Universitat Autònoma de Barcelona

Bacterial nanocellulose:

production optimization and application in plant materials

Bachelor's Degree Final Project

Bachelor's Degree in Nanoscience and Nanotechnology

Author: Oriol Muñiz Bàguena
Tutor: Dra. Núria Sánchez Coll and Dra. Anna Laromaine
Co-tutor: Dra. Agnese Rabissi
Institutions (Groups):

- Centre for Research in Agricultural Genomics (Bacterial Pathogens and Plant Cell Death)
- Institute of Materials Science of Barcelona (Group of Nanoparticles and Nanocomposites)

September 2019

Acknowledgements

I would like to thank Dra. Núria Sánchez Coll for accepting me into her research group (Bacterial Pathogens and Plant Cell Death at the Centre for Research in Agricultural Genomics) and introducing me to the Plant Nano-Healing project. I am also grateful to Dra. Anna Laromaine from the Group of Nanoscience and Nanocomposites at the Institute of Materials Science of Barcelona, which also participates in Plant Nano-Healing, for her insightful suggestions and guidance. In addition, I would like to express my gratitude to my fellow group members and staff from both research centers who helped me for their kindness and patience. I am also grateful to Miquel Segarra and the team at Forestal Catalana for making our shared work a very pleasant experience, to Roses Floriach, and to the rest of people and organizations who helped this project thrive. And above all I would like to thank Dra. Agnese Rabissi, with whom I have been working closely day to day, for her enduring support and teaching me so much.

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Abbreviations

AGAUR – Agency for Management of University and Research Grants
BC – bacterial nanocellulose
CECT – Spanish Type Culture Collection
CRAG – Centre for Research in Agricultural Genomics
CREDA – Center for Agro-food Economics and Development
CSIC – Spanish National Research Council
FIP – Frontier Interdisciplinary Projects
HS – Hestrin-Schramm
ICMAB – Institute of Materials Science of Barcelona
KX – *Komagataeibacter xylinus*
MINECO – Ministry of Economy and Finance
NAA – 1-Naphthaleneacetic acid
NCIMB – National Collection of Industrial Food and Marine Bacteria
OD600 – Optical density measured at a wavelength of 600 nm
PC – plant cellulose
SEM – Scanning electron microscope
UAB – Universitat Autònoma de Barcelona
UV-Vis – Ultraviolet-visible spectroscopy

1. Background

This Bachelor's Degree Final Project centered on bacterial nanocellulose, a biodegradable material that facilitates tissue regeneration. Specifically, the commercial viability of its application in plant materials (cut flowers and *Pinus pinea* grafts) was assessed, alongside an experiment studying its production by *Komagataeibacter xylinus*, with the aim of optimizing the production process in the laboratory.

1.1. Bacterial nanocellulose

Bacterial cellulose, also called bacterial nanocellulose due to the nanometric size of its microfibrils (Figure 1), is extracellularly synthesized by several gram-negative bacteria, mainly of the genera *Gluconacetobacter*, *Acetobacter*, *Agrobacterium*, *Achromobacter*, *Aerobacter*, *Sarcina*, *Azobacter*, *Rhizobium*, *Pseudomonas*, *Salmonella and Alcaligenes* [1].

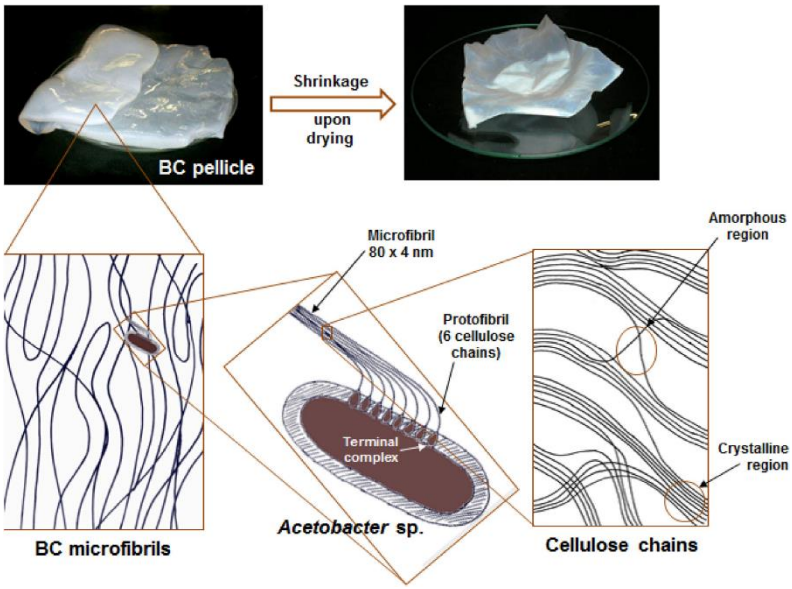


***Figure 1**. Schematic outline of the structural organization and synthesis of bacterial nanocellulose [5].*

It differs from plant cellulose in that it does not contain other components such as lignin, pectin or hemicellulose [2], and consists of a translucent and gelatinous membrane, formed by randomly distributed cellulose microfibrils. Some authors argue that two types of cellulose can be

distinguished according to their spatial configuration: Cellulose Type I presents a network of hydrogen bonds, hydrophobic and van der Waals interactions, so that it forms parallel chains [3]. However, when BC is treated with sodium hydroxide, it forms an antiparallel packing, the so-called Type 2 Cellulose, which unlike the previous one, is thermodynamically stable, and has a large Young's modulus of 118 GPa, almost comparable with that of Kevlar® and steel [4].

In addition, the reduced size of its microfibrils implies that BC has a large surface area and mechanical properties similar to those of soft tissue [1]. Other interesting properties of BC, which confer it a high applicability potential, are a high biocompatibility [2], flexibility, porosity [6], water retention capacity [7], crystallinity, mechanical stability, and hydrophilicity [8]. Another advantage is that, during the synthesis process, it is possible to control the shape, size or thickness of BC [1][2].

1.2. State of the art of bacterial nanocellulose applications

Thanks to the properties mentioned in the previous section, BC has numerous applications in biomedicine. It is used in dressings, as it allows rapid tissue regeneration compared to other commercialized alternatives [9], it does not need to be changed every day, it adapts to the surface of the wound and absorbs the exudates, maintains a humid environment, facilitates the removal of necrotic residues, and forms a physical barrier that protects against infections and reduces pain [10]. BC composites with biologically active components (drugs, polyelectrolytes, proteins, polysaccharides, nanoparticles [11], etc.) have been designed, which represent an advantage in dressings or expand the applications of BC, for instance, in diagnostic sensors and in the sustained release of drugs [5].

Its possible applications, however, are not limited to the medical field. For example, BC composites with graphene oxide have been prepared as possible conducting devices [12], with carbon nanotubes in thermoelectric generators [13], and with Al_2O_3 as a separator membrane in lithium-ion batteries [14]. Its use is also interesting in paper making: for example, damaged papers can be reinforced with BC if the latter is synthesized directly on the surface of the former [15]. In addition, the use of oxidized BC with laccase and covalently immobilized TiO_2 was shown to contribute to the degradation of dyes, which could help decrease water pollution by the textile industry [16]. In addition, among many other areas, the suitability of BC in the food industry has been studied, as a gelling and stabilizing agent [17] or in packaging [18]. However, its applications in agriculture have not been exploited yet.

1.3. *Komagataeibacter xylinus*

One of the bacterial species with a higher BC production yield is *Komagataeibacter xylinus* (formerly *Acetobacter xylinum* and *Gluconacetobacter xylinus*) [1]. For this reason, a *K. xylinus* strain, CECT 473 (NCIMB 5346), is used in Plant Nano-Healing, in a static culture. Its culture requires seven days of incubation at 30 °C and aerobic conditions. The capability of these bacteria to produce cellulose was discovered by A. J. Brown as early as 1886.

The thickness of BC membranes is important in some applications. Furthermore, it is advisable to produce BC as efficiently as possible in the laboratory. Therefore, one of the objectives of this work was to obtain a reproducible relationship between the time of KX culture growth and the resulting quantity of BC produced, as outlined in *Study of* Komagataeibacter xylinus *static culture growth* (page 5).

1.4. Plant Nano-Healing project

In 2016, the numerous applications of BC in biomedicine, and in many other fields, opened the door to the possibility that it could also be applied in plants, but the research in this field was scarce. This encouraged the Group of Nanoparticles and Nanocomposites of ICMAB and the Bacterial Pathogens and Plant Cell Death research group of CRAG to collaborate to investigate the potential of BC in the regeneration of plant tissues, forming the interdisciplinary work group Plant Nano-Healing, in which I have been taking part since summer 2018. This collaboration between the two CSIC research centers linked to the UAB resulted in a Spanish-level patent (ESP201630592), a method to regenerate damaged plant tissues based on BC, which is applied directly to the damaged area and allows rapid healing.

Additionally, the Plant Nano-Healing project is involved in the study of possible applications of BC in several fields that include plant materials, such as grafts, *in vitro* plant cell cultures, and cut flower preservation. In the future, it may also explore its use in post-pruning, coatings of fruit and vegetables after harvest (where its effectiveness has already been shown [21]), and cork-producing plants.

To decide what applications and experiments to pursue, internal investigations are conducted, and market surveys are ordered (for instance, one done by CREDA).

Plant Nano-Healing has thus far been financed through the following grants: Severo Ochoa (ICMAB-FIP), Llavor Grant (AGAUR), Gínjol Patents Fund (I-CERCA), National R&D&I Plan (MINECO) and Ajudes a les activitats de demostració de Transferència Tecnològica 2018 (Government of Catalonia, Ministry of Agriculture, Livestock, Fisheries and Food).

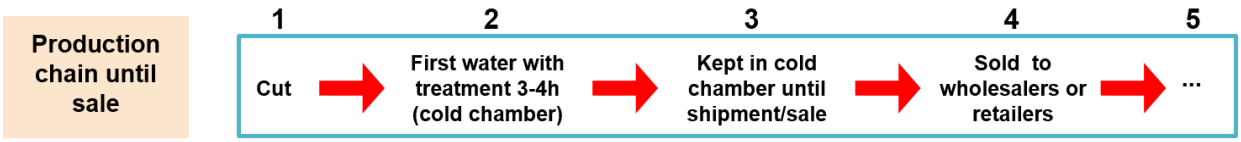
1.5. *Nicotiana benthamiana*

In order to test the quality of newly produced batches of BC (page 7), a tobacco-related plant species, *Nicotiana benthamiana*, is used. The reason is that previous Plant Nano-Healing experiments showed that BC accelerated the healing of wounds in its leaves [11]. Therefore, if this effect is not observed in the batches under testing, they must be discarded for future experiments. *Nicotiana benthamiana* was chosen in the first place because it is a model organism (i.e., it has been extensively studied and frequently used in plant research, especially in plant pathology and immunology, since it is vulnerable to infection from many types of viruses, bacteria, oomycetes and fungi) [19]. Another main reason for the ubiquity of *N. benthamiana* in research laboratories is the large size and smooth surface of its leaves, which are optimal for wound healing experimentation.

1.6. Current methods in cut flower production chain

The techniques used to produce cut flowers are very well established because they are relatively easy, cheap and effective enough. Consequently, any improvement provided by BC must be highly relevant, specific and/or of high economic value, since this material is still expensive to produce, in part because its industrial production is scarcely viable. For this reason, roses were chosen as the cut flowers to experiment with, since they are one of the most expensive and with a wider market, and quality is a vital attribute to their sale.

Mainly, the cut flower production process in garden centers or nurseries is the following:



Plant Nano-Healing aims at improving steps 2 and 3, as will be explained in coming sections of this work.

1.7. *Pinus pinea* grafting

Pinus pinea, commonly known as stone pine, is a Mediterranean tree from the pine family which is in great demand mainly because of its edible fruit, the pine nut. An experiment to determine whether BC increased the success rate of its grafting was conducted in collaboration with Forestal Catalana, a company owned by the Government of Catalonia involved in forestry, wildlife conservation and natural resource management, among other activities.

Grafting is an asexual vegetative propagation technique used since Ancient times which usually consists in joining two plant pieces, the 'scion' (shoot segment) and the 'rootstock' (root segment) [20]. If successful, the stone pine will reach maturity in five years at most (sometimes even producing nuts as early as two or three years) instead of the usual 15 to 20 years.

However, *Pinus pinea* grafting presents severe limitations. For instance, its success rates are very variable from one year to the next: they can range from more than 70% to as low as 20%. Moreover, the window of opportunity for grafting is quite short: it must be done from late March to early May, and very especially in the first half of April. Environmental factors have an important influence, and it is thought that humidity around the graft union is a relevant factor. For this reason, it was hypothesized that BC would be able to improve the success rates if placed in contact with the graft union, due to its wound healing and water retention properties described earlier (page 1). This would be especially important because of the high commercial value of grafted stone pine: each plant can be sold for up to 14€ and their demand is such that Forestal Catalana has orders covered for the next ten years.

1.8. Work Placement

I joined Plant Nano-Healing during the summer of 2018 in the framework of the optional subject 'Work Placement' included in the bachelor's degree in Nanoscience and Nanotechnology. During that time, I learned the BC production protocol in static culture, the quality test protocol, and did a preliminary experiment with cut flowers. In addition, I studied the effectiveness of BC as a support of *in vitro* plant cell cultures and performed a hormonal analysis of lyophilized BC membranes.

2. Initial hypothesis and objectives

2.1. Effect of bacterial nanocellulose on the preservation of cut flowers

- **Initial hypothesis**: the tissue regeneration properties of BC can help prolong the commercial lifetime of cut flowers while adapting to established procedures followed in flower gardens or nurseries.

- **Objective**: studying the effect of BC on the preservation of roses, with the aim of developing products based on this material that could slow flower decay.

2.2. Effect of bacterial nanocellulose on grafted *Pinus pinea* success rate

- **Initial hypothesis**: the tissue regeneration properties of BC could help increase the success rate of grafted *Pinus pinea* (i.e., the survival rate of stone pines after grafting).
- **Objective**: studying the effect of BC on the success rate of grafted stone pine. The BC is added as an extra layer, in direct contact with the graft union, and wrapped by an adhesive tape typically used by grafters to maintain the union close together. If the experiment has positive results, a BC-based product could ultimately be sold to grafters.

2.3. Study of *Komagataeibacter xylinus* static culture growth

- **Initial hypothesis**: a reproducible relationship exists between the stage of *K. xylinus* static culture growth and the quantity of BC that will be produced.
- **Objective**: being able to precisely control the quantity of BC (e.g., thickness of never-dried membranes) that will be produced in static culture in the laboratory by tweaking the time of growth of the *K. xylinus* pre-inoculum.

3. Materials and methods

3.1. Production and quality test of bacterial nanocellulose membranes

3.1.1. Hestrin-Schramm culture medium

The Hestrin-Schramm medium has the following composition (with ultrapure water as solvent):

- ✓ 20 g/L dextrose
- ✓ 1.15 g/L citric acid
- ✓ 6.8 g/L $Na_2HPO_4 \cdot 12H_2O$
- ✓ 5 g/L yeast extract
- ✓ 5 g/L bacteriological peptone

The dextrose solution was autoclaved separately to avoid its reaction with the protein components of the medium. When they were cooled down after autoclaving, the dextrose and the solution containing the remaining components were mixed together in a biosafety cabinet, to avoid contamination. The medium-containing bottle had to be tightly closed and, when it reached room temperature, it was stored at 4 °C in the refrigerator.

Additionally, when HS medium was needed in an agar plate, 15 g/L of agar were added to the solution that was autoclaved separately from the dextrose. Then, the mixing had to be performed before the temperature fell below 60 °C, because otherwise the agar would have solidified inside the bottle. Subsequently, 20 mL of the recently mixed warm medium were poured into each sterile Petri dish until the bottle was emptied, and they were left uncovered in the biosafety cabin. Finally, when the agar had solidified, the dishes were covered again with their lids, packed in plastic wrap and stored in an inverted position at 4 °C, in the refrigerator.

3.1.2. Laboratory production of bacterial nanocellulose membranes in static culture

BC was produced in static culture following a 'seed lot system', which consists in multiplying the culture through a series of controlled subculturing steps.

The BC production protocol started with the activated bacteria in a solid medium dish ('passage 0', or P0), which was bought from CECT, a public provider of microbial strains.

A first KX culture in HS liquid medium ('passage 1', or P1) was prepared from P0, and it was subsequently divided into several (in this case, fifteen) 1 mL microcentrifuge tubes, which were slowly frozen with glycerol and stored at -80 °C ('Master seed lot').

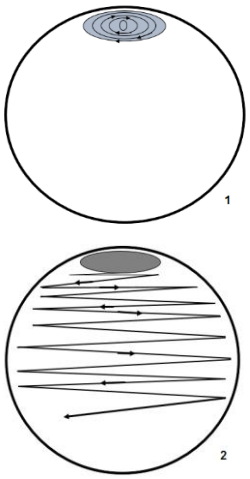


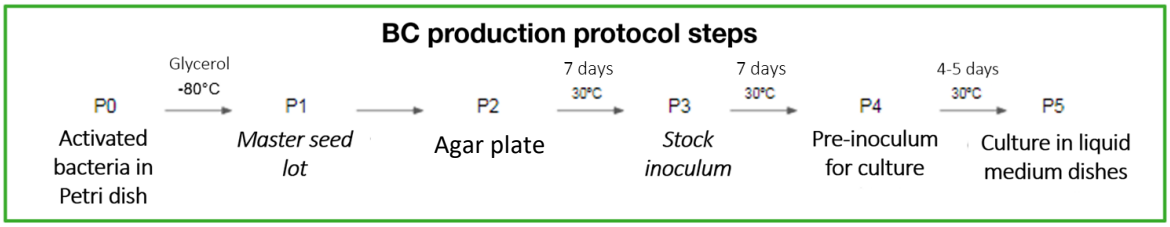
***Figure 2**. Drawing of the spread of KX bacterial culture over the surface of an agar plate.*

A volume of 330 µL of the P1 culture was placed on each agar plate (three plates in total), in the area marked in dish 1 of Figure 2, and spread over the entire surface in a zigzag motion, using a sterile inoculation loop (dish 2 of Figure 2). The plates ('passage 2', or P2) were incubated at 30 °C for 7 days, and then they were wrapped in parafilm and stored at 4 °C in the refrigerator up to two months until they were needed.

The Stock inoculum (passage 3, or P3) was the starting point every time a new batch of BC membranes was produced. Individual KX colonies were collected from a P2 agar plate and placed into each sterile 15 mL centrifuge tube containing 6 mL of HS liquid medium. Five to ten P3 tubes were prepared at once and incubated for seven days at 30 °C, with their caps left partially unscrewed in order to let oxygen in, since KX are aerobic bacteria. If they were not used straight away, P3 tubes were stored at 4 °C up to four weeks until a new BC batch was to be produced.

The remaining two steps of the BC production protocol were the most frequent because, unlike the previous ones, they had to be repeated for all new batches. A volume of 500 µL of a stock inoculum tube (P3) was added to each sterile 15 mL centrifugation tube containing 4.5 mL of HS medium. The necessary number of passage 4 tubes (the pre-inoculum) were obtained in this way, and they were incubated at 30 °C for around four days.

The appropriate volume of pre-inoculum (P4) was dissolved in HS medium in a 1:14 ratio. The resulting subculture (passage 5, or P5) was poured into different sterile dishes and plates, which served as molds that determined the shape and size of the BC membranes that would grow on their surface. After seven days in incubation at 30 °C, the BC membranes had already formed and had adequate thickness, so they were recollected and washed following the procedure detailed in the next subsection.



Each subculturing step and washing process was recorded in an online spreadsheet, registering the number of tubes produced, the date and batch number, and any other relevant information.

3.1.3. Protocol for washing BC membranes

This procedure was performed in a fume hood. First, the waste fiber sludge that surrounded BC membranes, which was rich in KX bacteria [22], was gently removed and discarded alongside the liquid culture. Subsequently, the membranes were immersed in ethanol 50 vol% for 10 minutes. Then, they were separated from the ethanol solution and boiled in agitation for 40 minutes in distilled water. Following this, they were heated at 90 °C in agitation in a NaOH 0.1 M solution for 20 minutes. Finally, the membranes were maintained in agitation in distilled water until their pH returned to neutral. These steps were followed to eliminate the bacteria and impurities.

After the washing steps, membranes were placed into properly labelled bottles (date, batch number, number of each type of membrane it contained, initials of the producer, etc.) in ultrapure water. The bottles were then autoclaved and stored at room temperature.

3.1.4. Quality test of bacterial nanocellulose batches

The quality test had to be performed on *N. benthamiana* plants ten to fifteen days old, which had not budded yet, but had leaves big enough to hold a few small BC membranes.

Using a blade, up to four cuts (approximately 1 cm in length) were made per leaf, for a maximum of three leaves per plant. Each BC membrane was applied on the cut, completely covering it.

After seven days, the BC membranes were moistened and delicately removed using tweezers. The regeneration r ates (length of the cut that had regenerated divided by total length) were recorded and compared to the positive control (i.e. BC membranes from a batch that had already shown a good regeneration capacity). A batch passed the quality test when the average regeneration rate was at least similar to the control average, and higher than 65%. No negative control was needed, since no significant regeneration has ever been observed in bare cuts after seven days [11]. This data was collected in an online spreadsheet where statistical tests could be performed.

***Figure 3**. BC membranes covering wounds on a* N. benthamiana *leaf during a quality test.*

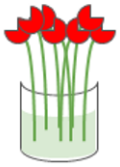
3.2. Effect of bacterial nanocellulose on the preservation of cut flowers

On **day 1** of the experiment (April 30, 2019), 35 roses were selected from the greenhouse at Roses Floriach SL, in Vilassar de Mar. In a nearby facility, their stem was cut diagonally to get a final length of 45 cm, so that they could be stored more easily. Additionally, the leaves from the last 20 cm of the stem were removed.

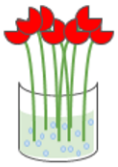
In general, the roses were of bad quality for the following reasons:

- Most leaves, and some stems, had powdery mildew and most leaves were withered.
- Many roses were infested with spider plague.
- Some outer petals were dry and decaying, so they were plucked.

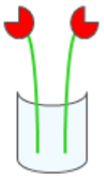
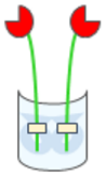
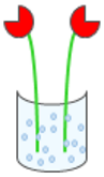
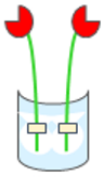
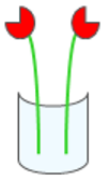
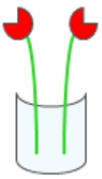
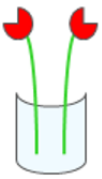
Twenty-five roses were added to a 5 L plastic beaker, containing 2 L of a 5 cm^3/L solution of **Viveflor Complet®**[1] in mineral water.



Ten roses were added to a 2 L plastic beaker, containing 1 L of a 5 cm^3/L solution of **Viveflor Complet®** and **ground BC**[2] in mineral water.



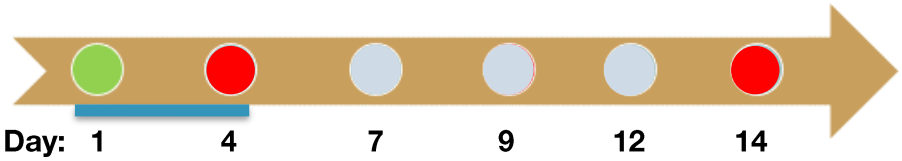
Back at CRAG, the two beakers (pre-treatments) were stored at 4 °C.

After 4h				After 24h	After 4h	After 24h
Five roses were added to a jar containing mineral water.  **Control 4h**	A BC membrane was attached in contact with the cut area of five roses, which were added to a jar with mineral water.  **BC membrane**	Five roses were added to a jar containing mineral water and **ground BC**.  **Ground BC**	An autoclaved PC sheet was attached in contact with the cut area of five roses, which were added to a jar with mineral water.  **Plant cellulose**	Five roses were added to a jar containing mineral water.  **Control 24h**	Five roses were added to a jar containing mineral water.  **Ground BC 4h pre-treatment**	Five roses were added to a jar containing mineral water.  **Ground BC 24h pre-treatment**

[1] **Viveflor Complet®** is currently one of the preservatives used for cut flowers in local garden centers. According to the manufacturer (DEYGEST, S.L.) "[Viveflor Complet can] increase the quality and duration of the flowers twice as long". More information at https://www.deygest.net/en/products/preservative-cut-flowers-viveflor/.

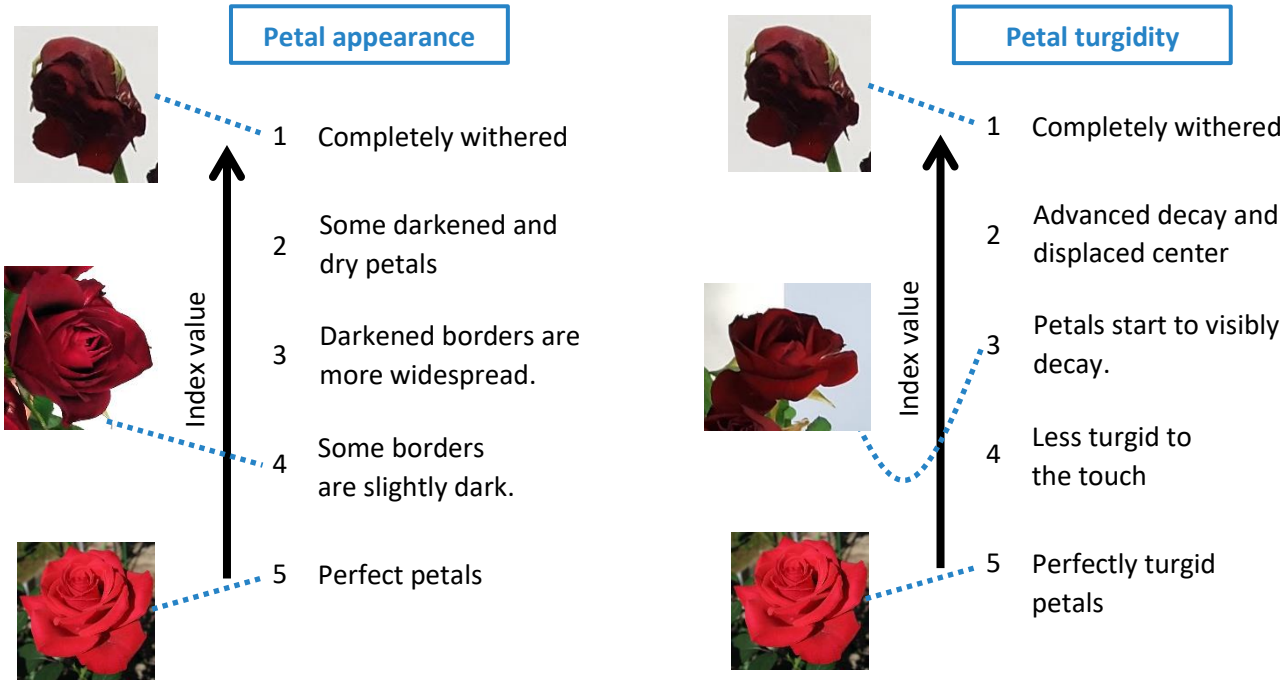
[2] **Ground BC** are never-dried BC membranes which were ground using a hand blender.

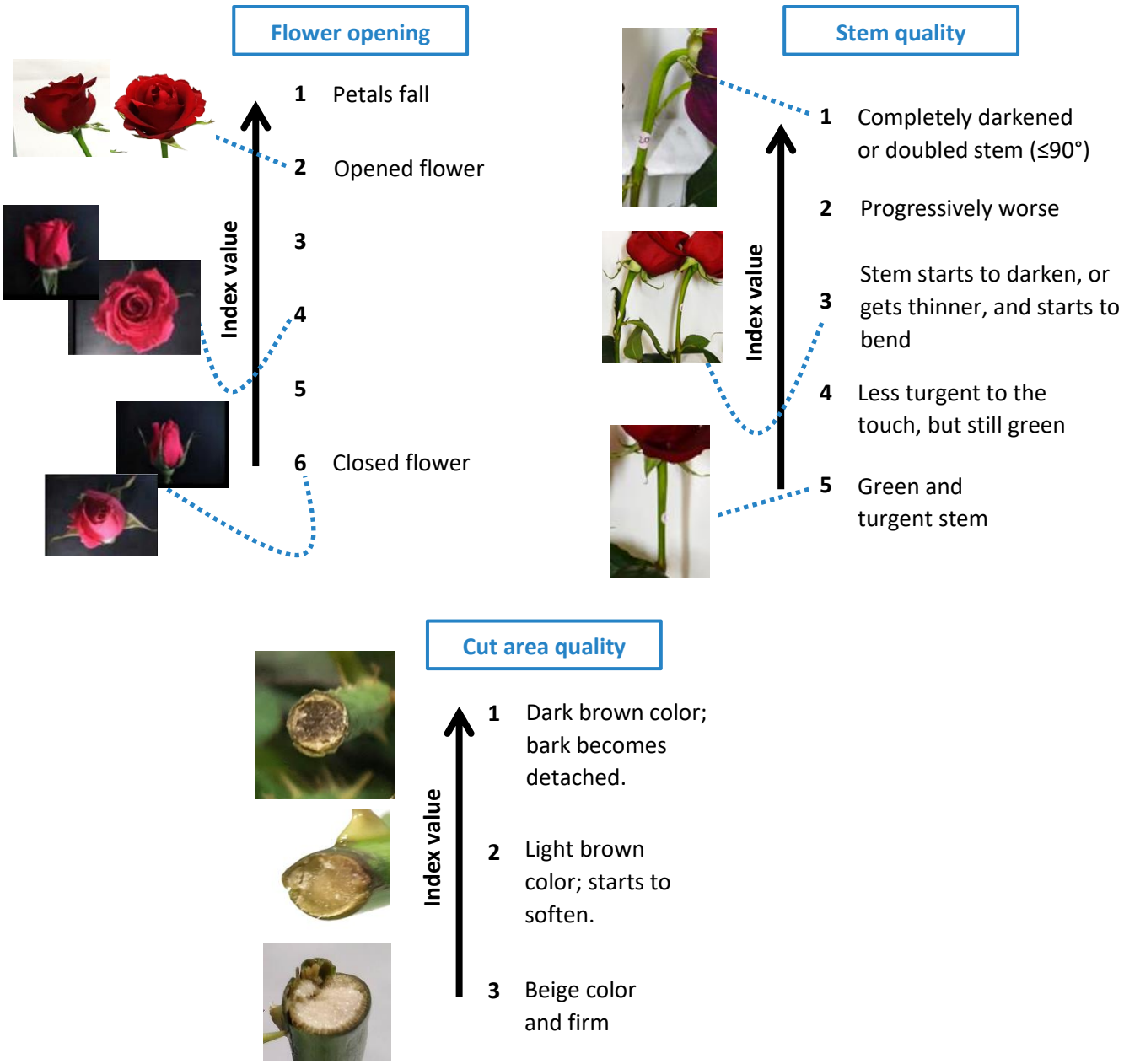
Roses were labelled using stickers and stored at 4 °C.
On days 1, 4, 9 and 14, pictures of all roses were taken.
On days 1, 2, 4, 7, 9, 12 and 14, assessments of petal appearance, petal turgidity, flower opening, stem quality and cut area quality were made following **quantitative indexes**.
On day 4, roses were transferred to a laboratory, at room temperature. BC membranes and PC sheets were removed from the roses of the **BC membrane** and **Plant cellulose** conditions, respectively. **Ground BC** condition was replaced with fresh, clean mineral water.
On day 14, the last of the experiment, sections around 5 cm thick were cut out of the ends of the stems of each rose, in order to examine the cut areas under the microscope. The sections were stored in properly labelled vials in ethanol 70 vol% for a few days prior to microscope observation.



Green: start (day 0), red: special task (see table), blue line: interval at 4 °C.

3.2.1. Quantitative indexes





3.3. Effect of bacterial nanocellulose on grafted *Pinus pinea* success rate

The experiment comprised two phases:

1. Addition of a BC membrane in the graft seals, as an extra layer. The success rate of grafted *Pinus pinea* was recorded at 10, 21, 30, 39, 50, 60 and 74 days after grafting. Additionally, the efficiency of the professional grafters was measured.
2. Some *Pinus pinea* samples were taken to the laboratory at 30 and 74 days after grafting for histological analysis. Thin sections were obtained from the graft unions in each plant, dyed, and examined under the stereo microscope. The removed graft seals were analyzed using a scanning electron microscope, in addition to the stereo microscope.

3.3.1. Addition of a BC membrane in the graft seals

This phase of the experiment was conducted in Forestal Catalana plantations in Riells i Viabrea (Selva, Girona). The rootstocks comprised a total of 306 *Pinus pinea* plants. The scions were

from two different *P. pinea* clones (no. 7 and no. 8) which had already shown good results in previous years. They were gathered on grafting day from a private property, with the proper permits. The grafts were performed on two consecutive days, one for each scion clone.

BC strips, 6.5 cm long and 1.5-2 cm wide, were prepared. The plants were divided into four conditions:

[1] **Control** (90 plants). The usual graft seals are used.
[2] **BC 74 days** (72 plants). Originally this condition was intended to last 21 days, because in a previous experiment with hazels it was observed that, if more time passed, the formation of callous not only sealed the graft union, but also prevented vascularization. However, it was planned that a change of duration could happen if need be (see Results and discussion, page 18).
[3] **BC 30 days** (72 pants). Same as condition 2, but the graft seals were left on the plant for a shorter period.
[4] **BC+hormones 74 days** (72 plants). The BC membranes used for this condition had not performed well in quality tests, so they were not expected to increase the grafting success rate by themselves. They were soaked in a 0.1 w% 1-Naphthaleneacetic acid (NAA) solution, which is a synthetic plant hormone in the auxin family that has been shown to induce tracheid differentiation in pine [23]. Since tracheids are xylem cells, it was hypothesized that this hormone would improve the grafting success rate by inducing vascularization.

In the conditions with BC, one BC band was wrapped around the graft union in each plant, and then an adhesive tape layer typically used for grafting was applied. While manipulating condition 4 BC strips, the professional grafters were advised to use gloves to avoid irritation caused by NAA. The top of the stone pine plants, which is the are a comprising the graft union and scion, was covered with plastic bags to avoid dehydration and offer some protection.

Environmental factors (temperature, humidity, amount of sunlight, etc.) change throughout the day, which can affect the grafting success rate. Therefore, the four experimental conditions were distributed among the professional grafters so that they were all grafted at the same time.

***Figure 4**. A professional grafter wrapping the graft union in a BC strip.*

The time that each professional grafter needed to complete their assigned grafts was measured to calculate their efficiency (minutes per graft).

Throughout the morning, different grafting times were measured randomly for each professional grafter when they prepared graft seals with BC bands. The resulting grafting efficiency calculations were compared to control stone pine graft data averages from previous years (16.6 grafts/person-hour).

Each grafted stone pine plant was properly labeled to indicate condition number (1 to 4), date, scion clone and professional grafter.

3.3.2. Microscope analyses

This phase of the experiment was done in the laboratory, mainly in CRAG. On days 30 and 74, sections were cut as thinly as possible out of the graft unions of samples from all conditions. A phloroglucinol solution was applied on the sections to detect lignin (vascularization), which becomes brilliant purple, or generally lighter purple in the case of newly formed lignified tissues. The microscopes used were Leica DM6 epifluorescence and Olympus SZX16 stereo microscope.

Finally, the graft seals were analyzed using an Olympus SZX16 stereo microscope and a SEMQUANTA FEI 200 FEG ESEM scanning electron microscope. The SEM is necessary due to the nanometric size of the BC microfibers, which cannot be seen in detail using optical microscopy. Six SEM simples were prepared:

1. Control: dried BC membrane
2. Control: dried BC membrane (different batch, therefore different thickness)
3. 30 days (autoclaved)
4. 30 days (ethanol 96 vol% + bleach + H_2O)
5. 74 days (autoclaved)
6. 74 days (ethanol 96 vol% + bleach + H_2O)

Samples 3–6 were sterilized using the methods indicated in parentheses. Pictures from these samples were first taken under the stereo microscope, and then under the SEM in ICMAB.

3.4. Study of *Komagataeibacter xylinus* static culture growth

First, 1 L of HS medium and several HS-agar plates were prepared following the protocol described in page 5. Subsequently, the BC production protocol described in page 6 was started from the P1-to-P2 step: from a single Master seed lot tube, three agar plates were prepared from it. After seven days incubating at 30 °C, the P2 plate which had grown the best isolated KX colonies was selected, and the other two were discarded. That same day, eight Stock inoculum tubes (P3) were prepared and incubated for seven days at 30 °C.

Then, 45 Pre-inoculum tubes (P4) were obtained and placed at 30 °C for incubation. The subculturing was performed so that a few mL of Stock inoculum remained in each one of the P3 tubes used. That same day, three different measurements of this leftover Stock inoculum, plus one of the recently made P4 tubes, were taken:

1. **pH**. A pH meter was used to measure the pH of each one of the P3 tubes and the single P4 tube.
2. **Optical density at 600 nm, prior to filtering**. 1 mL of KX culture from each tube was transferred to plastic cuvettes. A HS medium blank was also prepared. Their absorbance was measured using an UV-Vis spectrophotometer (Shimazu UV-2600) at 600 nm, which is the usual wavelength at which the growth of bacterial cultures is studied [24] [25].
3. **Optical density at 600 nm of filtered culture**. Given that KX produces fibrous residues that may distort absorbance results, pluriStrainer® cell strainers (pluriSelect Life Science) were used to filter the solution. Since the average length of KX bacilli is around 3 µm [26], and they are usually surrounded by fibrous structures that might increase their effective volume, a mesh size of 10 µm (i.e., the diameter of the filter pores) was chosen. The cell strainer is used by attaching it on top of a 50 mL centrifuge tube and then adding the

sample onto it, in this case the remaining Stock inoculum of each one of the P3 tubes and the remaining pre-inoculum subculture from the P4 tube. Once the filtration was completed, 1 mL from each centrifuge tube was transferred to plastic cuvettes. Their absorbance was measured alongside the unfiltered samples.

Subsequently, the steps outlined in Figure 7 were followed.

Finally, the following measurements of the BC membranes obtained in each batch were made:

1. **Fresh weight**. An analytical balance was used to measure the fresh weight (i.e., the weight of BC membranes which retain water, also called 'never-dried', which is their default state in storage) of seven of the 24 BC membranes obtained for each 24-well plate.
2. **Never-dried thickness**. The thickness of the previously weighted never-dried membranes was measured using an electronic micrometer. They were placed between two coverslips and the thickness measurements were taken at the middle of the membrane. The thickness at the center of the two coverslips was measured and later subtracted from the measurements.
3. **Dried weight**. The same BC membranes were placed in the laboratory oven at 30 °C, inside the wells of uncovered 6-well plates (one membrane in each properly labelled well). After two days, the membranes were completely dry and were ready to be weighted in the analytical balance.
4. **Dried thickness**. The exact same protocol for never-dried thickness measurements was followed.
5. **Rewet weight**. The dry BC membranes were returned to the properly labelled 6-well plates, and each well was filled with 5 mL of distilled water. The plates were left in agitation overnight and the resulting rewet membranes were weighted on the analytical balance.
6. **Rewet thickness**. The exact same protocol for never-dried thickness measurements was followed.

Figure 5. *A UV-Vis spectrophotometer (Shimazu UV-2600) was used to measure the OD600 of KX culture.*

Figure 6*. A micrometer (Mitutoyo QuantuMike Series 293-Coolant Proof Micrometer; top image) was used to measure the thickness of BC membrane samples. A cell strainer (pluriStrainer® 10 µm) was used to filter KX cultures.*

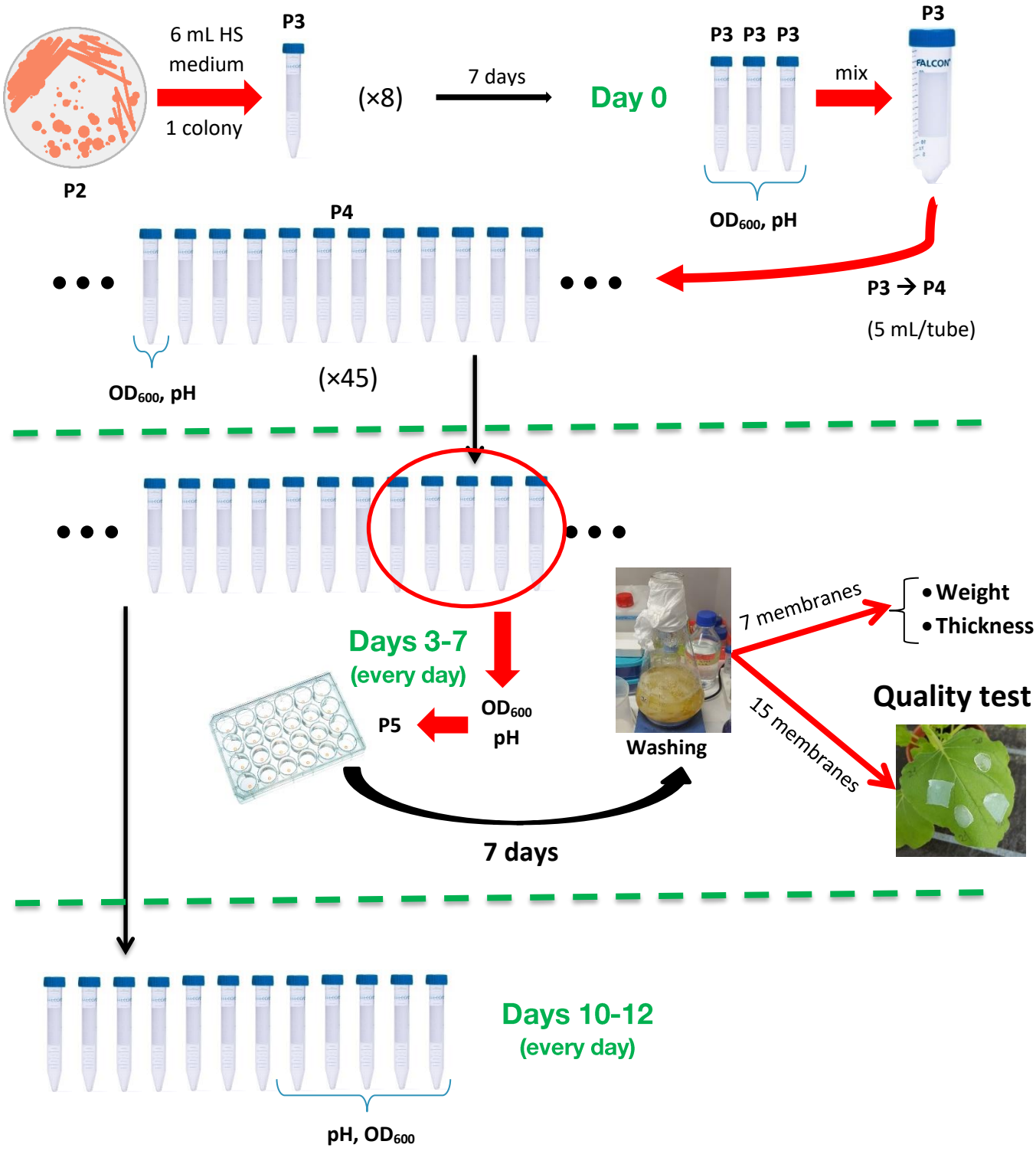


***Figure 7**. Diagram showing the protocol steps of the experiment to study* Komagataeibacter xylinus *static culture growth.*

4. Results and discussion

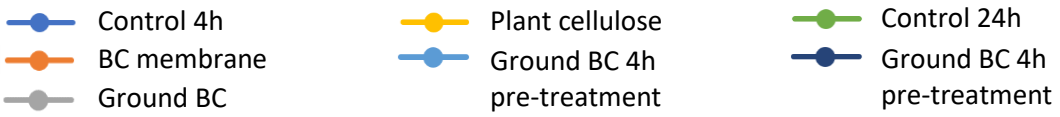
4.1. Effect of bacterial nanocellulose on the preservation of cut flowers

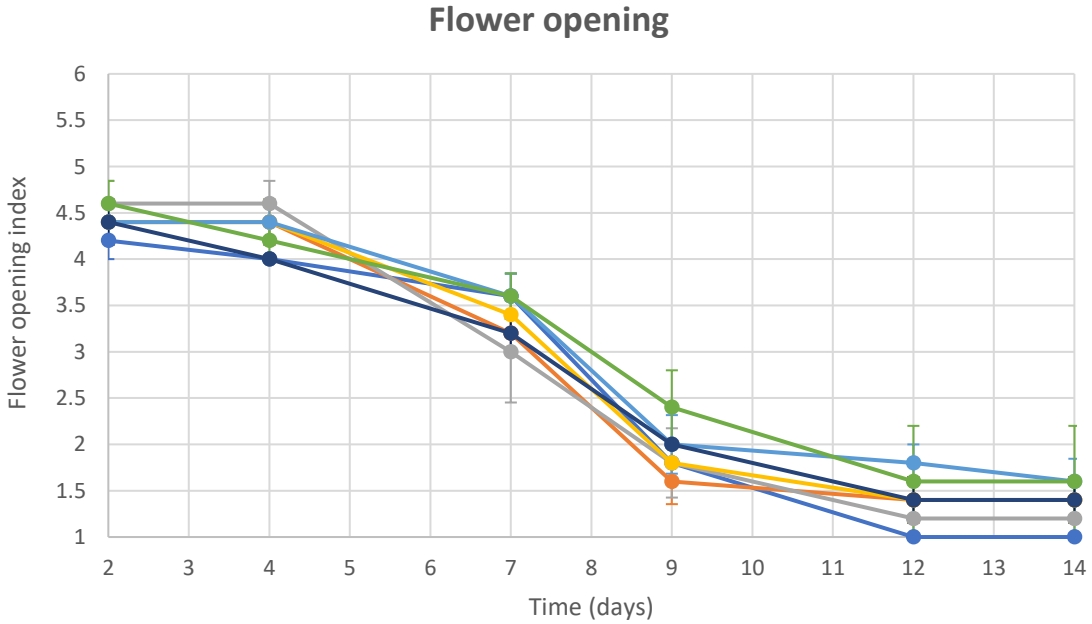
Unfortunately, the initial bad quality of the leaves and the powdery mildew and spider plagues prevented to obtain neither conclusive nor relevant results, since roses must be perfect to have any commercial value. Therefore, in real conditions BC would never be applied to such low-quality roses.

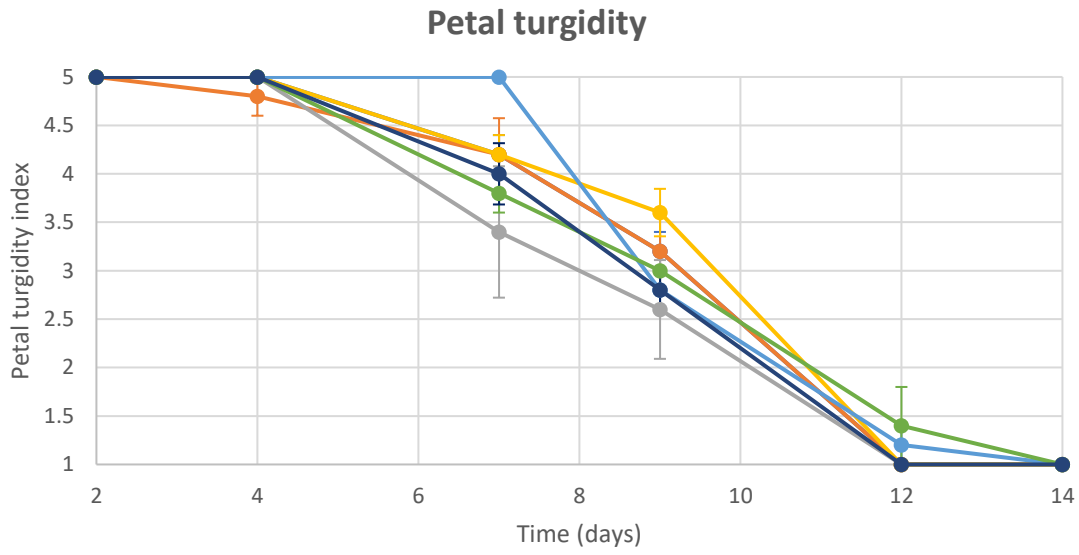
***Figure 8**. Cut areas at day 12 of* Control 4h *(top) and* Ground BC *roses. The latter, especially the three on the right, are visibly less brownish than the former.*

Statistical analysis (Kruskal-Wallis test and Dunns posttest, since the data failed the normality test) revealed no significant differences between controls and BC-treated flowers. Nevertheless, we could clearly observe that the 'Ground BC' treatment (page 8) improved cut area quality (the least commercially relevant of the rose traits being assessed), as compared to the 'Control 4h', after 12 days (Figure 8).

The error bars from the following quantitative result graphs represent the standard error for n=5. Legend:






Petal turgidity
Petal turgidity index
5
4.5
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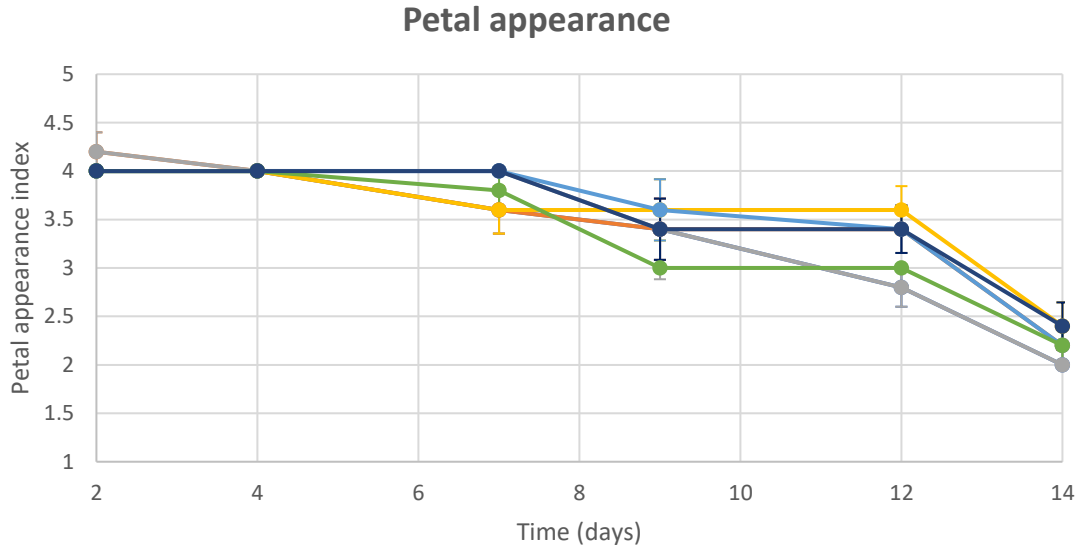
3.5
3
2.5
2
1.5
1
2
4
6
8
10
12
14
Time (days)


Petal appearance
Petal appearance index
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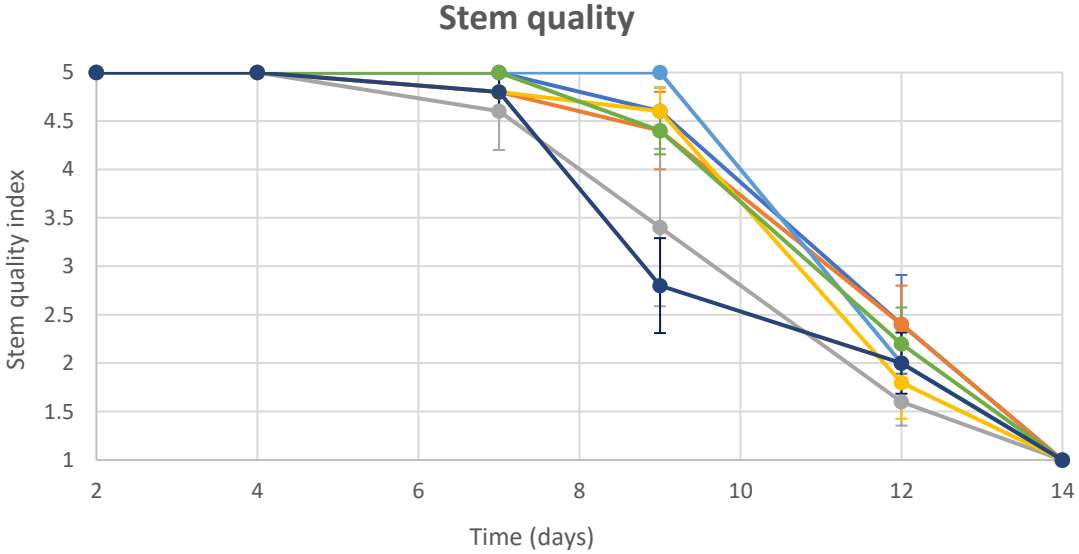
14
Time (days)


Stem quality
Stem quality index
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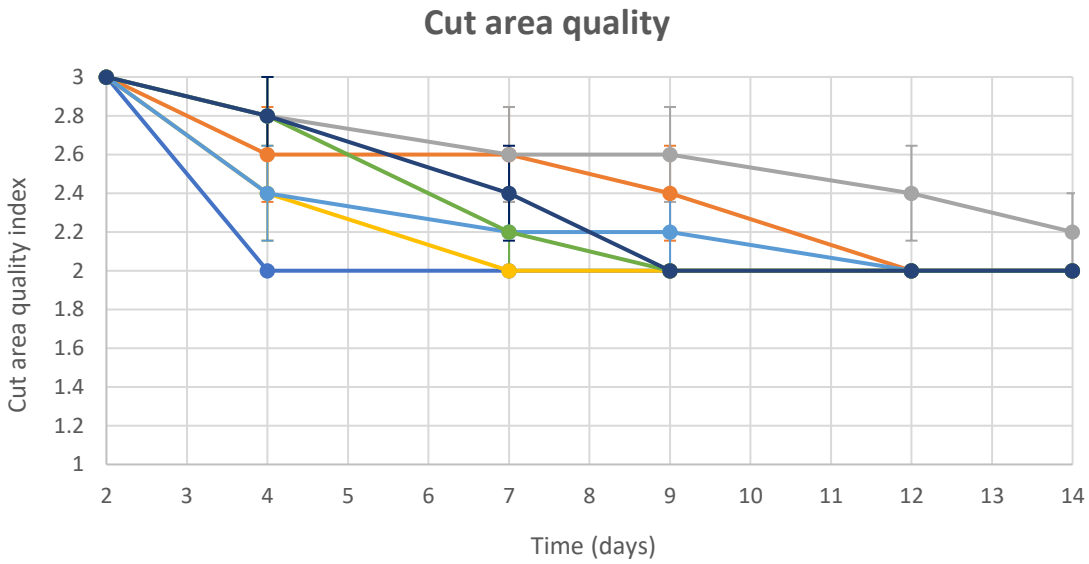
Time (days)



4.2. Effect of bacterial nanocellulose on grafted *Pinus pinea* success rate

4.2.1. Grafting efficiency

Clone no. 7 grafting
Sky: cloudy
Temperature at 11 a.m.: 14 °C
Temperature at 13 a.m.: 14 °C
Relative humidity at 11 a.m.: 76%
Relative humidity at 13 a.m.: 72%

Clone no. 8 grafting
Sky: cloudy
Temperature at 11 a.m.: 14 °C
Temperature at 13 a.m.: 16 °C
Relative humidity at 11 a.m.: 59%
Relative humidity at 13 a.m.: 56%

Professional grafter	Efficiency 1 (minutes/graft)	Efficiency 2 (minutes/graft)	Efficiency 3 (minutes/graft)	Mean (minutes/graft)
Manel	4.50	4.80	3.83	4.38
Jesús	3.00	3.50	-	3.25
Rafel	4.25	4.13	-	4.19
Montse	4.40	4.00	-	4.20
				4.00

Total grafts per grafter: 38

$$\text{Total time per grafter} = 4\,\frac{\text{minutes}}{\text{graft}} \times 38\ \text{grafts} \times \frac{1\ \text{hour}}{60\ \text{minutes}} = 2.54\ \text{hours}$$

$$\text{Efficiency per grafter} = \frac{38\ \text{grafts}}{2.54\ \text{hours}} = 14.99\ \frac{\text{grafts}}{\text{hour·person}}$$

$$\text{Efficiency decrease from control} = \frac{16.6 - 14.99}{16.6} \times 100 = 9.7\%$$

For better results, it would be advisable to incorporate BC bands in the adhesive tape, so that no extra step would be necessary during grafting. Nonetheless, these results are positive according to Forestal Catalana, since that efficiency decrease is not excessive to them.

4.2.2. Observations and modifications

Day 21

The original plan was to unwrap the graft seals of all condition 2 plants. However, after initially removing them from 2-3 plants of both the condition 1 (control) and condition 2, which were visibly alive, it was observed that the graft unions from both conditions were identical (Figure 9). Consequently, it was decided to maintain the seals of the remaining condition 2 grafts to see if differences emerged after a longer period of BC contact (74 days). The control graft seal (adhesive tape only) was easier to unwrap than the one of condition 2, and it was more flexible. This could mean that BC is more likely to strangulate the plant, which would be detrimental because of an increase in calli production.

***Figure 9**. No differences were observed between condition 1 (left) and condition 2.*

A professional grafter noticed a slightly higher amount of resin at condition 2 plants compared with the control (where resin is localized over the graft union only), which may suggest BC favors its diffusion.

Day 30

Graft seals were removed from condition 3 plants. A total of 14 plants from all conditions were taken as samples for histological analysis, half of them from each clone, eight of them with alive grafts, and six with dead grafts. The following day, the graft seals were removed from all samples and stored. Sections as thin as possible were cut out of the graft union area and stored in ethanol 70 vol%. Pictures of the dead grafts were taken because the graft union was completely dry and detached, usually with fungal contamination, so only two of the dead samples could be cut into (very fragile) sections. This was true for both the control and the BC-containing conditions, so BC does not prevent contamination of the graft union. The contaminations could be either a cause or a consequence of the failed graft.

Day 60

It was decided to leave the graft seals two more weeks (until day 74). Instead, a longitudinal cut was made to open the seals and reduce their constriction, which could result in strangulation as the plant grows. Some pictures were taken.

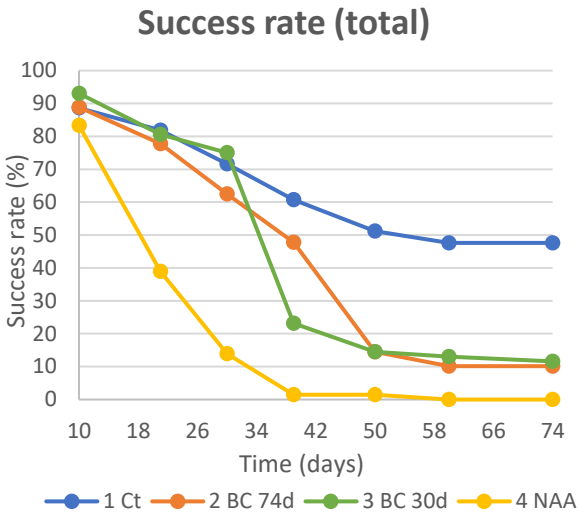
Day 74

Graft seals were removed from conditions 1, 2 and 4. Six plants were selected in total, two from each of the conditions 1, 2 and 3 (there were no successful grafts of condition 4), and half of them from each of the two scion clones. Four days later, sections were made and stored in the same way as day 30.

4.2.3. Grafted *Pinus pinea* success rate

Table 1. *Success rates (alive grafts / total grafts * 100) for all conditions at different times from the day grafting was performed.*

Success rate (%)				
Time (days)	**1 Ct**	**2 BC 74d**	**3 BC 30d**	**4 NAA**
10	88.6	88.9	93.1	83.3
21	81.8	77.8	80.6	38.9
30	71.6	62.5	75.0	13.9
39	60.7	47.8	23.2	1.5
50	51.2	14.5	14.5	1.5
60	47.6	10.1	13.0	0.0
74	47.6	10.1	11.6	0.0

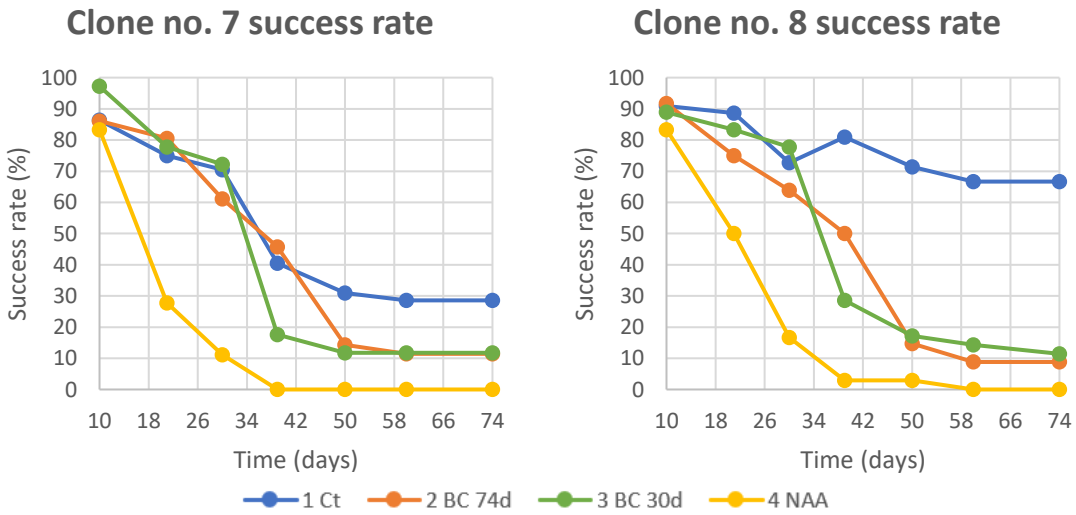


No improvements in grafting success rate were observed when including a BC band in contact with the graft unions. In fact, the BC rates were worse than the conventional graft seals (control), especially from day 30. It was observed that graft unions wrapped in BC showed more pathogen contamination and decay.

From BC+NAA (condition 4) results, it is evident that the NAA hormone had a negative impact on the success rate of grafted *P. pinea*. The concentration used might have been excessively high.

The clone no. 8 control had higher success rates than that of clone no. 7, whereas from 50 days onwards both clones had similar results in the conditions with BC.

On day 30, removing the graft seals from condition 3 plants clearly impacted the survival of the grafts, whose success rates showed a sharp decrease. This could be explained by assuming that their graft unions were not strong enough to sustain the lack of constriction and hydration resulting from seal removal, even if they had seemed alive. Another reason could be an excessively rough manipulation when the seals were removed.



On day 39, the plastic bags covering the top of the plants were removed from all conditions. This particularly affected the condition 2 of clone no. 7, because up until that point it had

presented a similar success rate than the control. It may have been caused by adverse environmental factors for which plastic bags could have offered some protection, or because of irrigation from above. Finally, no changes are observed from day 60 to 74, so 60 days can be considered the minimum time necessary to have reliable definitive success rate results.

Almost all condition 2 grafts, successful or not, were darkened under the seals (contamination or decay). This was not nearly as evident in control grafts. It is possible that water leaked into the seals once the plastic bags had been removed because of the irrigation from above, and it was retained by the BC strips, which could have accelerated decay and provided an ideal environment for fungi growth. This could have been a factor in the death of many condition 2 grafts.

When removing the graft seals, differences were also noted. The adhesive tapes were generally unwrapped quite easily, they had kept clean and outgrown pine needles had perforated some of them. On the other hand, to remove the adhesive tape + BC strip it was necessary to make a cut along the seal: the BC was very attached to the graft union and adhesive tape, thus preventing it from stretching (that is, it would probably strangle the area).

Clone no. 7 success rate (%)				
Time (days)	1 Control	2 BC 74d	3 BC 30d	4 PNH NAA
10	86.4	86.1	97.2	83.3
21	75.0	80.6	77.8	27.8
30	70.5	61.1	72.2	11.1
39	40.5	45.7	17.6	0.0
50	31.0	14.3	11.8	0.0
60	28.6	11.4	11.8	0.0
74	28.6	11.4	11.8	0.0
Clone no. 8 success rate (%)				
Time (days)	1 Control	2 BC 74d	3 BC 30d	4 PNH NAA
10	90.9	91.7	88.9	83.3
21	88.6	75.0	83.3	50.0
30	72.7	63.9	77.8	16.7
39	81.0	50.0	28.6	2.9
50	71.4	14.7	17.1	2.9
60	66.7	8.8	14.3	0.0
74	66.7	8.8	11.4	0.0

4.2.4. Histological analysis

Day 30

Clone no. 8 scions performed better than the ones of clone no. 7. No relevant differences are observed between the controls (condition 1) and the conditions with BC strips but without hormone (2 and 3), neither in the quantity of undifferentiated tissue generated to form the graft union, nor the quantity, quality and situation of new vessels.

The condition 4 plant with clone no. 7 scion was dead and presented fungal contamination, even though it was initially deemed successful due to its green color. Conversely, the condition 4 plant with clone no. 8 scion was indeed alive and was virtually identical to the other conditions.

In most grafts there was a noticeable accumulation of lignin or darkened tissues (oxidized, probably by phenols) on the cut surfaces, specifically around free vascular cambiums (i.e., the ones which have not become formed the graft) of both the scion and the rootstock. Therefore, it could be tried to treat the scion and rootstock with antioxidants, for instance through the BC strips.

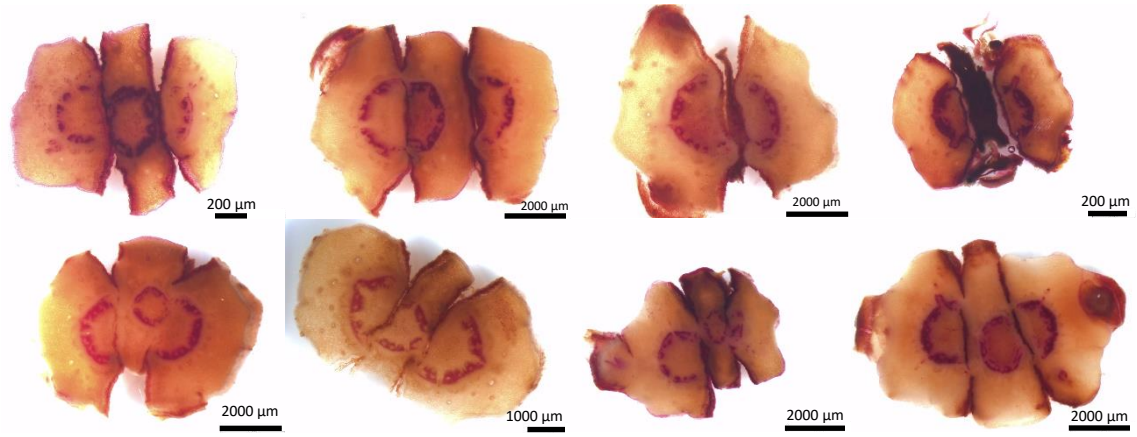


***Figure 10**. Top row: clone no. 7 samples; bottom row: clone no. 8 samples; columns from left to right: conditions 1 to 4. Images taken using Olympus SZX16 stereo microscope and software Cell^D.*

Day 74

In the conditions with BC, the quantity of newly formed tissue was greater than the controls. However, this did not result in a better union between scion and rootstock. In fact, the extra layers of undifferentiated tissue that BC provided could have affected the viability of the grafts negatively. The reason is that too large a quantity of vessels would be necessary to bridge the increased distance between scion and rootstock, so the former would not be able to get enough nutrients from the latter.

At the controls, new vascularization seemed more uniformly distributed around the old

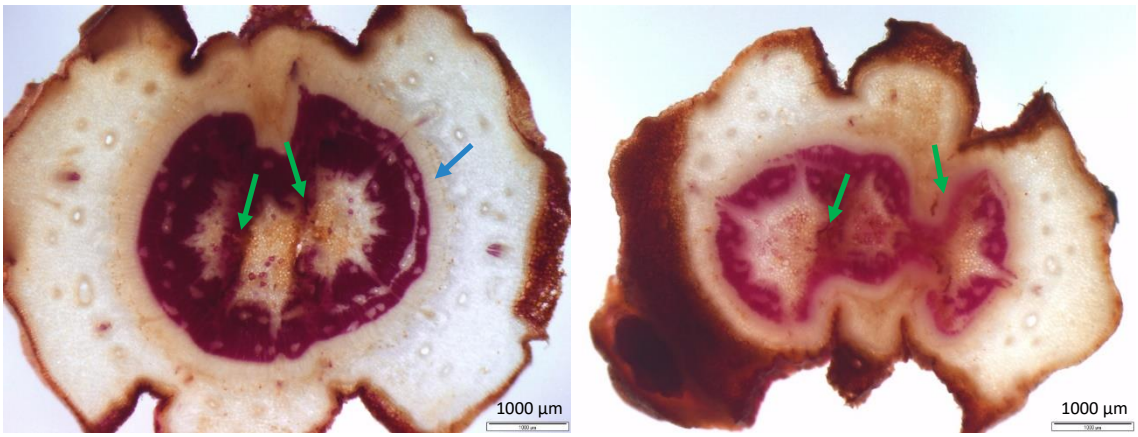


***Figure 11**. Condition 1 (control) clone no. 7 (left) and condition 2 (BC 74 days) clone no. 8 samples. **Green arrows**: newly formed vascular tissue. **Blue arrow**: new circle of vascular tissue around the original ones of the scion and rootstock. The purplish areas are lignified differentiated tissues arising from vascularization. Images taken using Olympus SZX16 stereo microscope and software Cell^D.*

vascular cambiums, but it also depended on how the scion was cut and joined to the rootstock.

No noticeable differences between conditions 2 (74 days wrapped in BC) and 3 (30 days in contact with BC). In general, on day 74 the vascular tissue was well formed, the old tissue being clearly distinguishable from the new one in many images. No condition 4 images were taken under the microscope because there were no surviving grafts.

The same oxidation described on day 30 was observed, but it was not as widespread. This may be the case because some of the apparently alive oxidized grafts did not survive to day 74, which would imply that this oxidation was another cause of graft failure.

4.2.5. Graft seal examination under stereo microscope and SEM

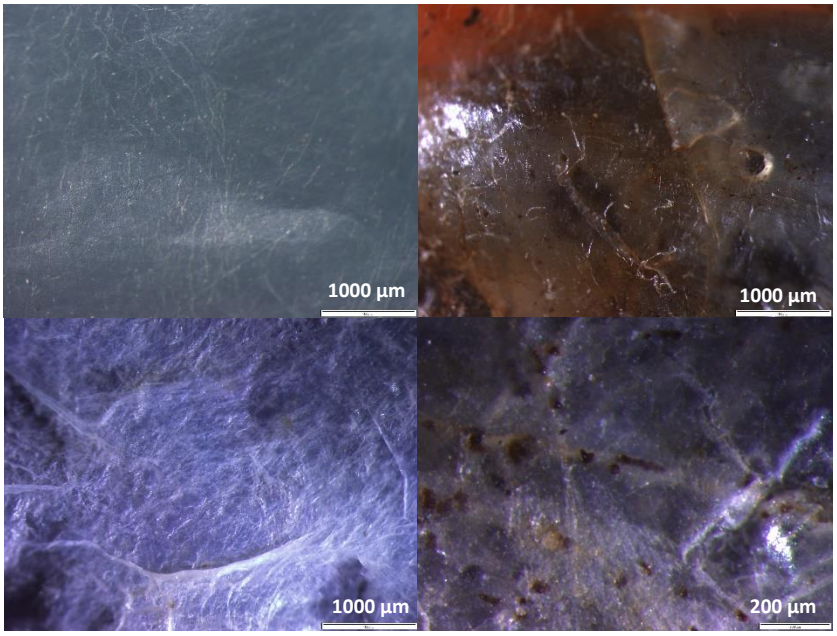


***Figure 12**. Clockwise from top-left: control (dried BC membrane); autoclaved graft seal from condition 3 (BC 30 days); graft seal from condition 2 (BC 74 days) treated with ethanol and bleach; graft seal from condition 3 treated with ethanol and bleach. Images taken using Olympus SZX16 stereo microscope and software Cell^D.*

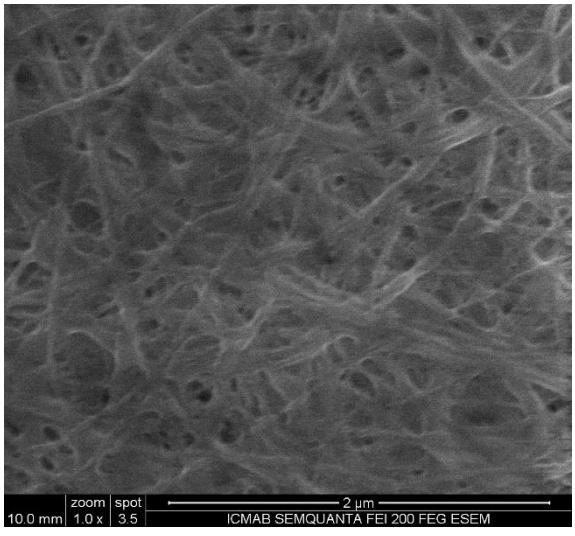


***Figure 13**. SEM image of a dried BC membrane, where its nanometric microfibers can be observed. Low vacuum, 10 kV, spot size 3.5, working distance 10 mm, magnification 100000X.*

On the surface of the graft seals that were in contact with BC some deposited material was observed, mainly brown in color, which could be spores, sclerotia and hyphae from fungal contaminations. There was no visible plant material (fragments of pine needles had been removed before the preparation of samples from BC strips and, slightly less frequently, adhesive tape).

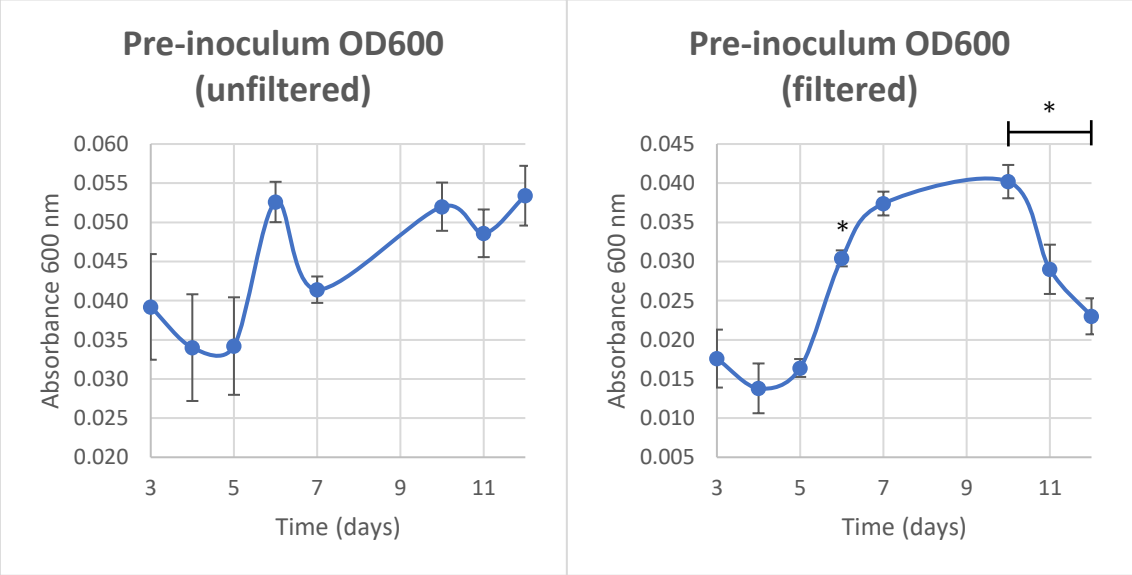
The samples that were (unsuccessfully) sterilized using ethanol and bleach had lost most color. On the other hand, the autoclaved samples had more hyphae remaining on their surface (especially

evident in condition 2 graft seals). BC was more wrinkled because it became closely attached to the adhesive tape, which was thawed and compacted due to the high temperatures of that sterilization method.

No conclusions could be reached regarding differences in BC structure between the different conditions. Under the stereo microscope, the hypha and other contamination made it difficult to discern the actual structure of BC under them. Furthermore, SEM images had low resolution because the samples retained too much humidity, so a low electron beam energy had to be used to avoid sample damage. The structures could be observed anyway, but no conclusive differences could be inferred from them.

4.3. Study of *Komagataeibacter xylinus* static culture growth

The KX strain (NCIMB 5346) growth curve was obtained by measuring the optical density of the pre-inoculum culture (P4) at 600 nm wavelength:



From these results, it was confirmed that the filtered samples were more reliable than the unfiltered ones, since they had less standard deviation and the curve obtained was much closer to what would be theoretically expected. The error bars represent the standard error (from 5 replicates) and asterisks on the growth curve from filtered samples indicate that the data point below them is significantly different from the previous one, or from the one at the start of the marked range ($p<0.05$). Normality tests, one-way ANOVA test and Turkey post-tests were used to verify this, using the software GraphPad Prism. Basically, then, the growth curve shows that the bacterial cell number stays constant until the fifth day after subculture (lag phase), at which point it increases

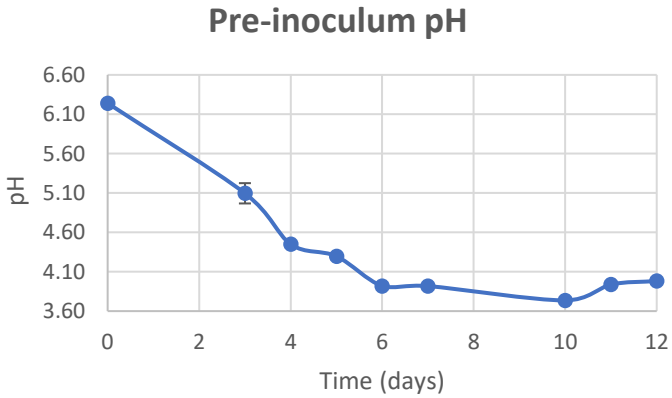


***Figure 14**. Pre-inoculum pH as a function of time of culture growth in days.*

rapidly (log phase). On day six, it has already reached the stationary phase. Finally, the death phase begins after day nine.

Another proxy for KX cell culture growth is the pH (Figure 14). This result suggests that pH decreases at an approximately constant rate until day six, when It stabilizes. It could mean that the medium is depleted of glucose around the sixth day, which coincides with the start of the KX culture stationary phase.

The pH and OD600 of the Stock inoculum (P3) used to subculture the pre-inoculum was also measured on day zero (95% confidence intervals for n=8): 4.29 ± 0.05 (pH); 0.027 ± 0.002 (unfiltered OD600); 0.018 ± 0.002 (filtered OD600).

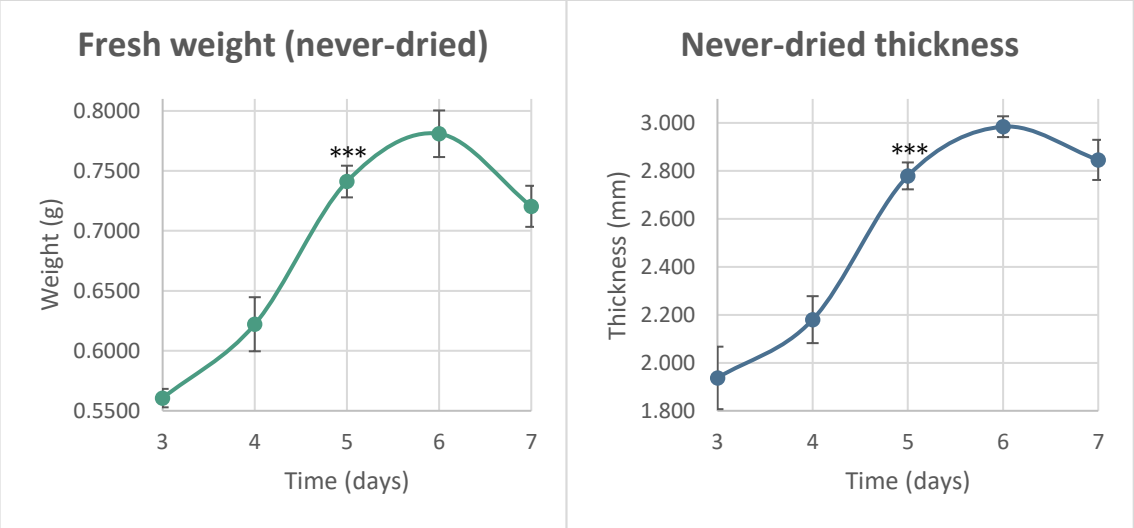


Figure 15. *Graphs showing fresh weight in grams (left) and thickness in millimeters of never-dried BC membranes against the time of pre-inoculum (P4) culture growth, in days. Error bars represent standard errors for n=7. Asterisks indicate a significant difference (determined using one-way ANOVA and Turkey post-test) between a data point and the previous one, or the one at the other extreme of the drawn range, with *, ** and *** meaning 0.01<p≤0.05, 0.001<p≤0.01 and p≤0.001, respectively.*

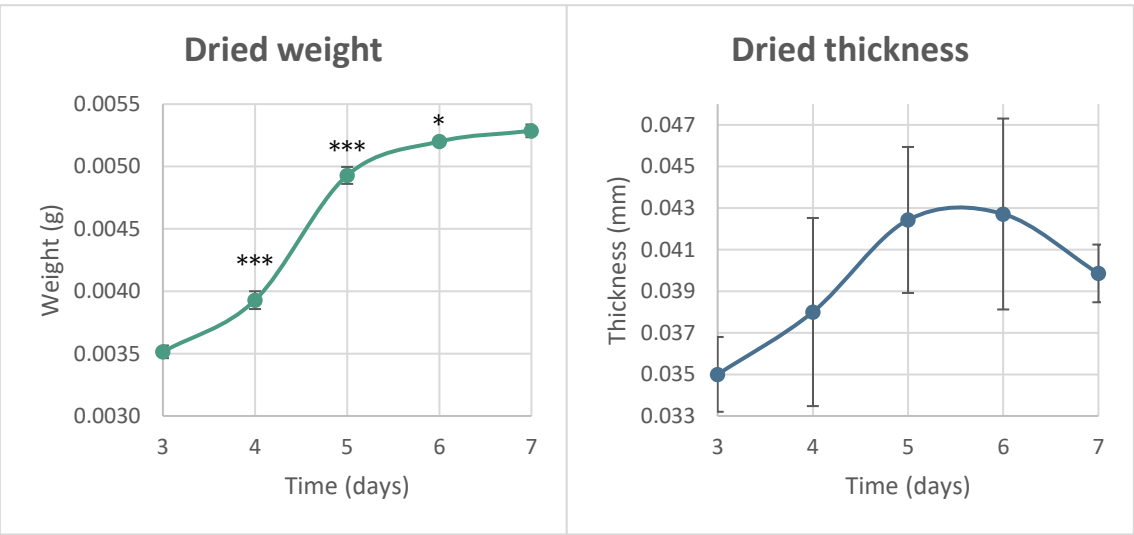


Figure 16. *Graphs showing weight in grams (left) and thickness in millimeters of dried BC membranes against the time of pre-inoculum (P4) culture growth, in days. Error bars represent standard errors for n=7. Asterisks indicate a significant difference (determined using one-way ANOVA and Turkey post-test) between a data point and the previous one, or the one at the other extreme of the drawn range, with *, ** and *** meaning 0.01<p≤0.05, 0.001<p≤0.01 and p≤0.001, respectively.*

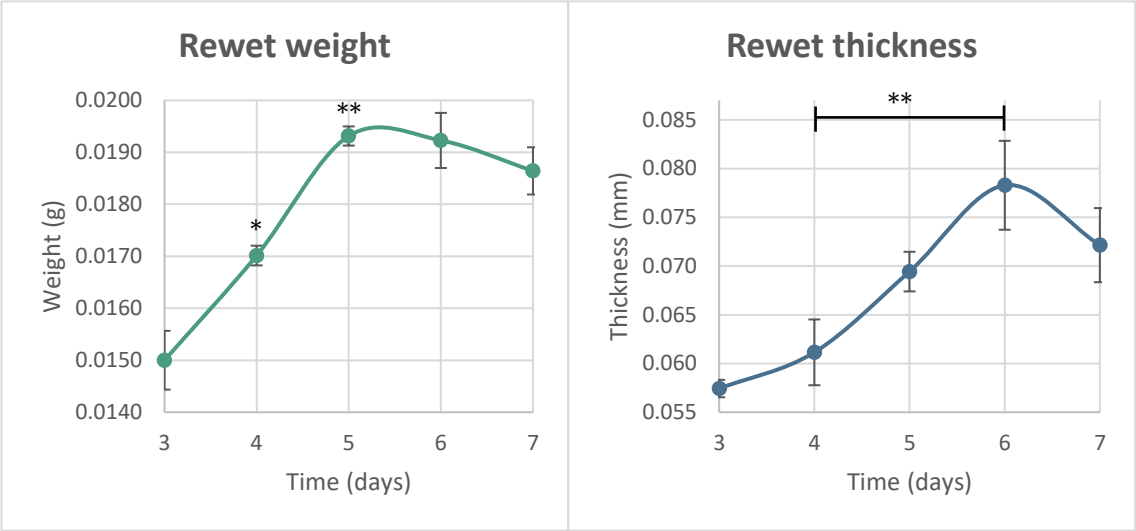


Figure 17. *Graphs showing weight in grams (left) and thickness in millimeters of rewet BC membranes against the time of pre-inoculum (P4) culture growth, in days. Error bars represent standard errors for n=7. Asterisks indicate a significant difference (determined using one-way ANOVA and Turkey post-test) between a data point and the previous one, or the one at the other extreme of the drawn range, with *, ** and *** meaning 0.01<p≤0.05, 0.001<p≤0.01 and p≤0.001, respectively.*

The fresh weight and thickness of never-dried BC membranes (Figure 15), which grew on the surface of P5 cultures in 24-well plates, increase exponentially from days three to five (pre-inoculum growth time), then the pace of growth slows until day six, when they start to decrease again. However, the most significant trend is that these properties reach their maximum around day five (significance level of α=0.05).

Note: the y-axis does not start at zero in the previous nor the following graphs because, for comparison purposes, the shape and trends are deemed more relevant than the actual values.

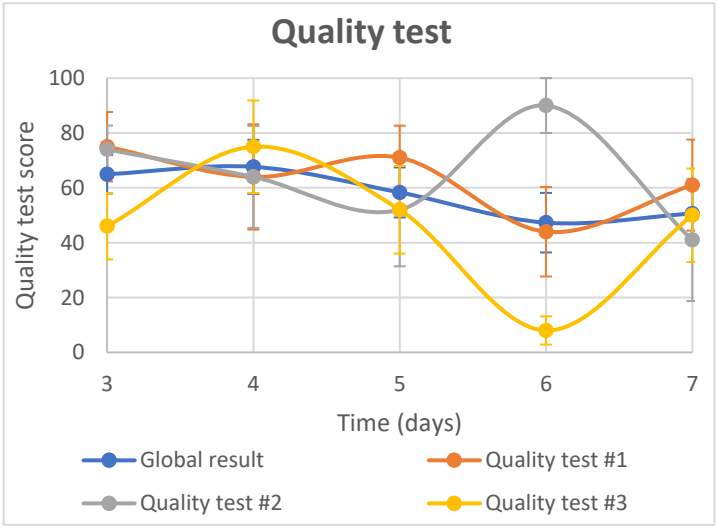


Figure 18. *Graph showing quality test scores of BC membranes against the time of pre-inoculum culture growth. Three quality tests were performed at different times with five membranes for each data point (n=5). Therefore, for the global result, n=15. The error bars represent the standard error. The quality test score is the average regeneration rate (length of cut regenerated divided by total length and multiplied by 100). The control batch membranes scores were 90.8, 60.7 and 58.3 in quality tests #1, #2 and #3, respectively.*

Likewise, the weight of dried membranes (Figure 16) increases exponentially until the fifth day, at which point the growth rate slows and eventually stabilizes after day 6. The thickness of dried membranes changes with time following the same shape of the measurements of never-dried BC membranes (i.e., it decreases from day six to day seven), however the standard error is so large that the pattern cannot be validated statistically.

The graphs showing weight and thickness of rewet membranes as a function of pre-inoculum growth time are, again, similar in shape as when the membranes were never-dried and dried. The

main difference is that rewet weight shows an approximately constant growth rate until day five, and afterwards it starts to decrease slowly.

The quality test results (Figure 18) do not reveal conclusive patterns, even though the global result resembles the thickness measurements as far as the shape of the graph is concerned, but inverted (decreases until day six and then starts to increase again). This would suggest that the thinner the BC membrane, the better it regenerates cuts in *N. benthamiana* leaves. This is further supported by the fact that the control membranes, which were visibly thinner than those of the experiment, presented a higher quality test score (69.5 in the global result).

5. Conclusions

5.1. Effect of bacterial nanocellulose on the preservation of cut flowers

5.1.1. General conclusions

- Since the quality of the roses used in the experiment was insufficient to have commercial value, the results obtained were neither representative nor reproducible.
- BC did not provide statistically significant improvements in rose quality and lifetime.
- Therefore, the results obtained could not support the initial hypothesis because the roses used had little commercial value from the start, and they did not show any commercially relevant improvement nonetheless.
- On a positive note, the Ground BC treatment visibly slowed the darkening of the cut area of the roses.

5.1.2. Future directions

In the future, a new experiment to test BC healing potential on cut roses may be considered, being conducted by rose producers on the field, as they are better equipped to discern any improvements BC may offer. Then, they could evaluate whether the results they get are worth the extra investment required to use BC.

5.2. Effect of bacterial nanocellulose on grafted Pinus pinea success rate

- The results obtained from this experiment led to the rejection of the initial hypothesis: BC did not improve the success rate of stone pine grafts, quite the opposite.
- Nevertheless, it provided some valuable information that Forestal Catalana will consider for future grafting campaigns:
    1) As fungal contamination was seen to be related to graft failure, they have planned to clean the scalpels they use for grafting more thoroughly, for instance with ethanol, and alternate them to keep them disinfected for a longer period.
    2) They have also considered sending some plants to a laboratory to identify the type of fungi, so that more specific actions can be made.
    3) In addition, they have entertained the possibility of watering the plants from below instead of irrigating them from above.
    4) Finally, they could pre-treat the scion and rootstock with antioxidants before joining them to improve the chances of graft union success, as reasoned previously (page 21).

- If a new experiment was conducted, a lower dose of NAA could be used; the BC strips could be coupled with the adhesive tape to eliminate grafting steps; and ways to prevent BC strangulating the graft union should be considered.

5.3. Study of *Komagataeibacter xylinus* static culture growth

5.3.1. General conclusions

- The results showed a relationship between the time of *K. xylinus* static culture growth and the quantity of BC that would ultimately be produced, with the caveat that it is only reproducible for the KX strain used (NCIMB 5346) and the procedure followed.
- Consequently, the hypothesis was supported by the results, but it cannot be generalized to all strains and methods.
- The results provided relevant information to improve the BC production protocol in the laboratory:
    - to optimize efficiency (i.e., to produce the maximum possible quantity of BC in the shortest possible time) it would be advisable to grow the pre-inoculum for five days. This is because the weight and thickness graphs (page 23) have shown that these properties grow significantly from days four to five, but the difference between days five and six is much smaller, so it may not be worth the wait.
    - Instead, if the overall final quantity of BC produced is deemed more important than efficiency, waiting for six days would be advisable.
- These results can be useful to researchers who use this strain and protocol and want to maximize the production of BC or modulate the thickness of membranes based on OD600 measurements.

5.3.2. Future directions

For more quantitatively reproducible and accurate results, more independent iterations of the same experiment should be conducted. Additionally, to generalize the findings to more bacterial strains and production methods, several different experiments could be done to look for common patterns.

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