is composed of Boric acid 0,05 g, Sucrose 50 g, and distilled water 500 ml. 3 mg of pollen were set to germinate in 4 ml of this solution. Results showed a germination ratio of 42%, much lower than those obtained in *in vitro* germination with a solid culture medium. These results can be due to the lack of nutrient on this medium. By Comparing the solid culture medium composition to the liquid medium solution, it is understandable that the liquid medium solution is quite poor on nutrients. In An attempt to maximize pollen germination ratio on a liquid medium, a new solution was tested. The solution developed on this research, named Nutrient rich solution, was composed of sucrose 6%, Boric acid 5 mg/l, Calcium Chloride 15 mg/l, Potassium Nitrate 10 mg/l and distilled water. This new solution obtained germination ratios of 63%, somewhat higher than those obtained with the Séverine solution, but still lower to those obtained with a solid culture medium.

Liquid medium has been considered an ideal technique for mass production as it reduces manual labor and facilitates the medium exchange (Teisson & Alvard, 1999), although the need for a big apparatus, which is often not easy to operate, and the unsatisfying germination ratios are unsolved problems of this method.

Several authors refer to the importance of effective pollination, in order to obtain an adequate fruit set and how natural pollination is usually not enough to achieve optimized productivities (Razeto *et al.,* 2005; Costa *et al.,* 1993; Goodwin *et al.,* 2013). Pollination in kiwi is mostly anemophilous (Intoppa & Piazza, 1990; Costa *et al*., 1993) and bees, *Apis*

melifera (Vaissiere *et al.,* 1996). In Portugal, the winter climate can be harsh for *Actinidia* species. Rimes or strong winds frequently cause major damage on the plantations, resulting in losses in the production. To avoid these problems, is common to install structures to protect the plantations. These structures will deny most of the action of the wind, leaving the pollination almost exclusively by bees (Antunes, 2008).

As it was mentioned before, pistillate flowers present a low level of attractiveness, which will limit the efficiency of the bees' pollination (Valenzuela *et al.,* 1991). Artificial pollination is essential to produce kiwifruit of marketable size and shape (Yano *et al.,* 2007), and it is commonly enhanced with the usage of a pollen extender, a mixture of a thickening agent and pollen (Yano *et al.,* 2007). This work focused on the development of a new pollen extender formula, which adds vegetable hormones to the thickening agent and to the sucrose mixture. During this work, hormones were selected and tested in order to evaluate its effect both on germination ratio and pollen tube growth.

Hopping (1976) made an investigation where different hormones were applied exogenously to identify their effect on the fruit set. This work used the same hormones and applied them in the culture medium where pollen will be germinated to verify if they had enhanced germination rates. Our results showed that the combination of 2,4-D + IBA was able to get the best payoff on pollen germination ratios, obtaining an astonishing 91% germination ratio on stored pollen. In 2014, Mohammad published an article regarding the effect of hormones on seed germination. That article was the basis to the plant growth regulators assumption of this thesis. Mohammad described the effects of the major hormonal groups on seeds. With that knowledge we choose which individual hormones and combination of hormones to apply. Also, to assure more reliable results, all hormones tested had up to 3 replicas from which 3 counts were made.

The increase on pollen germination its only as important as it is to guarantee the adequate growth of the pollen tube. Therefore, pollen tube growth was also measured and taken into consideration when choosing the hormones to use in the pollen extender.

Comparing the results on pollen tube growth, it was found that BAP+GA_3 had the longest pollen tubes reaching up to 1458 µm while the 2,4-D+IBA combination reached 1396 µm.

Considering these two hormone combinations, which obtained the best results in pollen tube growth, it's easy to understand that the 2,4-D+IBA combination is the best choice since BAP+GA_3 only presented a germination ratio of 46%. Since auxins are known to promote cell division and elongation, these results were expected.

Although there are no studies about the effect of *in vitro* germination of *Actinidia* pollen the obtained results, are not a surprising since its widely known that auxins promote cell growth.

The next reasonable step in the process of this thesis was to research for a thickening agent. At the moment, the most common thickening agent being used is *Lycopodium* powder (Oliveira, 2009). Even though Lycopodium powder has shown some good results, it is quite expensive, and a cheaper solution is needed (Yano, 2007).

In 2007, Yano and et al. tried to produce a liquid pollen extender using polysaccharides as thickening agents. That research led to the conclusion that agar, carrageenan and xanthan gum can be used as thickening agents and obtain some good results. Yano also pointed out the fact the hormonal growth factors could be added to the mixture to increase even more its effect.

During this thesis, Yano's research was discussed and continued, and one conclusion was that carrageenan 0,5% was the best option as a thickening agent. This decision relied not only on the fact that carrageenan can maintain the high rates of pollen germination, but also the fact it presents a viscosity liquid enough to be expelled via a sprayer. After all different parts of the pollen extender being singularly tested, the mixture was made and tested. The tests evaluated pollen germination, pollen tube growth and storage capability. Pollen extender tests were carried out by adding pollen extender mixture with the pollen to a culture medium and analyzing the germination ratios. Pollen extender was able to obtain germination ratios of 93 %. Comparing this result with the other treatments applied (hormones and control) we can understand observe that both pollen extender and hormones impact positively pollen germination. This fact is due to the increase on the cell division and elongation promoted by the auxins. This result not only was expected but also reinforces the idea that pollen extender might be the solution to the lack of effective pollination on this species.

Regarding pollen tube growth, it reached a maximum length of 1302 µm. This value was higher than the normal pollen tube growth obtained when pollen is set to germinate solely, concluding that pollen extender has a positive impact on pollen germination.

The stored pollen extender was able to maintain a germination ratio as high as the fresh pollen extender, even after being stored in -80 ºC for 31 days. Further tests should evaluate pollen extender effectiveness when the mixture is stored for a longer period of time.

The results of these three tests support the hypothesis that a pollen extender combined with hormones can enhance pollen germination and improve *Actinia* yields.

Field tests with pollen extender must be done, in order to assure its effectiveness *in vivo* and testing the effects of an extended application on the vines.

More so, the fact that the pollen extender can be stored can be an important factor when idealizing the marketable concept of this product. After testing its effectiveness on the field, pollen extender can present itself as a great business idea. Pollen extender would benefit not only the fruit producers that could guarantee higher productivity but could possibly

begin a new era of *Actinidia* culture, where vines would specialize in planting males in order to produce pollen for the manufacture of this product.

5. Conclusions and future work

In this thesis, we addressed the problem regarding pollination in *Actinidia sp.* The main contributions of this work were to identify when pollen produced from male flowers was more able to germinate and develop a new pollen extender formula that can boost artificial pollination.

Different types of *in vitro* germination were also tested. Even though the results showed that liquid germination is possible, the germination ratios were lower than those expected. Further investigation must be done in order to develop a liquid medium solution capable of reaching higher germination values.

This work showed that both branches' age and flowering stage affect pollen germination. Younger branches produce pollen with more capability to germinate and flowers on earlier stages of their development have pollen with higher quality. This knowledge will be of great aid, in terms of collecting pollen produced by male flowers to perform artificial pollination.

Regarding the pollen extender, this research identified that a combination of 2,4-D and IBA in concentrations of 1mg/L each promote pollen germination while also increasing pollen tube length. This factor shows great importance because, when combined with carrageenan 0.5%, acting as a thickening agent, and sucrose 2 M, the germination ratios kept being impressively higher than when pollen was applied exclusively. This increase in germination can lead to an increase in the yields production.

The evaluation of the viability of storing the pollen extender mixture was an important step to guarantee the effectiveness of this solution for a long period of time. The capability of maintaining its germination boost potential when properly stored can promote its transition into a marketable product.

On the future, field tests with the pollen extender formula can successfully verify its effectiveness and revolutionize the pollination on *Actinidia sp.* Effective pollination period it's a well-known term although, some scientific information is still lacking on this subject. A well-described method to determine when female flowers are on their peak to receive the pollen can also translate into a perfect pollination that can boost even higher the production and solve one of the main problems concerning this culture.

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