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The use of cryoprotectants in unrooted cuttings of *Pelargonium zonale*, in order to increase their life expectancy

Användning av cryoprotectants i orotade Pelargonium zonale sticklingar för att utöka deras hållbarhet

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Abstract/Sammanfattning

The rooting capacity of an unrooted *Pelargonium zonale* cutting decreases immensely 96 hours after being cut. The aim of this paper was to evaluate if two different cryoprotectants, 25% glucose and 30% trehalose, with the help of vacuum impregnation and pulse electric fields could result in an increase in the life expectancy of these cuttings. This was established by testing four different vacuum protocols were the average weight gain was noted. The optimal protocol was the first one where the average weight gain of the cutting was 17.13% when treated with glucose and was 24.75% when treated with trehalose. Pulsed electric fields parameters were optimised through trial and error were the optimum voltage was determined to be at 800V. Moreover, after treating the *P. zonale* cuttings with both vacuum impregnation and pulsed electric fields, they were stored at 6 ℃ for approximately 7 days and then they were planted in Jiffy-7 pots. This was repeated three times. Further, the root development was measured daily after approximately two weeks. The data was later analysed using a binary logistic regression where it illustrated that there was a significant difference between the untreated cuttings and the ones treated with 30% trehalose. A comparison between the two sugars conveyed that the cuttings treated with trehalose rooted better than those treated with glucose.

Målet med detta kandidatarbete var att testa och se om man kan utöka hållbarheten hos orotade *Pelargonium zonale* sticklingar med hjälp av vacuumimpregnering, puls elektriska fält och frysskyddsmedel. De två frysskyddsmedel som provades var 25% glukos och 30% trehalos. Fyra olika vacuum protokoll testades där vikt ökningen mättes. Det optimala protokollet visade sig vara det första där hela sticklingsvikten ökade med 17.13% när den behandlades med glukos, och med 24.75% när den behandlades med trehalos. Parametern för de puls elektriska fälten optimerades med hjälp av trial and error. Den optimala spänningen låg på 800V. Därefter, behandlades sticklingarna med både vacuumimpregnering samt puls elektriska fält. De behandlade sticklingarna plus några obehandlade förvarades i ungefär en vecka vid 6 ℃ i ett kylskåp. Därpå planterades de i Jiffy-7. Detta upprepades tre gånger. Rottillväxten mättes ca. två veckor efter planteringen. Det insamlade data analyserades med hjälp av en binär logistik regression där man såg en signifikant skillnad mellan de obehandlade och de som hade behandlads med trehalos. En jämförelse mellan de två behandlingarna visade att de sticklingarna som hade behandlads med trehalos hade rotad sig betydligt bättre än de som hade behandlads med glukos.

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1. Introduction

Bedding plants are any herbaceous plant that is grown under controlled environmental conditions and is onwards sold to either home gardeners or landscapers for outdoor use (Carlson & Johnson, 1985). A commonly grown bedding plant is a geranium. According to Miller, (1996) "the genus *Pelargonium* includes well over two hundred distinct species". There is a vast variety in the anatomy, morphology and taxonomy of the *Geraniaceae* family where the *Pelargonium* genus is included and thus this diversity leads to extraordinary variations.

They are perennial herbs that have plenty of trichrome on both their leaves and stems. This is an advantage for the plant as it can act a defence mechanism against an insect or a pathogen attack. Additionally, they inhibit water loss and increase the plant's tolerance to abiotic stress (Yang & Ye, 2013).

In any production system, there a number of different variables that need to be taken into consideration and some of which can limit the capability of meeting consumers' expectations. The propagation of fresh *Pelargonium zonale* cuttings is not without its fair share of problems.

About a year ago word about a company by the name OptiFreeze AB came into the picture. They have a patented method where with the help of cryoprotectants, vacuum impregnation and pulse electric fields the freezing tolerance of leafy greens have been improved immensely (Anon., n.d.). An example is their success with freezing rucola and still having viable cells after thawing. This project started with the hopes of being able to increase the freezing tolerance of unrooted *Pelargonium zonale* cuttings with the aid of cryoprotectants. As time went by, it became clear that in this limited time span much needed to be accomplished. After contemplating it was decided that it would be best to test if there were alive cells after treatment and freezing. This was achieved, partially, though with a few difficulties. The problem was that the after thawing the cutting would lose all its liquids and as such would mould when planted. This indicated that the cells were dying and thus lysis was occurring. This changed the course of the project. The aim went from increasing the unrooted cuttings freezing tolerance to increasing their shelf life without freezing.

2. Aims

The aim of the project was to see if it was possible to increase the life expectancy of unrooted *Pelargonium zonale* cuttings with the aid of vacuum impregnation (VI), pulse electric field (PEF) in combination with different cryoprotectants. Additionally, it is to try and keep the cutting's cells alive after a longer period in a refrigerator. By testing different parameters and treatments, their cells should still be viable after a week in the refrigerator and root after being planted.

2.1 Research question

Can the life expectancy of unrooted *P. zonale* increase with the aid of VI, PEF and cryoprotectants?

2.2 Implementations & limitations

In order to achieve the aims of the project, there are several objectives that needs to be met. Firstly, there is a need to optimise the VI and the PEF parameters. There is a need for the optimisation of the sample handling before and after pre-treatments as to their storage conditions. Lastly, there needs to be a combined application of the VI and PEF prior to storage and checking of the viability of the tissue.

As in any project, there are a few limitations that need to be accounted for. Time is always a limiting factor and being that this is a bachelor thesis; there might not be enough time to carry out as extensive tests as needed. Another limitation is that of the ageing of the cuttings. From the moment that they are cut from the mother plant their rooting capability decreases after 96 hours (Johnsen, 2017). This means that if the cuttings are cut in Africa on Sunday they must be planted the latest on Thursday. There is also a risk that this method might not be applicable on *P. zonale* cuttings.

3. Background

3.1 The genus

One way to describe *Pelargonim* as stated by Berninger (1993) is that it is has been one of the backbones of the bedding plant business for decades. There is a tradition that comes with *Pelargonium*.

> The pelargonium is a plant for the crowded future, for the gardens of today and the windowsills of the world where gardening will live on, in an age of towering flats and tiered roads, where time is money and too much of this is need not to be devoted to getting a striking display of colour over a long period; it is the flower that will cover the crematoriums of the world in years ahead, it has already begun this brilliant infiltration into parks and cemeteries where the magnificent colours predominate all summer long. Where else can such beauty be so abundantly found? (Wood, 1966)

They are perennial herbs with a vast variety of different leaf formations and types (Dole & Wilkins, 1999). The shrubbery can vary immensely as it may be a combination of greens, whites, ivories, bronzes, yellows and reds. Flowers can be arranged in umbels and can be either single or double (Dole & Gibson, 2006).

3.2 Anatomy and morphology

The genus *Pelargonium* consists of a vast array of different cells were the epidermis cells form the thumping majority. In figure 1 the epidermis is primarily visible as the utmost cell layer; further in the cross-section can one see an array of cells. Its principal function is to act as a protective shield against a variety of disease vectors as well as from drying out (Adams, 1971).

There is a substantial difference between the epidermis covering aerial parts and that which covers the roots; as the latter is more permeable to water and thus thinner in comparison to the aerial one. Furthermore, “appendages of epidermis are called trichomes and appear in a variety of shapes ranging from quite hair-like projections to […] large enough to be viewed with the unaided eye” (Adams, 1971).

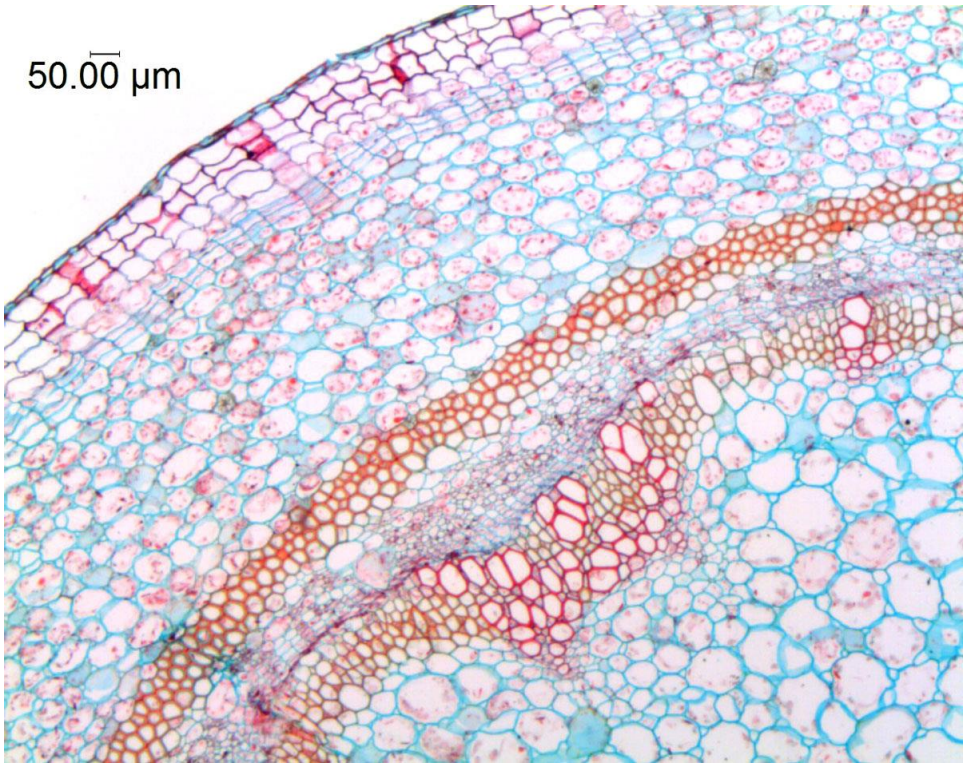

Figure 1: Cross-section of a *Pelargonium zonale* stem. Credit Nickrent, D.L., Costea, M., Barcelona, J.F., Pelser, P.B. & Nixon, K. (2006 onwards) PhytoImages. Available from: http://www.phytoimages.siu.edu

According to Adams, (1971) *Pelargoniums* are characteristically pubescent, i.e. covered with epidermal hairs. Everything from the leaves to the young stems and even some flower parts are covered by them. There are two types of epidermal hairs. Some act as protective hairs whereas others are glandular hairs. As stated by Rajeswara, (2002) it is due to their glandular hairs that some *Pelargonium* varieties make it possible to produce expensive oils used in amongst other in perfumes, flavour and pharmaceutical industries.

Stomata cells are another group of specialised epidermal cells. There are a variety of stomata types that regulate an assortment of things amongst other leaves' transpiration rates. The prime function of stomata is to adjust leaves diffusive conductance (Buckley, 2005). This, in turn, influences their carbon gain and water loss.

According to Buckley, (2005) "reducing atmospheric humidity shifts hydraulic balance towards demand, which reduces leaf water status; however, stomata respond by reducing their apertures, which restricts water loss and mitigates the potential decline in water status". This means that on a warm sunny day the stomata tend to be shut and thus reduce water loss; whereas had it

been humid and cloudier they would be open. There are certain plants' stomata which open only during the night hours, so to reduce water losses during the day due to transpiration.

Moreover, *Pelargonium* leaves are mostly hypostomatic; meaning that they are located on the abaxial surface which is the lower surface (Adams, 1971). The primary root body of *Pelargoniums* consist mainly of epidermal hairs and are cells of utmost importance. *Pelargoniums* have an awfully efficient root system due to the fact that it produces vast numbers of small lateral roots which consequently result in functional root hairs.

The leaf tissue consists of an array of cells and as such the mesophyll of leaves is the most photosynthetic tissue (Taiz, et al., 2015). Additionally, the leaf is the "most highly variable vegetative part of a plant", it can rapidly respond to environmental changes (Adams, 1971, p. 7). *Pelargonium* leaves tend to be morphologically stable and as such the adaxial (upper) surface tends to have a thicker epidermis in comparison to the abaxial (lower) surface.

3.3 Production of unrooted cuttings

Vegetatively propagated perennials, bulbs, trees and shrubs are a custom, as they are exact clones of the mother plant (Brown, 2002). *Pelargoniums* are mostly propagated by vegetative cuttings but *Pythium* and *Botrytis* infections can cause serious damage (Hartmann, et al., 2002). Other alternative ways include propagation through seeds; however, "the germination may be erratic" (Hartmann & Kester, 1968, p. 679). This though not the norm though landscapers prefer them for mass plantings (Oglevee, et al., 1998).

According to Ogelevee, (1998) "the ideal [*P. zonale*] cutting for propagation is a 2- to 3-inch (5-8 cm) terminal with an active growing tip and no physical damage". Growers prefer terminal cuttings as they finish two weeks earlier than leaf bud or heel cuttings. In figure 2 one can see a *P. zonale* terminal cutting. A terminal cutting is one that has been directly attached to the mother plant i.e. a side shoot. It is of the highest importance that any kind of knives or clippers used for cutting the cuttings are sharp so not to leave any jagged edges. It is also of importance that all utensils used are disinfected and changed every 10 minutes (Oglevee, et al., 1998).

Figure 2: A terminal *P. zonale* cutting. Photograph: Maria Hellström.

A production cycle in Africa begins with the planting of the mother plants. This is usually done in week 30 and the first cuttings are taken during week 45. Between weeks 30 and 45 all the mother plants are topped so to produce as many side shoots as possible. The cutting season is between week 45 and continues until the end of week 15. Furthermore, the peak of the season is between weeks 52 and 8, where an approximately 70% of all *P. zonale* cuttings are propagated (Johnsen, 2017). Each mother plant produces 2 – 2.5 cuttings per week.

In a year, approximately 400 – 500 million *Pelargonium* are cultivated from cuttings and then sold onwards in Europe; where 50% of this are *P. zonale*. 90% of the *P. zonale* are produced in Africa (Johnsen, 2017). Before the processing of the propagation begins, cuttings are stored in refrigerators at temperatures between 4-8 ℃ for one or two days (Hartmann, et al., 2002), before they are onwards transported to the rooting nurseries. A way to access the quality of an unrooted cutting is whether it roots after planting. Also, another way is to see how quickly it develops visible roots; when using paper pots.

3.4 Problems

The biggest issue is that once cut, a *P. zonale* cutting has a life expectancy of 96 hours; i.e. the rooting capacity of an unrooted P. zonale cutting decreases immensely 96 hours after being cut (Johnsen, 2017). Another factor that adds to this issue is that 90% of *P. zonale* cuttings are produced in Africa, see figure 3. This means that once cut they need to be packed and stored in a refrigerator between 2-5 ℃ (Johnsen, 2017). Soon after they are flown to the Netherlands

where upon arrival they are distributed to brokers who see to them reaching their destination. During this, it is vital that the transportation chain remains intact, so not to risk spoiling the cuttings.

The cuttings used in this thesis were flown from Kenya on Sunday evening/ Monday morning and where in the Netherlands on Monday, see figure 3 illustrating how many kilometres the *P. zonale* cuttings have travelled. On Monday, they were then loaded onto a truck and driven up to the south of Sweden. They were then received on Tuesday afternoon/night. It takes approximately 72 to 96 hours from the moment they are cut to when they are received by a customer (Johnsen, 2017). This is reflective of the importance of getting the cuttings in time for the plane, truck etc. If they were to be delayed for a day or so this may lead to losses.

As illustrated in the above section 3.3 Production of unrooted cuttings; mother plants are planted week 30 and the first cuttings are cut week 45. In order to build up the mother stocks, there is approximately a 30% overproduction of cuttings. This is to be able to produce the maximum amount possible during the peak of the season (Johnsen, 2017). This results in wastage. One should consider the fact that there is a non-seasonal dependency. This highlights the importance in developing new methods that may lead to an improved transportation mean or in new cultivation techniques. A common concern during transport is maintaining the cold chain (Friedman & Rot, 2005). This is as the temperature can fluctuate between the transits and may result in losses.

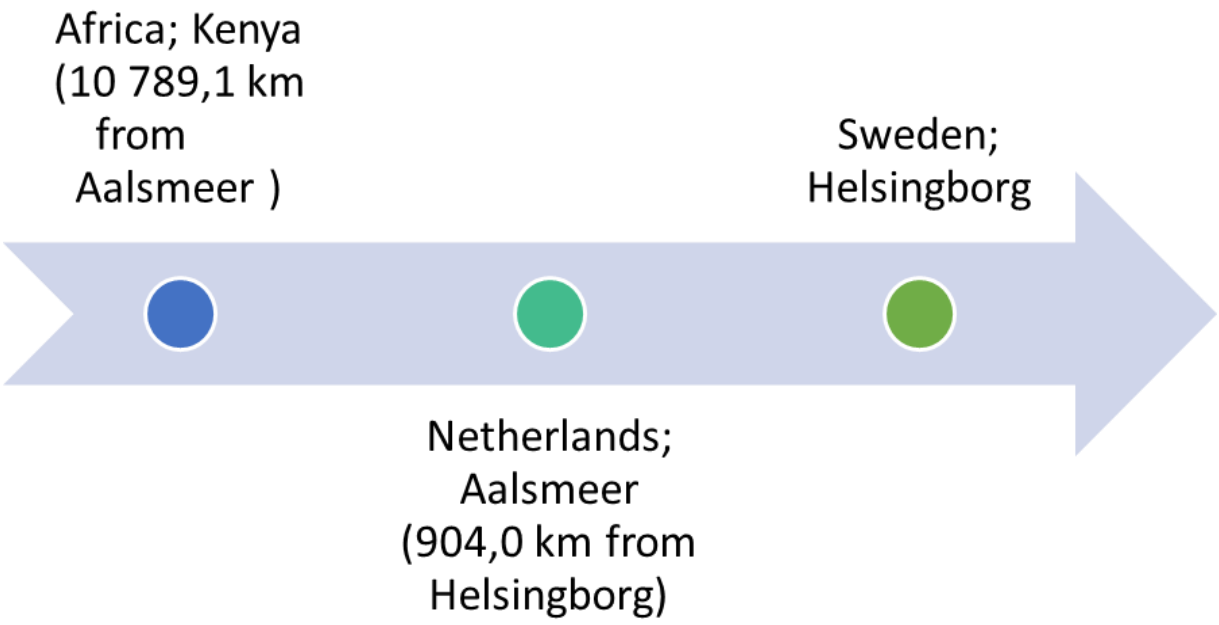

Figure 3: A schematic diagram showing the distance *P. zonale* travel. Distances calculated by Google Maps, Google Inc.

3.5 Cryoprotectants

Since the late nineteenth century and early twentieth century, plant physiologists have studied the nature of freeze injuries and have investigated how changes occur during freezing, freezing storage and thawing of plant tissues (Joslyn, 1966).

Cryoprotectants are substances that aid cell structures during cooling and freezing. This is due to water inside the cells expands in the form of ice crystals upon freezing and thus leads to cell death. They increase the total concentration of all solutes in any given system. This results in a reduced amount of ice crystal formation; “but to be biologically acceptable they must be able to penetrate into the cells and have low toxicity” (Pegg, 2007).

Per definition, cryopreservation is when small molecules enter cells so to prevent the formation of intracellular ice crystals and dehydration which can occur during freezing (Yawn, 2010). Common cryoprotectants are glycerol, dimethyl sulfoxide, ethanediol, propanediol and trehalose (Pegg, 2007; Beattie, et al., 1997). Additionally, cryoprotectants may also have a role in the metabolic activity of the cells and therefore can make the cells last longer.

3.6 Vacuum impregnation (VI)

Vacuum impregnation (VI) is a common technique used to introduce external solutions into porous structures. It has a wide spectrum of use such as in the fruit and vegetable industry (Zhao & Xie, 2004). It all begins with the immersion of the porous material into a liquid of choice and upon immersion, it is subjected to a decrease in pressure and then an increase in pressure.

Firstly, the pressure decreases so to force out any gas from the pores of the material immersed in the liquid. This is done “until mechanical equilibrium is established” (Dymek, 2015). The second step consists of restoring the atmospheric pressure. Fito et al., (1996) described that this is due to an action of a hydrodynamic mechanism, the encapsulated material’s pores are filled with the external liquid. Depending on “pore size, surface tension of the liquid and the wetting angle between” the pores are affected by capillary pressure (Tylewicz, et al., 2012).

This means that depending on the plant's cell structure a longer period of time might be necessary or a lower pressure so to impregnate it. Four different protocols with varying times and pressures were tested during the course of this thesis.

3.7 Pulse electric field (PEF)

High-intensity pulsed electric field (PEF) processing is when a product such as a *P. zonale* cutting is placed in a medium between two electrodes and then pulsed at a certain voltage. According to Barbosa-Canovas, et al., (2000) “PEF treatment is conducted at an ambient, sub-ambient, or slightly ambient temperature for less than one second and thus energy loss due to heating of [the product] is minimised”.

When using PEF it results in the electroporation of the cell membrane and therefore it becomes permeable (Dymek, et al., 2015). This, therefore, allows molecules that under other circumstances would be unable to penetrate the membrane pass into the cell (Dymek, 2015). There is a variation of pulses that may be used during PEF. As reported by Barbosa-Canovas, et al., (2000) they may ”be applied in the form of exponentially decaying square wave, bipolar, or oscillatory pulses”. In this thesis, solely bipolar pulses were used.

Continuing, the difference between the three types are that the square pulses are more energy efficient though they are lethal. The least efficient pulses are the oscillatory decay pulses due to them stopping the cell from a continuous exposure to a high-intensity electric field over a prolonged period of time; leading to the prevention of an irreversible breakdown of the cell membrane (Barbosa-Canovas, et al., 2000). Irreversible breakdown of the cell membrane is used in the food processing industry as it inactivates microorganisms (Dymek, 2015; Everndilek, et al., 1999); though it kills the cells and therefore is not optimal if the aim is to keep the cell viable. Bipolar pulses enhance the cell membranes electrical breakdown as they produce alternating changes in the movement of charged molecules and thus cause a stress in the cell membrane (Cueva, 2009).

Electroporation may be reversible. This is when the pores which were induced by the PEF under a very short period of time, are allowed to reseal (Benz & Zimmermann, 1981). As it has been shown by Kotnik, et al., (2001) when using bipolar pulses a lower concentration of metal ions were found in the solutions tested when compared to unipolar pulses having the same amplitude and duration. It was the electrodes that had released the metal ions.

3.8 Chilling injury

According to Purvis (1999), there is direct evidence that soluble carbohydrates increase plants resistance to chilling injuries. This has been proven in studies involving the application of exogenous carbohydrates to plants and there upon been exposed to a chilling period. An example is that of rice seedlings treated with glucose and fructose prior to their chilling, were a higher percentage of those who were treated survived the chilling (Tajima & Kabaki, 1981).

A direct effect of low temperature is the alteration of several biochemical and physiological processes in the plant (Lyons, 1973). " Thus, prolonged chilling leads to metabolic imbalances, enzyme inactivation, membrane damage, and eventual cell death" (Purvis, 1999, p. 214). This is why sensitive plants can benefit from soluble carbohydrates; as they can directly or indirectly protect the cellular constituents of these plants (Purvis, 1999).

As stated by Levitt (1966), a plant is winter hardy if it has properties that aid it in surviving harsh severities of winter. Furthermore, it has been suspected that a plant's survival may be linked to its ability to avert excessive water loss from its extremities as it cannot translocate due to other parts being frozen.

4. Materials & method

4.1 Plant material

Each week 100 new *P. zonale* 'Classic Dolce Vita' cuttings were received from Syngenta; Flori Pro Services. They were all harvested in Kenya and were flown into the Netherlands. Upon arrival, they were then transported by truck to the South of Sweden. The first instalment was received in week 4 and the last was in week 14, 2017. In average the weight (g) was 2.3 ± stdv g per cutting and had a height (cm) of 8.7 cm. This was calculated using 50% of the cuttings received during week 14. Once received they were stored in a refrigerator at 6-7°C.

4.2 Isotonicity

It all began with the determination of the isotonic solution for the *Pelargonium* cuttings using different cryoprotectants. One of the important factors in VI is the selection of the tonicity of the solution (Demir, 2012). There is a variation in the solute concentration within and outside the cell membrane depending on the solution. Conforming to Zhao & Xie, (2004) if it is a hypertonic solution then the solute concentration is higher in the solution than in the cell membrane; the opposite occurs when it is a hypotonic solution. When it is an isotonic solution then both the solute concentration is equal on the inside and the outside of the cell membrane (Zhao & Xie, 2004).

Figure 4: Beakers filled with different sugar concentrations with immersed *Pelargonium* cuttings. Photograph: Maria Hellström.

Additional tests were conducted for 15% and 20% sucrose and 40% trehalose.

Different concentrations were made ranging from 0% to 30%, see figure 5. Six beakers were filled with 400 ml tap water. Each cryoprotectant was carefully weighed with an analytical balance and mixed in the water using an agitator. In total six different cryoprotectants were tested. D-mannitol 99%, D-fructose 99%, D-(+), glucose 99%, sucrose 99% were purchased from Alfa Aesar, Thermo Fischer, Germany. The trehalose, used was from Cargill Deutschland GmbH and lastly, the glycerol 99,5%, was from Creme Glyc Refined, Hamburg, Germany. Two cuttings were placed in each solution. Their weight was recorded with an analytical balance prior to their immersion. They were weighed after one hour, two hours and the next day (kept in a refrigerator overnight). The weight gain/loss was recorded and using excel could an isotonic solution for each cryoprotectant be calculated.

4.3 Vacuum impregnation (VI)

The treatments were conducted using 30% trehalose (Tre) and 20% glucose (Glu). The two sugars and their concentrations were determined after conducting the isotonic tests. In total four different vacuum impregnation protocols were tested, see figure 6. Before each test, each *P. zonale* cutting was weighed carefully using an analytical scale. Each treatment required three cuttings, three stems and three leaves. They were weighed after each treatment and a percentage increase was calculated. Plastic nets were used in the beakers so to keep the cuttings from floating in the beakers.

In figure 5 can the experimental setup for the VI be viewed. A beaker filled with 400 ml of a cryoprotectant, cuttings and green plastic nets, can be seen in figure 6, to the far right.

Figure 5: The VI setup at OptiFreeze. To the far left is the computer which is connected to the vacuum pump and on the floor, is the chamber. Photograph: Maria Hellström.

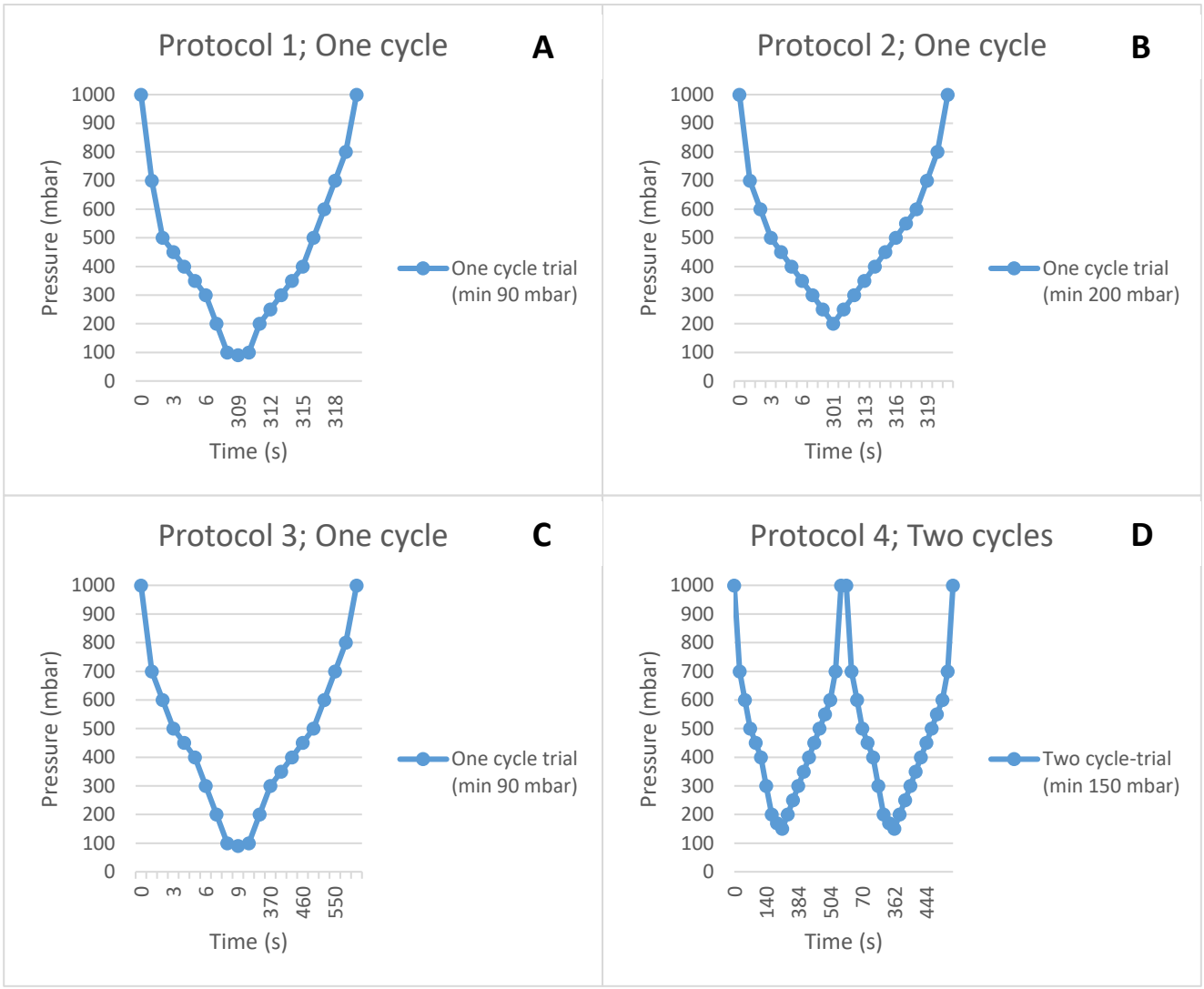

Figure 6: Four different protocols; times and pressures that where tested for the impregnation of *P. zonale* cuttings. *min: minimum

The first protocol is shown in figure 6A. The pressure gradually decreased from 1000 mbar to 90 mbar in 5 minutes (min), kept at 90 mbar for 5 min, then it gradually increased to atmospheric pressure for 2 min and kept at atmospheric pressure for 2 min. In total 14 min were needed to complete the first protocol. The second protocol is displayed in figure 6B. The pressure gradually decreased from 1000 mbar to 200 mbar in 4 min, kept at 200 mbar for 5 min, then it gradually increased to atmospheric pressure for 3 min and kept at atmospheric pressure for 2 min. In total 14 min were needed to complete the second protocol.

The third protocol can be viewed in figure 6C. The pressure gradually decreased from 1000 mbar to 90 mbar in 5 min, kept at 90 mbar for 5 min, then it gradually increased to atmospheric pressure for 12 min and kept at atmospheric pressure for 2 min. In total 24 min were needed to complete the third protocol. The fourth protocol is illustrated in figure 6D. The pressure gradually decreased from 1000 mbar to 150 mbar in 11 min, kept at 150 mbar

for 1 min, then it gradually increased to atmospheric pressure for 7 min and kept at atmospheric pressure for 13 min. Then the cycle repeated itself. In total 64 min were needed to complete the fourth protocol.

After each treatment was done, each leaf, stem and cutting were gently blotted with a piece of paper. There upon, they were weighed again using an analytical scale and an average weight gain (%) was calculated. The most appropriate protocol was deemed to be P1 because average weight gain (%) was the highest in comparison to the other ones.

4.4 Optimisation of PEF Parameters

The optimisation of the PEF protocol for non-impregnated and impregnated cuttings began by measuring the electrical conductivity of propidium iodide (PI) so that it was 250 μS. The PI was used as a control for electroporation as it stains the nuclei (Demir, 2012), and so was used to see if the plant cells had resealed or if they had died after being electroporated, with the help of a microscope.

During the optimisation of the PEF parameters the chamber used was 10cm*5cm*0.5cm. Where it was length*width*gap. Different voltages were tested from 100-1000 V and 1500 V, in 100 V intervals. Two cuttings were used for each trial. The parameters used were experimentally obtained to have an optimum effect in plant cell reversible permeabilization in the previous studies at OptiFreeze; pulse width: 100 μs, pulse space: 1000, the number of pulses: 50, trains: 1, bipolar pulses and the machine used was Cept® ArcAroma Pure, Lund, Sweden.

Additionally, another twelve cuttings were used to determine which voltage to use. The two cuttings were placed in a conductive solution at 250 μS and were treated at 100 V, 600 V, 1500 V and 2000 V. Then the eight treated ones, the two negative controls and the two positive controls were placed in lunch boxes overnight. Later next day they were immersed in a fluorescein diacetate (FDA) (Sigma-Aldrich, 2017) solution for approx. 15 min. The FDA is a cell viability assay which is used see if the electroporation if reversible (Demir, 2012). Before immersing the cuttings into the FDA, a slight cut was made at their base so to make it easier to see the cell structure.

4.5 Combination of VI and PEF

After concluding that the optimal VI protocol was the first one and that the optimal PEF parameters were at 800V saw the start of the self-life extension phase. In total forty (40) *P. zonale* cuttings were treated each week, for three weeks. Twenty were immersed in 30% Tre and twenty were immersed in 25% Glu. First, they were treated using the first VI protocol. After their treatment, they were immediately blotted on four layers of paper. Thereupon, they were electroporated at 800V, in a conductive solution at 250 μS using the parameters stated in 4.4. The PEF chamber used was (30cm*30cm*2cm). They were blotted once more and then placed in closed plastic boxes where the saturated environment was ensured with a wet tissue. They were stored in a refrigerator at 6 ℃; the next day they were placed in closed (grippie) bags. This was repeated for three weeks.

4.6 Storage, planting and rooting of treated cuttings

After being placed in the plastic bags they were transported from Lund to SLU, Alnarp, were the treated and control cuttings were stored in a refrigerator at 6.2℃ and 66.5% RH for a week. Thereupon they were planted in a single glassed venlo greenhouse that had a temperature of 18- 20℃.

Untreated controls were planted the next day after each shipment, to check to see their viability and root ability. One week after each treatment both treated (20 pieces) and untreated (10 pieces) *P. zonale* cuttings were planted in Jiffy-7 paper pots, Jiffy Products International BV, Netherlands. They were planted so that they had one space between one another. Under each tray there was a thick white horticultural textile, to keep the moisture. Each planted tray was covered with clear plastic which had holes. They were covered until 50% of all the plugs had visible roots.

Directly after planting they were heavily watered. They were watered twice a day the first four days after planting and then watered once a day.

4.7 Statistical tests

After approximately two weeks' after planting the root measurements began. Each plug was thoroughly checked and a scale of; 0, 1-5, 6-10 and >10; was used in the taking of the measurements. The treating, planting and measuring was repeated three times.

All statistical tests were conducted using Minitab 16, 2010, Minitab Inc.; were a binary logistic regression was used. The data used was from the 25th day after planting from all the three different weeks and respectively the three repetitions. The binary logistic regression compared if there was a significant difference between the control and the two treatments. The values from 0-5 were added together and everything that was more than 6 was added together.

5. Results

5.1 VI

Table 1 demonstrates the results from the four different VI protocols tested. The average weight gain (%) is presented. After looking at the data it was determined that the most optimum protocol was number 1; as the average weight gain (%) was the highest in comparison to the other ones, see table 1.

Table 1: Average weight gain (%) after testing all four protocols. All had one cycle, except for protocol 4 which had two cycles. *L= only the leaf used, S= only the stem used

	Protocol (P)				
	1	2	3	4	
Glucose 25%	17.13	4.72	11.09	12.89	5.60
Glucose 25% (L)	12.72	11.83	10.04	13.20	3.80
Glucose 25% (S)	8.68	2.79	5.53	7.11	3.37
Trehalose 30%	24.75	13.29	15.00	13.63	10.09
Trehalose 30% (L)	13.65	15.11	16.45	22.96	8.54
Trehalose 30% (S)	9.79	7.33	4.79	6.95	4.57

5.2 PEF

A visual observation was made on the hardness of the stems and the hardest ones were those treated at 600 V and the softest were those treated at 2000 V. This showed that the higher voltage had caused irreversible damage to the cell structure. After a closer inspection under the microscope, this was proven to be right.

After looking under the microscope one could see that over 1000 V there were little to none cell nucleuses visible, see figure 7. This illustrates that the voltage was far too high and thus there was no impregnation occurred. Also, by looking at picture B one can see that there was positive electroporation; whereas in picture A there are minimal signs of viable cells, see figure 7. After this test, it was decided that the voltage needed is between 650-900 V. Further testing was conducted and it was decided that the most appropriate voltage was at 800 V.

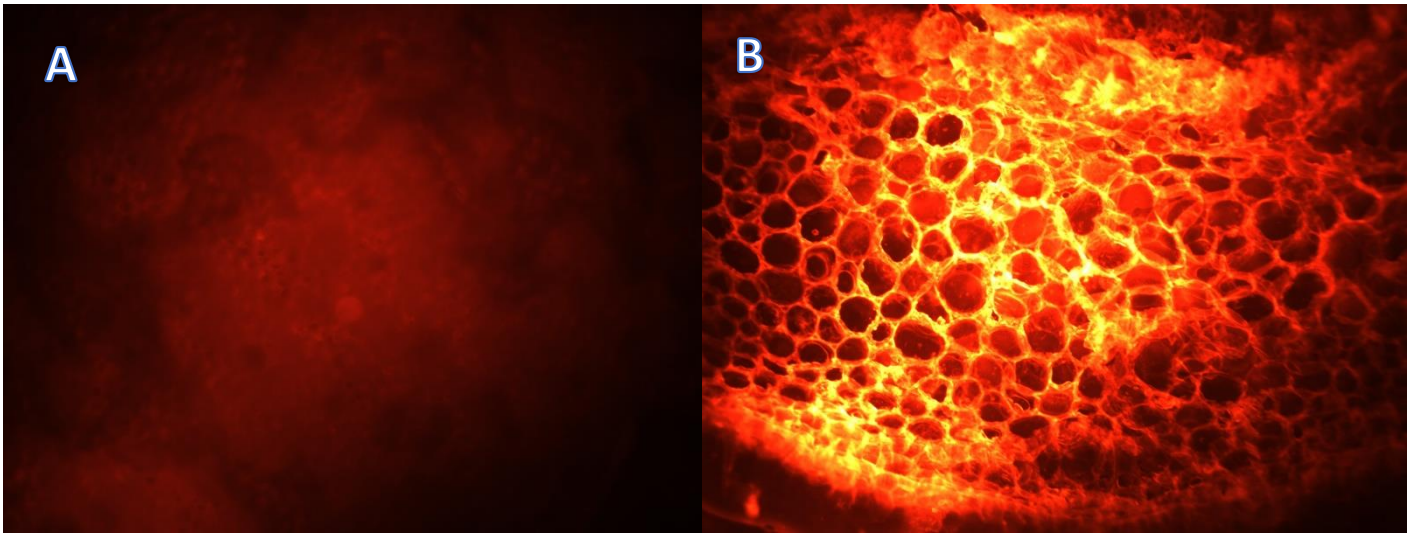

Figure 7: Microscopic pictures of a cutting after PEF. In picture A is at 1500 V and picture B is a cutting after 800 V. Taken 10/2/17.

5.3 Measurements

Measurements were taken at different intervals after the planting of the untreated (cont.) and the treated cuttings. In the figures below can the measurements that were taken be seen. The columns are representative of the amount of rooted or unrooted cuttings (%). In table 2 one can observe when each the unrooted cuttings were received from Kenya and when the untreated control and the treated ones were planted.

Table 2: When the unrooted cuttings were received and planted.

Week:	Received:	Planted:
12	23/3	31/3
13	31/3	7/4
14	5/4	18/4

5.4 Statistical tests

The 25th day is circled as it is those values that will be used in the statistical analysis. The colour blocks represent the number of roots visible through the Jiffy-7 pots, see figures 9-11. After conducting a binary logistic regression, see table 3, it was conveyed that there was a significant difference between the untreated ones when compared to the ones treated with 30% Tre. This is because the p-value was at 0.001.

Moreover, by comparing the untreated ones to the ones treated with 25% Glu, no significant difference could be proven as the p-value exceeded the 5% limit, see table 3. A third comparison was made between the two treatments where it showed that there was a significant difference between them; as the p-value was at 0.011. This proved that the cuttings treated with 30% Tre had rooted better than the ones treated with 25% Glu, after 25 days of being planted. Additionally, by comparing the constant coefficients with the coefficients for each comparison made, the same conclusions could be drawn, see table 3.

Additionally, by using this test a comparison of the two treatments was made. The results are conveyed in table 3.

Table 3: Results indicating the p-value and the coef. from each comparison made using binary logistic regression.

Control vs Glucose	P: 0.212	Coef.: 0.583225	Constant Coef.: 0.726278
Control vs Trehalose	P: 0.001	Coef.: 1.79436	Constant Coef.: 0.726278
Trehalose vs Glucose	P: 0.011	Coef.: -1,21114	Constant Coef.: 2.52064

5.5 Graphs from the measurements

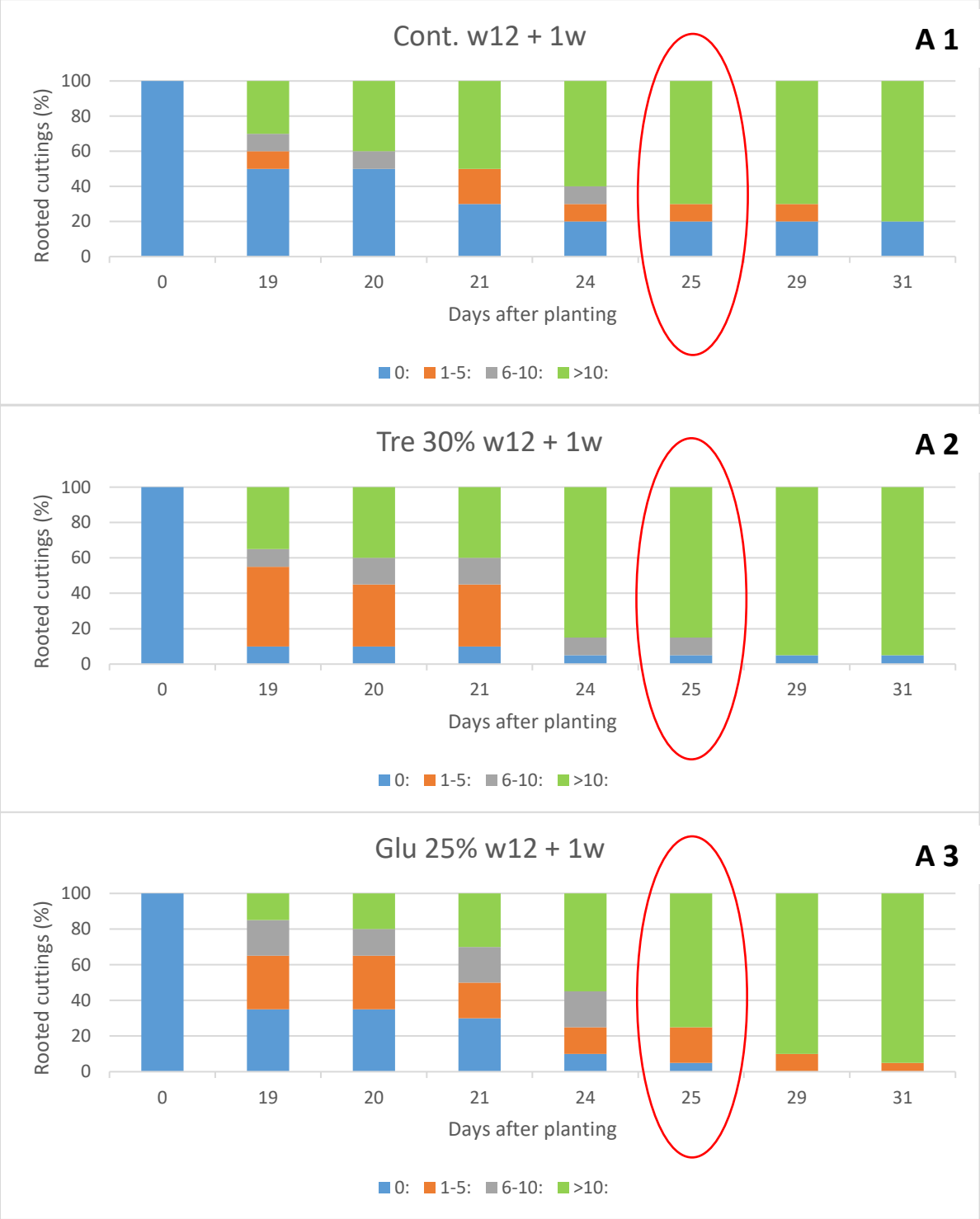

Figure 9: Three graphs, cuttings received w12 and planted w13, illustrating the percentage of rooted *P. zonale* cuttings. A 1: Are untreated cuttings. B 1: Are cuttings treated with 30% Tre. C 1: Are cuttings treated with 25% Glu.

In figure 9, A1; can be seen that 20% of the cont. cuttings did not root; whereas in A2; a total of 5% did not root. In A3, 30% of the cont. cuttings planted in the beginning of w16 did not root.

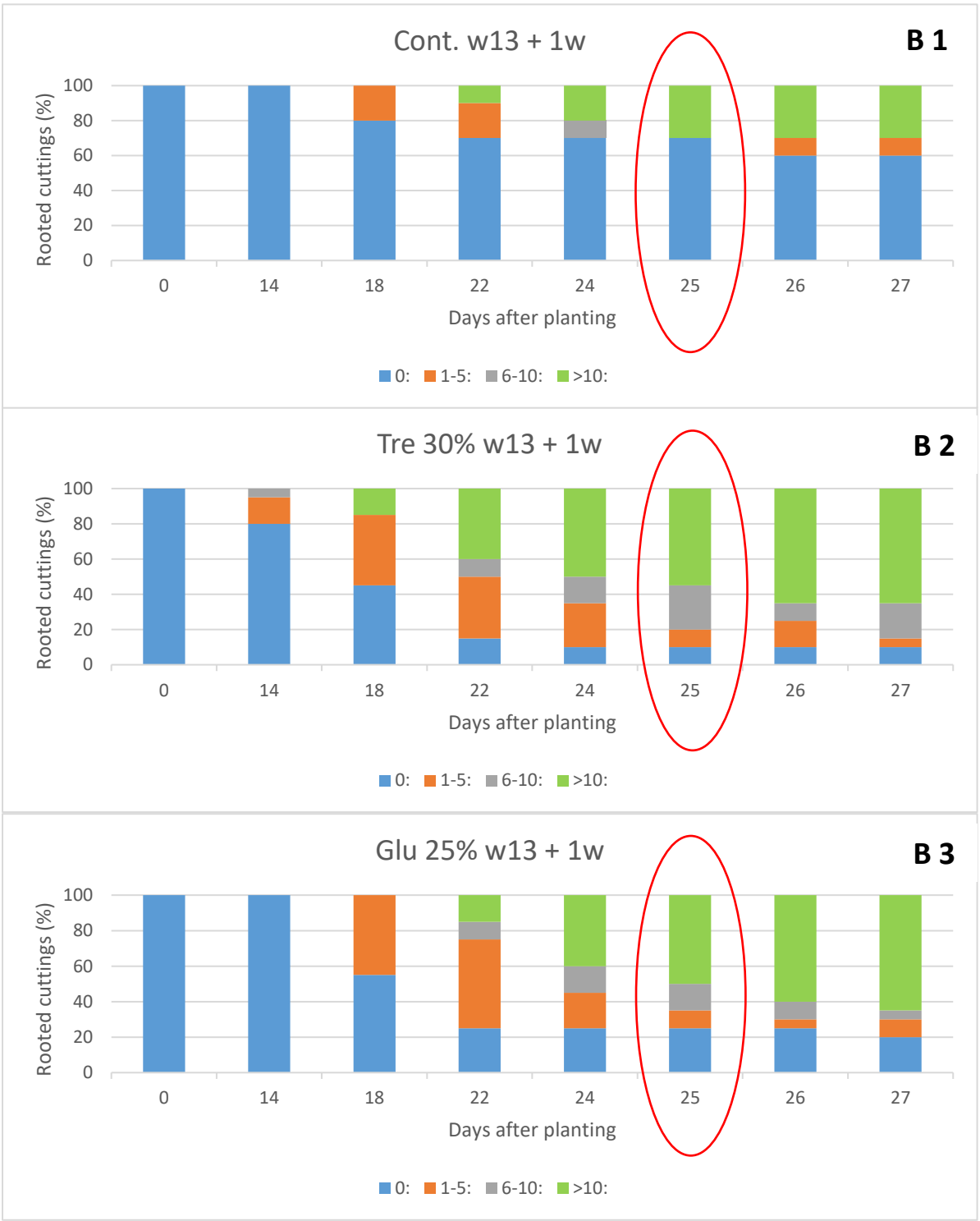

Figure 10: Three graphs, received w13 and planted w14, illustrating the percentage of rooted *P. zonale* cuttings. B1: Are untreated cuttings. B2: Are cuttings treated with 30% Tre. B3: Are cuttings treated with 25% Glu.

In figure 10, B1; can be seen that 70% of the untreated cuttings did not root; whereas in B2; a total of 10% did not root. In B3, 15% of the cuttings treated with 25% Glu, 25% did not root.

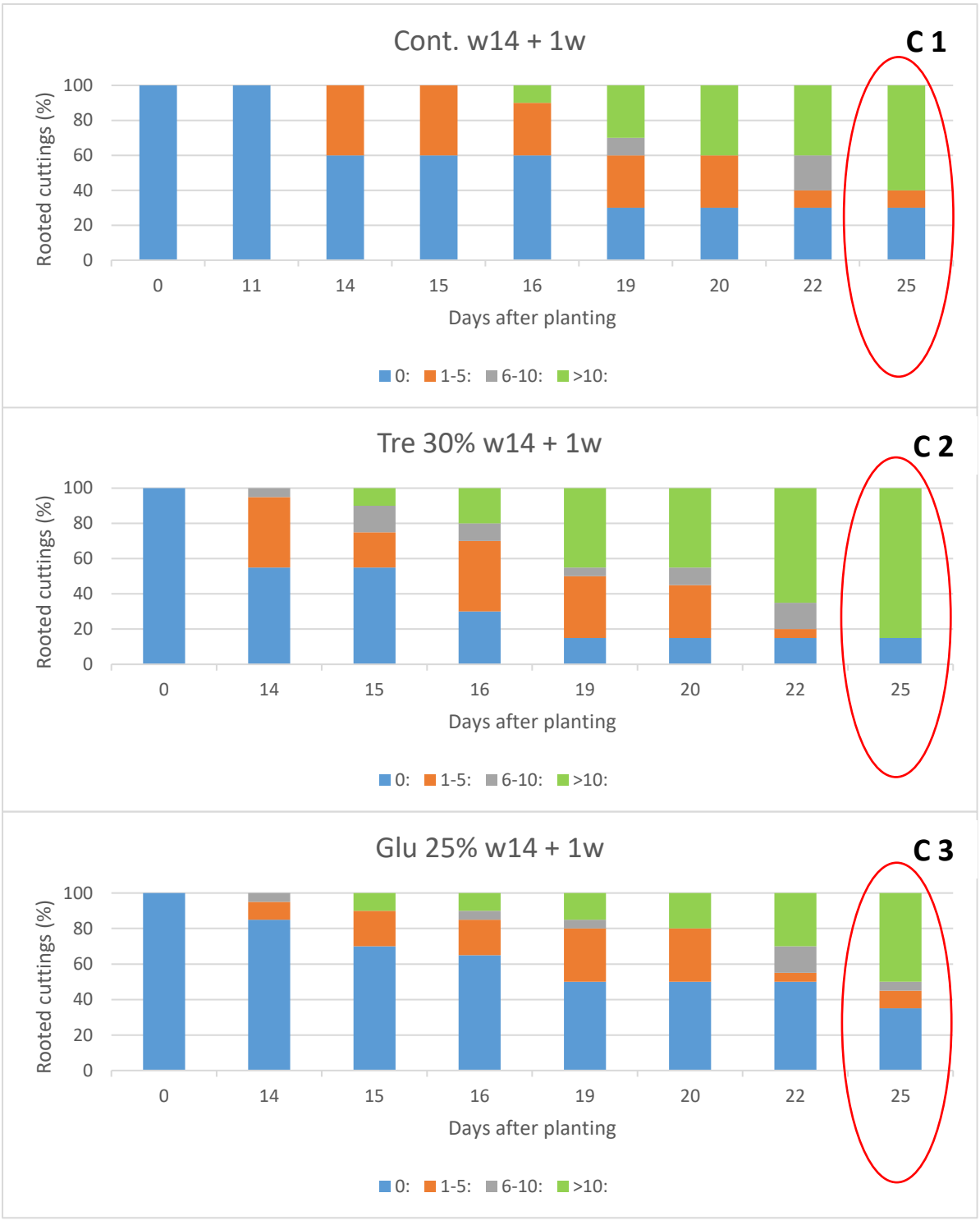

Figure 11: Three graphs, are cuttings received w14 and planted w16, illustrating the percentage of rooted *P. zonale* cuttings. A 3: Are untreated cuttings. B 3: Are cuttings treated with 30% Tre. C3: Are cuttings treated with 25% Glu.

In figure 11, C1; can be seen that 30% did root; whereas in C2, treated with 30% Tre; a total of 15% did not root. In C3; 35% of the cuttings treated with 25% Glu, 35% did not root.

6. Discussion

The aim of this thesis was to see if it was possible to increase the life expectancy of unrooted *P. zonale* cuttings with the aid of VI and PEF; in combination with two different types of cryoprotectants. The question that needs to be answered is if the life expectancy can increase by applying the method presented in previous sections and if this method has aided in the rooting of the treated cuttings.

Firstly, when adding any form of substance to a malleable entity there are risks involved, especially when it comes to living plant tissues. A recent review by Galindo & Yusof (2015) of the latest within VI, stated that little attention has been given to the consequences of VI on how it affects the metabolism of the impregnated product. According to them, three of the consequences are that the impregnation provokes structural changes, the molecules impregnated "and/or anaerobic stress". This might be a limiting factor when trying to achieve a longer shelf life with the aid of VI; as it may cause deterioration of the treated product. What was observed is that there were no visible markings indicating any form of stress. On the contrary, when treated with the correct amount of cryoprotectant solution the cuttings showed visible signs of perkiness and thus illustrated a positive trend.

One factor that may have affected the degree of impregnation is the fact that as per Adams, (1971) *Pelargonium* are covered with epidermal hair. Their main function is to act as a protective shield against unwanted "intruders". This may hinder the cryoprotectants from entering the plant tissue, and as such lead to an unnecessary stress moment. Also, this would mean that *P. zonale* might not be treatable with this method. The PEF treatment aids as it results in the opening of the stomata cells and thus allows the intracellular cells to be filled with the cryoprotectant solution (Demir, 2012).

As the sugar content of plants tends to increase when there is a temperature change; this could mean that by externally adding sugars one could aid the plant tissue. This is since studies have shown that after being exposed to non-freezing low temperatures e.g. cabbage seedlings had acquired a higher freezing tolerance due to the accumulation of the sugars (Sasaki, et al., 1996). Furthermore, this was proven as "In some plants (e.g. mulberry) any treatment that increases the sugar content increases hardiness and any treatment that decreases the sugar content lowers hardiness" (Levitt, 1966, p. 528).

This is applicable in this study, as by observing the results in table 1; P1, there was an average weight increase of the whole stem with 17.13% when treating the cuttings with 25% Glu. The

average weight gain of the whole stem when treated with 30% Tre was 24.75%. This means that the cuttings stem where filled with the cryoprotectant solution and thus that the sugars had entered the plant tissue. Therefore, this may have aided in increasing the life expectancy of the unrooted *P. zonale*.

Continuing, when testing the different voltages, only bipolar pulses were used. This was because when treating a sample with electrical pulses an imbalance of ions is created. If the cuttings were treated with monopolar pulses this would have resulted in a one-sided imbalance. Furthermore, this imbalance can also cause irreversible electroporation which is unwanted; as it may lead to cell death (Barbosa-Canovas, et al., 2000). Figure 7 A; is illustrative of a poor permeabilization as there are minimal visible cell membranes and thus showing that at 1500 V there was no survival. Whereas at 800 V there are visible cell membranes and some nuclei proving that positive permeabilization had occurred, see figure 7 B.

Additionally, due to the fact that *Pelargonium* are hypostomatic (Adams, 1971), this may have affected both the degree of impregnation and also how well the cuttings took to the PEF treatment. This is because the stomata are mainly located in the lower part of the plant. One of the many reasons to why PEF is used is because it results in the electroporation of the cell membrane and therefore it becomes permeable (Dymek, et al., 2015); thus, opening the stomata. Furthermore, by using bipolar pulses this resulted in the opening of the stomata on both the upper and lower parts of the plant tissue, meaning that the PEF treatment was successful. Another aspect that needed to be taken into account is the fact that the leaves of the *Pelargoniums* tend to have a thicker epidermis on the upper part of the leaves than on the latter; therefore, it was wise to use bipolar pulses in the treatment of the unrooted *P. zonale* cuttings.

PEF "has been presented as advantageous in comparison to, for instance, heat treatments, because it kills microorganisms while better maintaining the original color, flavor, texture, and nutritional value of the unprocessed food" (Mohamed & Eissa, 2012, p. 275). This could be a possible way to kill any bacteria or microorganisms that are attached on the unrooted *P. zonale* cuttings and therefore could minimise the risk of transporting unwanted pathogens.

By observing the results illustrated in figure 9-11; the controls which were also one week old when planted, there is a significant percentage of 40% of the untreated cuttings that did not root after 25 days of planting. This was calculated by summarising the blue columns for day

25, of all three replications. On the contrary, when observing the total that did not root after being treated with 30% Tre the percentage was at 10%, and for the 25% Glu it was 23%, see figure 9-11. Additionally, by looking at figures 9-11, it can be stated that there is a higher percentage of more than 10 roots for the treated cuttings when compared to the untreated ones. This conveys that not only did the treatment aid in increasing the life expectancy of the unrooted cuttings it also assisted in the rooting of them.

The aim of this thesis was to see if by applying these treatments, the life expectancy of the unrooted *P. zonale* cuttings could be increased and by looking at the results this was achieved. The cuttings were received four days, 96 hours, after being cut. According to Johnsen, 2017, the rooting capacity of an unrooted *P. zonale* cutting decreases immensely 96 hours after being cut. After this experiment, the life expectancy and rooting capability was increased by approximately another 170 hours. This could result in major changes to the production cycle and transportation of these unrooted cuttings.

7. Conclusion

In conclusion, the aim of this project was met. With the help of OptiFreeze's AB patented method, the life expectancy and rooting ability of the unrooted *P. zonale* increased from 96 hours to approximately 270 hours in total. This is very promising. By extending the time frame this could mean that the percentage of wasted *P. zonale* could decrease; the handling i.e. the cutting process could change as also the transportation means. What could be of interest is to test and see if there is a difference between freshly cut cuttings and how they would react to the treatment. The next step would also be to test and see if the treated cuttings could be stored even longer or at different temperatures.

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9. Appendix

Appendix 1: Interview with Lennart Johnsen; EAME Grower Services Lead, 11/01/2017

1) How many unrooted *Pelargonium zonale* (*P. zonale*) cuttings are produced per year and then sold onwards in the European Union (EU)?
   Around 400 – 500 million unrooted *P.* cuttings are sold to the EU each year, including ivies and cascades. The estimated total volume of zonale is approximately 50% of the above.

2) Where are they produced?
   90% of all *P. zonale* cuttings are produced in Africa.

3) When do you start with the production? When does the production end? How does the production cycle look like?
   The planting of the mother plants begins during week 30. The cuttings are taken between week 45 and until the end of week 15. The production peak is between weeks 52 – 8, where 70% of all the cuttings are taken for that particular season.

4) What percentage of unrooted *P. zonale* are thrown away during a production cycle?
   Around 30% of the unrooted cuttings taken during one season are thrown away. This is so to build up the mother plants and thus be able to achieve maximum number of unrooted cuttings produced during the peak of the season.

5) Approximately how many cuttings can one mother plant produce per week?
   It can produce 2-2.5 cuttings per week. This means that it is a costly production.

6) How does the demand for *P.* zonale look like?
   It is the no. 1 plant sold in most European countries.

7) To which degree are the cuttings tested for different viruses, bacterium and at which point in the production cycle are they tested?

   All production mother stock starts from SEE level (Super Extra Elite) which is grown in Europe. From the SEE plants, cuttings are harvested for the build up of EE level (Extra Elite) at the URC production location. From the EE plants, cuttings are harvested for the build up of commercial stock.

   All levels are tested for the important diseases before and during production;

   SEE = 100% of plants tested, EE = 100%, Production stock = 10%

8) Storing of unrooted cuttings:
   - How long are they stored for The rooting capacity of an unrooted *P. zonale* cutting decreases immensely 96 hours after being cut.
   - Which temperature? 2-5 ℃

9) Would it be of any interest if the durability of the unrooted cuttings was increased so that they would survive up to 10 days after being cut?
Yes, as this would reduce the time press as would also enable the use of other transportation means. Additionally, this would also mean that we can make use of surplus production one or even two weeks prior to the main season.

Appendix 2: Statistical results

Binary Logistic Regression: Rotad, Sticklingar versus Vecka, Behandling

```
Link Function: Logit


Response Information

Variable     Value      Count
Rotad        Event        108
             Non-event     42
Sticklingar  Total        150


Logistic Regression Table

                                                    Odds     95% CI
Predictor         Coef   SE Coef      Z      P  Ratio  Lower  Upper
Constant      0.726278  0.483878   1.50  0.133
Vecka
 13          -1.02598  0.493278  -2.08  0.038   0.36   0.14   0.94
 14          -0.724748  0.500215  -1.45  0.147   0.48   0.18   1.29
Behandling
 Glucose      0.583225  0.467281   1.25  0.212   1.79   0.72   4.48
 Trehalose     1.79436  0.537915   3.34  0.001   6.02   2.10  17.26


Log-Likelihood = -80.127
Test that all slopes are zero: G = 17.632, DF = 4, P-Value = 0.001


Goodness-of-Fit Tests

Method           Chi-Square  DF      P
Pearson             2.13286   4  0.711
Deviance            2.18250   4  0.702
Hosmer-Lemeshow     0.94811   5  0.967


Table of Observed and Expected Frequencies:
(See Hosmer-Lemeshow Test for the Pearson Chi-Square Statistic)

                            Group
Value         1     2     3     4     5     6     7  Total
Event
  Obs         9    13    12    22    16    17    19    108
  Exp       9.3  11.4  12.8  22.5  16.3  17.2  18.5
Non-event
  Obs        11     7     8     8     4     3     1     42
  Exp      10.7   8.6   7.2   7.5   3.7   2.8   1.5
Total        20    20    20    30    20    20    20    150


Measures of Association:
(Between the Response Variable and Predicted Probabilities)
```

Pairs	Number	Percent	Summary Measures	
Concordant	2987	65.9	Somers' D	0.42
Discordant	1087	24.0	Goodman-Kruskal Gamma	0.47
Ties	462	10.2	Kendall's Tau-a	0.17
Total	4536	100.0		

Binary Logistic Regression: Rotad, Sticklingar versus Vecka, Behandling

Link Function: Logit

Response Information

Variable	Value	Count
Rotad	Event	108
	Non-event	42
Sticklingar	Total	150

Logistic Regression Table

Predictor	Coef	SE Coef	Z	P	Odds Ratio	95% CI Lower	95% CI Upper
Constant	2.52064	0.521378	4.83	0.000			
Vecka							
13	-1.02598	0.493278	-2.08	0.038	0.36	0.14	0.94
14	-0.724748	0.500215	-1.45	0.147	0.48	0.18	1.29
Behandling							
Control	-1.79436	0.537915	-3.34	0.001	0.17	0.06	0.48
Glucose	-1.21114	0.474834	-2.55	0.011	0.30	0.12	0.76

Log-Likelihood = -80.127
Test that all slopes are zero: G = 17.632, DF = 4, P-Value = 0.001

Goodness-of-Fit Tests

Method	Chi-Square	DF	P
Pearson	2.13286	4	0.711
Deviance	2.18250	4	0.702
Hosmer-Lemeshow	0.94811	5	0.967

Table of Observed and Expected Frequencies:
(See Hosmer-Lemeshow Test for the Pearson Chi-Square Statistic)

	Group							
Value	1	2	3	4	5	6	7	Total
Event								
Obs	9	13	12	22	16	17	19	108
Exp	9.3	11.4	12.8	22.5	16.3	17.2	18.5	
Non-event								
Obs	11	7	8	8	4	3	1	42
Exp	10.7	8.6	7.2	7.5	3.7	2.8	1.5	
Total	20	20	20	30	20	20	20	150

Measures of Association:
(Between the Response Variable and Predicted Probabilities)

Pairs	Number	Percent	Summary Measures	
Concordant	2987	65.9	Somers' D	0.42
Discordant	1087	24.0	Goodman-Kruskal Gamma	0.47
Ties	462	10.2	Kendall's Tau-a	0.17
Total	4536	100.0		