Sterilization method of perennial ryegrass tillers for callus induction and downstream applications

Bachelor thesis

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Karjamaa-raiheina võsude steriliseerimise meetod kalluse indutseerimiseks ning allavoolu protsessideks

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Declaration

Hereby I declare that I have compiled the paper independently and all works, important standpoints and data by other authors have been properly referenced and the same paper has not been previously been presented for grading.

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Abstract

Perennial ryegrass (*Lolium perenne* L.), as an important forage crop, needs to be able to withstand the change in weather conditions caused by climate change. Normally, classical breeding is used in crops to provide plants with traits such as abiotic stress resistance. This cannot be easily done with perennial ryegrass due to its out-crossing reproductive nature. Genome editing technologies could help to produce perennial ryegrass plants that are able to cope with, for example drought and frost. One of the most used systems to transform a plant genome is with *Agrobacterium tumefaciens*, and in the case of CRISPR-Cas9 technology, the effector complex is delivered to the plant cells via agrobacteria. Transformed plants can be obtained by targeting meristematic tissue (e.g., shoot apical meristems), abundance of which can be generated quickly by inducing callus from it.

The goal of the current thesis was to develop sterile *in vitro* cultures of tillers and calli from several perennial ryegrass genotypes using soil-grown tillers as a starting material. The specific genotypes are used in an international project (EditGrass4Food) related to CRISPR-mediated edition of *Lolium perenne* L. for its better adaptation to frost and drought in Northern Europe.

Four sterile *in vitro* cultures of tillers and five cultures of calli were accomplished, after testing different sterilization methods. Additionally, a transformation vector containing the CRISPR-Cas9 complex including a specifically designed guide RNA sequence was obtained. Said vector can be used to obtain genome edited perennial ryegrass plants in the near future.

Abbreviations

2,4-D – 2,4-dichlorophenoxyacetic acid

BAP – 6-benzylaminopurine

bp – basepair

C – Cytosine

Cas – CRISPR associated protein

CDS – Coding sequence

CIM – Callus Inducing Media

CRISPR – Clustered regularly interspaced short palindromic repeats

crRNAs – CRISPR RNAs

G – Guanidine

indel – insertion or deletion

IPTG – Isopropylthio-β-D-galactoside

kb – kilobase

LA – Lysogeny agar

LB – Lysogeny broth

MM – Maintenance media

PAM – Protospacer Adjacent Motif

PCR – Polymerase chain reaction

RNP – Ribonucleoprotein

SAM – Shoot apical meristem

SCM – Subculture media

TaU6 - *Trititicum aestivum* U6 Pol III promoter

tracrRNAs – trans activating CRISPR RNAs

T-DNA – transfer DNA

X-Gal – 5-bromo-4-chloro-3-indolyl- β -D-galacto-pyranoside

Introduction

The EditGrass4Food project's mission is to improve the cold and drought tolerance of *Lolium perenne* L. (perennial ryegrass) with CRISPR-Cas9 technology, so it can be grown in the Northern-Eastern European countries, including Estonia. With the ongoing climate change, reduced precipitations and warmer temperatures are expected. This implies less snow covering the perennial plants, including ryegrass, which leads to increased frost damage. The present thesis is one part of the project, focused on the creation of *in vitro* cultures of tillers and calli for plant transformation using the CRISPR-Cas9 system.

For the CRISPR-Cas9 technology to work, it needs to be introduced into the plant cells. This can be done using biolistics, pollen, silicon carbide whiskers or the bacterium *Agrobacterium tumefaciens* (now known as *Agrobacterium radiobacter*). This crown-gall causing bacterium can target various parts of the host plant, such as roots, shoots, seeds or meristematic tissue. Gall causing DNA from the bacterium's tumor-inducing plasmid enters the plant cells and integrates into their DNA. Modifying parts of this mechanism, a binary vector method was developed that allows insertion of almost any DNA of interest, including genes coding for the CRISPR-Cas9 complex. The introduction of this complex into plant cells makes genetic engineering possible.

Targeting highly differentiated tissue, from which edited DNA would not be transmitted further on, would give only a partially edited plant, which is why quite often dedifferentiated tissue (e.g., seeds, embryos or meristematic tissue) is targeted. One way to get a continuous source of meristematic tissue is by induction of callus—a mass of disorganized, often undifferentiated cells.

Developing and optimizing a successfully working transformation system (e.g., an *A. tumefaciens*-mediated CRISPR-Cas9 system) requires a great amount of targetable tissue. Callus induction and *in vitro* culture establishment for the plant of interest can help overcome this limitation.

The goal for this thesis was to start *in vitro* tiller and callus cultures for several ryegrass genotypes, for use as a clean *in vitro* plant collection and as a source material for callus induction. For that, a reliable method for sterilization of soil-grown perennial ryegrass tillers had to be developed. Moreover, the first steps of the downstream processes needed for the obtention of a transformed plant were to be tested.

In the literature review section, the topics of perennial ryegrass, tissue culture, CRISPR-Cas9 system and plant transformation using *Agrobacterium* are discussed. The materials and methods section describes the sterilization method, starting and maintenance of *in vitro* tillering and callus cultures and assembly of a plasmid containing a guide RNA for transforming plants with CRISPR-Cas9 technology.

A successful *in vitro* culture was established for 6 ryegrass cultivars and a plasmid containing a guide RNA for further *A. tumefaciens* and CRISPR-Cas9 mediated plant transformation was acquired.

1. Literature review

1.1. Perennial ryegrass

Perennial ryegrass (*Lolium perenne* L.) is an important grass growing in temperate regions. This significance is related to some of the plant main characteristics, such as its ability to quickly germinate, strong seedling vigor, rapid establishment and tolerance against wear [1]. These traits explain why it was probably the first herbage grass that became a crop plant [2] and its wide use for cattle grazing, feeding, and amenity (e.g., for sports turf). [3]

Although perennial ryegrass is also present in Northern Europe, the genetic diversity of the populations growing in the Nordic-Baltic latitudes is restricted since it is not a native species of such regions [4].

As for its reproduction, perennial ryegrass is a wind-pollinated, outcrossing and highly self-incompatible species [5]. The self-incompatibility system of ryegrass, as in other angiosperms, has the goal to avoid the production of zygotes after self-fertilization of flowers. This prevents inbreeding which would result in a decrease in genetic variation among populations. The decrease will in turn diminish the chance of said population to cope with environmental changes [6, 7].

When working with crops, the self-fertilization or selfing of an organism is a procedure commonly used in classical breeding. Among other purposes, selfing allows to preserve a line with interesting traits and to generate new organisms that present homozygous genotypes regarding a specific characteristic. Since this approach is highly inefficient in ryegrass, classical breeding methods are more limited and slower than when comparing with other crops [2].

Perennial ryegrass is phylogenetically related to other important crops, such as barley (*Hordeum vulgare*), Rye (*Secale cereale*), common wheat (*Triticum aestivum*), rice (*Oryza sativa*), corn (*Zea mays*) and fescue (*Festuca* spp.) [8]. The molecular mechanisms underlying stress tolerance are similar in these crops. Therefore, strategies aiming towards the modification of said mechanisms can be used as a reference when improving ryegrass.

Because of its important uses and the aggravation of climate change [9], which brings extreme fluctuations in temperatures, causing frequent cold waves and periods of prolonged drought, there is a need to improve the resistance of ryegrass, so that it can withstand these challenging conditions.

While there is already substantial effort invested to genetically modify perennial ryegrass, the overarching problems of technical complexity and laborious procedures remain. In the end, commonly genetic transformation relies on callus induction, propagation, and regeneration of explants [10].

1.2. Tissue culture

Manipulation of plant cells and tissues *in vitro* (“under glass”) is called tissue culture. It implies culturing plant tissue/cells on nutrient medium under sterile conditions. Tissue culture is a keystone in plant biotechnology since it is used for many applications like conservation of germplasm or for transgenic plant production [11].

The phenotypical similarity of ryegrass’s relatives helps to develop *in vitro* culture methods meant specifically for perennial ryegrass; for example, specific hormone concentrations can be extrapolated to perennial ryegrass tissue culture.

1.3. Callus

Callus is a mass of disorganized cells [12]. It can also be described as a transient tissue, similar to the blastema of animals [13]. One of the earliest callus tissue culture was done by Gautheret in 1939, when he managed to grow *in vitro* a carrot root [14]. During the last 40 years, calli have been used to regenerate whole transgenic plants with the help of *Agrobacterium tumefaciens*.

Root is not the only explant tissue from which calli can be obtained from. For example, perennial ryegrass callus has been induced from immature inflorescence, mature seed, leaf base, meristem tip and axillary bud [5]. Calli can be induced from shoots or tillers. For this, the sterilization of seeds from which the germinated tillers can be used is a crucial step [15]. Tillers are new grass shoots formed by sections known as phytomers, which originate from a common apical meristem. Each of these phytomers are composed of a stem, leaves (blade and sheath), roots nodes, and axillary buds. It is worth mentioning that phytomers are structures exclusives of the Poaceae family [16].

Ryegrass, as an outcrossing species, cannot be crossed with another similar individual, which means sexual reproduction is not an option for conserving the genotype. Grasses have shoot apical meristem (SAM) in their tillers. These meristems do not stay at the tip of the shoot, therefore shoot tip culture is not possible. Moreover, when ryegrasses sexual reproduction is unsuitable, techniques such as embryo rescue and seed culture are not available. So, there are only a few options left for calli induction, one of which is induction of meristematic calli from tillers.

Callus can be divided into categories according to different criteria [5, 12, 17–19], but most commonly it is broadly classified as friable or compact [12]. The type of generated calli depends on the composition of media used for inducing them. The concentration of two types of phytohormones in the media, auxins and cytokinins, play a big role in obtaining calli efficiently [20] and stopping it from differentiating into specific tissues [12].

1.4. Plant regeneration

As with callus, it is possible to regenerate a whole plant from different plant source material. Ryegrass has also been successfully regenerated from cell suspensions and protoplasts [3]. Independently from the starting material used, the balance between auxins and cytokinins remains crucial for a successful regeneration [3].

1.5. Genetical variation

As the cultivars of perennial ryegrass differ amply genetically [5], there is a need to test the efficiency of the developed methods on more than one genotype. Additionally, the plant source material used for the induction of calli determines the genomic profile of calli itself [5]. Meristems excised from clones will generate into genetically uniform calli, while calli derived from seeds or gametes will generate into genetically diverse calli [10]. To preserve the genetic background of a particular cultivar or genotype, vegetative explants should be used. This decreases the variability among calli that could interfere with the genotypes and phenotypes of later regenerated plants [5]. Besides the source of callus, variation in media composition adds more layers to this complexity. All in all, it is necessary to optimize tissue culture conditions, such as genotype, explant tissue, culture media and supplements to transform perennial ryegrass successfully, without genetic instabilities [3].

1.6. Clustered regularly interspaced short palindromic repeats

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR associated protein (Cas) are parts of an adaptive immune system, present in some bacteria and archaea, first discovered in 1987. Bacteria use it to degrade foreign DNA coming from subsequent infections of a bacteriophage. Targeting of said DNA is possible thanks to the fragments of DNA, protospacers, that were integrated into the bacterial genome during the previous infection by the same bacteriophage. Although the system was discovered at the end of the 80s, this system's capability to work as genome editing tool was not proposed until 2012 [21, 22]. To date, two CRISPR systems have been discovered (types I and II) depending on the structure and function of their CRISPR associated protein (Cas) [23]. In the type II systems, such as the one isolated from Streptococcus pyogenes, the CRISPR arrays are transcribed and processed into small interfering CRISPR RNAs (crRNAs). These small RNAs combine with the trans activating CRISPR RNAs (tracrRNAs) to activate and guide the nuclease Cas9. All nucleases need a Protospacer Adjacent Motif (PAM) downstream of the target DNA. Each Cas protein has a specific PAM sequence that lets it bind and cleave the target DNA sequence (e.g., the Streptococcus pyogenes Cas9, the PAM sequence is 5'-NGG-3') generating a double strand break in case of the Cas9 nuclease. This particularity allows the use of the CRISPR type II system to generate changes in the genome of an organism. Virtually all organisms can repair DNA damage by using a mechanism known as nonhomologous end-joining. This mechanism is error prone, which means that it can lead to the insertion or deletion (indels) of nucleotides in the repaired DNA region. These changes can be exploited to generate mutations in a gene, for example knocking it out. The CRISPR II system has been engineered to perform genome editing in organisms. The modified system uses a chimeric RNA, known as guide RNA (gRNA) that mimics the function of the bacterial crRNA:tracrRNA. The gRNA is formed by a variable 20 nucleotides target-specific adaptor sequence and a non-variable RNA scaffold, which is derived from the crRNA:tracrRNA duplex [23].

1.7. Agrobacterium tumefaciens

Agrobacterium tumefaciens (*A. tumefaciens*, now known as *Agrobacterium radiobacter*) is a plant pathogen bacterium that produces crown gall tumors in plants. This bacterium delivers a segment of DNA present on a plasmid, known as the tumor-inducing (Ti) plasmid, when infecting plant cells. The Ti plasmid has different regions, including the *vir* section that encodes for the virulence (*vir*) genes, and the transfer DNA region (T-DNA). The T-DNA is the part of the plasmid that is transferred into the infected plant cells, and integrated into their genome, when the *vir* genes are activated. This activation happens when *A. tumefaciens* detects molecules secreted by wounded plant tissues. Using this mechanism of the plasmid Ti, a plant transformation method known as binary vector system was created [24]. In this system, *Agrobacterium* strains have been engineered to encode a plasmid that only has the *vir* genes and not the T-DNA (known as the helper plasmid). These strains can be transformed with a vector (named binary vector) that has a region of interest flanked by the borders of the T-DNA (Left and Right borders). The presence of said borders allows the transfection of the flanked region into plant cells when these are infected with *Agrobacterium* in the presence of certain elicitors like Acetosyringone. Virtually anything flanked by the T-DNA borders can be transferred into a plant cell and later integrated into its genome.

The binary vectors are used to insert exogenous DNA or ribonucleoproteins (RNPs) complexes (e.g., CRISPR-Cas complex) into the host plant or plant material such as protoplasts or *calli* (e.g., plasmids coding for a CRISPR-Cas complex).

2. Aims of the Study

The main goal of the thesis was to develop a sterilization method for the tillers of soil grown *Lolium perenne* L. plants.

The main goal implies:

- To establish a treatment for the *in vitro* tillering of *Lolium perenne* L. to asexually propagate different plant lines.
- To establish a treatment for callus induction and callus maintenance of different *Lolium perenne* L. plant lines.

3. Materials and Methods

3.1. Plant material

Throughout the experiments, 6 cultivars/ecotypes with varying drought resistance were acquired from the Lithuanian Agriculture and Forestry Sciences Center (LAMMC). Researchers from this institute are part of the EditGrass4Food project (more information can be found in Appendix 1). For simplicity and consistency, in this thesis "cultivar" or "ecotype" is referred to as line. The tillers used for *in vitro* cultures were kept in a growth chamber with the following setup: 21 °C, 60% humidity, and 26 µmol*m^{-2}*s^{-1} of light intensity. The calli was grown in the same growth chamber as the tillers, but in dark conditions instead than with light. For this, the plates with calli were placed inside a paper box.

3.2. Sterilization experiments of soil-grown tillers

A pilot experiment was performed to determine the parameters that should be used during the sterilization of tillers from the different plant lines. To preserve these lines, tillers from a different variety, Veja, were used. Seeds from this Lithuanian variety were provided to us by our colleagues from the LAMMC. In the pilot experiment we tested a sterilization method that involved using a 3-minute wash with 70% ethanol followed by a 45-90 minute wash with a bleach solution. The bleach solution—0.01% Tween-20, 50% liquid commercial bleach (ACE) and autoclaved distilled water—was prepared with liquid bleach whose active compound was at a concentration of 5%. The final step of the sterilization method involved washing the tillers with sterile water 4 times, 5 minutes per wash, to get rid of any remaining ethanol or bleach solution. The ethanol, bleach and water washes were performed inside a laminar using sterile 50 mL Falcons, to ensure sterile conditions. Different sterilization times using bleach were tested and it was decided that this step of the sterilization method would range between 45 and 90 minutes. These were the minimum required bleaching time to not observe contaminations of sterilized soil-grown tillers from Veja and the maximum time at which the tillers were still alive and growing one week after the sterilization was done, respectively. Determining these two time points allowed us to select the length of bleaching according to the state of the plant when performing the later sterilization experiments. If a plant had visible contaminations, it was decided to use a bleaching time longer than the minimum required 45 minutes.

Before the sterilization, the tillers were extracted from the pots (in which they had been growing) and individually separated. A brush was used to eliminate as much soil as possible, and the top part of the tillers together with most of the roots were cut. Then, the cut tillers were peeled by eliminating as many leaves as it was possible without damaging or breaking the tiller. Finally, the shoot part of the tillers was further cut leaving tillers of ~2 cm of total length. The ~2 cm tillers were divided into two groups. One group was used for the *in vitro* propagation of the line, and the other to induce the formation of meristematic calli.

For this thesis, 6 different lines (3177, 22, 3944, 3948, 3575 and 3776) were sterilized following the previously established method.

An additional 10-20 minute wash using a fungicide solution—0.1% of Switch® (Syngenta) and autoclaved water—was used for the last 4 sterilization experiments of this thesis. For these experiments, after the bleach wash 2 washes with water were done. Then, the tillers were immersed in fungicide solution that was followed by two final water washes. The additional fungicide wash was done to increase the chance of having non-contaminated tillers that would be

kept for the *in vitro* tillering. Lines 3177, 3575 and 3776 were sterilized using this additional fungicide wash.
The only line which was used in multiple different sterilizations was line 3177. This was because that line was the one growing the fastest in pots, which meant we had an abundance of it.

3.3. *In vitro* tillering

The tillers that were used for the *in vitro* propagation of the plant lines were processed after the sterilization to ensure the fast and easy discard of possible contaminated ones. This would allow us to preserve enough plant material to establish the *in vitro* tillering culture. The processing involved peeling them to eliminate as many dead leaves as possible to decrease the possibility of contamination. This, and all following handling of the tillers, was done working in a laminar in sterile conditions and using sterile scalpels and tweezers. The tools were re-sterilized using a glass beads sterilizer (Glass bead sterilizer 220-230 V, Duchefa Biochemie) so that each individual tiller was handled using sterilized tweezers and scalpels. Moreover, sterile petri dishes were used to provide a supporting surface to perform the "peeling" of the tillers. Once a tiller was processed, it was placed into solid Maintenance Media (MM)—30 g/L sucrose (Fisher BioReagents), 4.3 g/L Murashige & Skoog basal salts (Duchefa Biochemie), 8.8 µM BAP (Sigma-Aldrich), 2 mg/L glycine (AppliChem GmbH), 1 mg/L thiamine·HCl (Serva), 0.5 mg/L nicotinic acid (Sigma), 0.5 mg/L pyridoxine (Sigma), distilled water; final pH = 5.7-5.9—petri dish. The plate was sealed using Micropore™ tape (3M) to favor the gas exchange inside of the petri dish.
For the last line that was used in this thesis, 3776, a modified media composition was used. Half of the MM plates used for this line were supplemented with cycloheximide (10 mg/L initially, later replated to 25 mg/L) to counteract the high number of fungal contaminations previously observed after sterilizing tillers from the other lines.
After the tillers were placed in MM media, they were regularly checked to detect possible contaminations. Five to seven days after the sterilization, the tillers would normally present contamination. The contaminated tillers were then discarded, and the "clean" ones were passed into fresh MM plates. This was repeated until no further contamination was observed. After that, the tillers were passed into new MM plates every 3-4 weeks. When passing tillers after the first 3 weeks from the sterilization, part of their leaves was trimmed to both trigger their growth and to ensure that they would physically fit inside the media plates.

3.4. Callus induction

The tillers that were used for the *in vitro* propagation of the plant lines were processed after the sterilization to increase the chance of inducing meristematic calli. For this, the topmost part of the tiller and any remaining roots were sectioned. Then, a longitudinal cut was done in the tiller to damage the meristematic region. This "wound" was done to trigger the regeneration of the meristematic tissue that at the same time would lead towards the induction of calli. This processing, and all following handling of the tillers/calli, was done working in a laminar in sterile conditions and using sterile scalpels and tweezers. The tools were re-sterilized using a glass beads sterilizer so that each individual tiller/calli was handled using sterilized tweezers and scalpels. Moreover, sterile petri dishes were used to provide a support surface to perform the sectioning of the tillers. Once a tiller was processed, it was placed into solid Callus Inducing Media (CIM) callus induction medium—30 g/L maltose (Duchefa Biochemie), 4.3 g/L Murashige & Skoog basal salts, 0.6 mg/L $CuSO_4$, 5 mg/L 2,4-D (Serva), 1.03 g/L Murashige & Skoog vitamin mixture (Duchefa Biochemie), distilled water;

final pH = 5.7-5.9—petri dish. The plate was sealed using Micropore tape to favor the gas exchange inside of the petri dish.

After the tillers were placed in CIM media, they were regularly checked to detect possible contaminations. Five to seven days after the sterilization, the tillers would normally present contamination. The contaminated tillers were then discarded, and the "clean" ones were passed into fresh CIM plates. This was repeated until no further contamination was observed. Once calli was formed, it was passed into new Subculture media (SCM) subculturing medium—30 g/L sucrose, 4.3 g/L Murashige & Skoog basal salts, 8.8 µM BAP, 0.6 g/L CuSO4, 3 mg/L 2,4-D, 0.103 g/L Murashige & Skoog vitamin mixture, distilled water; final pH = 5.7-5.9—plates every 3-4 weeks. When passing stablished calli, any shoots or roots present were sectioned form the mass of calli. If the calli was dry or presented a dark brown coloration, indicating possible oxidation, it was discarded to avoid it from excreting any metabolites that could the overall health of the surrounding calli.

3.5. Calculations concerning contamination and tillering rates, calli induction and calli growth

Contaminations were recorded when they were visually detected. For *in vitro* tillering, the end point of contaminations and survivability data is the moment before the splitting of clean tillers. For calli, the recorded data reached the end of the experiment. For the tillering rate (reproduction rate) at a certain time point, the total number of tillers was divided by the number of tillers at the start point. The number of observed calli per treatment was divided by the number of tillers used for callus induction. The tillers that got contaminated were not considered. The capacity of calli to grow was estimated as the average area covered by callus at a certain time point, considering the number of tillers used for the induction. On each passage to fresh medium, the area of the plates covered by calli was approximately estimated and divided by the number of alive and clean tillers. Only three treatments of specific lines could be compared because of the available recorded data. In the case of these three lines and treatments, no contaminated callus was observed during the period of recording data.

In this thesis, graphs have been made using GraphPad Prism version 9.00 (GraphPad Software, LaJolla California USA).

3.6. Designing the guide RNA

The guide RNA (gRNA) used in this thesis was designed using the CRISPOR online tool [25]. In this program the region of the first coding sequence (CDS) of the *VERNALIZATION INSENSITIVE 3* (*VIN3*) gene of *Lolium perenne* L. was used as a query. The gRNA that was close to the start codon of the CDS and that had good efficiency and specificity values, was selected. Then, two oligonucleotides containing the gRNA (sense and antisense) and 4 base pairs (bp) overhangs (ACTT for the sense gRNA and AAAC for the antisense gRNA) were commercially synthesized (Microsynth AG, Switzerland) (the gRNA sequences can be found in Appendix 2). The overhangs were complementary to the sequence of the *Trititicum aestivum* U6 (TaU6) Pol III promoter, that controls the expression of the gRNA in the pDIRECT_25F transformation vector (available at Addgene) [26]. The *AarI* enzyme used for the vector enzymatic restriction (digestion), creates sticky-ends in the vector sequence that are complementary to these overhangs. This allows the correct ligation of the gRNA into the vector. Additionally, an extra guanidine (G) was added to the beginning of the sense

gRNA sequence and a cytosine (C) was added to the antisense gRNA sequence. This extra base is needed for the correct transcription of the gRNA when using the TaU6 promoter.

3.7. Assembly of a single guide RNA spacer into the pDIRECT_25F vector

A NanoDrop™ (Thermo Scientific™ ND-2000C) spectrophotometer was used to measure the approximate concentration of DNA solutions (gRNA and plasmid concentration).

The method used for the assembly of the transformation vector (vector including the designed gRNA) is based on the protocol 2B described by Čermák et al. [26] in their supplementary materials. The phosphorylation and hybridization (annealing) of the gRNA oligonucleotides was performed as described in the previously mentioned protocol. Conversely, the Golden Gate reaction was separated into two different steps: enzymatic restriction and ligation. This was decided to improve the efficiency of the ligation of the gRNA into the transformation vector (pDIRECT_25F) (the vector map can be found in Appendix 3).

3.7.1. Phosphorylation and hybridization of the guide RNA

The commercially synthesized gRNA oligonucleotides were phosphorylated for protection against degradation and to enhance the annealing to the unphosphorylated transformation vector. A mix of T4 DNA ligase buffer (10x), sense gRNA oligonucleotide (100 µM), antisense gRNA oligonucleotide (100 µM), and T4 polynucleotide kinase (10 Units), was incubated in a heat block (Eppendorf AG 22331) at 37 °C for 1 hour. Then, the gRNA was denaturated and gradually cooled down using a heat block and following the PCR program described in the protocol 2A described by Čermák et al. [26] (Appendix).

3.7.2. Enzymatic restriction of the transformation vector

The transformation vector (pDIRECT_25F) was enzymatically restricted (digested) by mixing the plasmid (1 µg), the *AarI* enzyme (1 Unit), the buffer *AarI* (10X) and the oligonucleotide present in the *AarI* kit (contains the AarI recognition sequence and enhances the activity of the enzyme) (0.025 mM). The mix was incubated 1 hour at 37 °C (optimal enzyme activity temperature) followed by 20 minutes at 65 °C (to inactivate the enzyme).

3.7.3. Gel purification of the digested transformation vector

The product from the enzymatic restriction was used loaded in a gel (1% agarose) and run at 75 V for 45 minutes. GelRed® (Biotium) was used to dye the gel before casting. Then, the gel was observed in a transilluminator to check if the expected fragment of 456 bp that was digested by the *AarI* enzyme was present in the gel. If the fragment was observed, the band corresponding to the digested transformation vector (16.8 kilobases, kb) was cut using a sterile scalpel blade and placed in a sterile Eppendorf. The digested plasmid was purified using the GeneJET Gel Extraction kit (Thermo Scientific), following the protocol described by the manufacturer. In brief, this kit uses silica-based columns to purify the DNA embedded in agarose. First, the fragment is mixed with a special buffer and melted at 55 °C for 10 minutes. Then, the solution containing the melted gel fragment is loaded into a spin-column that will be used to wash (with an ethanol containing buffer) and bind the digested transformation vector. Finally, the vector was eluted using sterile nuclease-free water instead of the elution buffer provided with the kit. This was done to improve the efficiency of the ligation step.

3.7.4. Ligation of the guide RNA and the transformation vector

The previously hybridized gRNA and the enzymatically digested and gel purified transformation vector were used in this step. The insert:vector ratio was adjusted to be 7:1 to increase the efficiency of the ligation, due to the size of the insert being only 24 nucleotides. The ratio was calculated using New England Biolabs® Ligation calculator (https://nebiocalculator.neb.com). For the ligation, the digested and purified vector (36 ng) was mixed with the hybridized gRNA (0.36 ng), T4 DNA ligase (1 Unit) and T4 DNA ligase buffer (10x). The mix was incubated overnight at 4 °C. The low temperature is supposed to decrease the kinetics of the enzymatic reaction and therefore enhance the ligation efficiency.

3.7.5. Transformation of *Escherichia coli* with the ligation product

Chemically competent cells of *Escherichia coli (E. coli)* DH5α strain, were transformed using the heat shock method [27]. An aliquot of competent cells was thawed on ice for ~20 min, after which 5 µL of the overnight ligation reaction product was added to *E. coli* and left to incubate on ice for 30 min. Next, *E. coli* was heat shocked for 45 s at 42 °C in a prewarmed heat block, and then incubated 3 minutes on ice. After the 3-minute incubation, 500 µL of S.O.C. medium (Invitrogen) was added to the tube and incubated at 37 °C 180 rpm for 1 hour in an orbital shaker-incubator.

Meanwhile, two plates of lysogeny agar (LA) supplemented with kanamycin (50 µg/ml), IPTG (isopropylthio-β-D-galactoside, 1 mM) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside, 100 µg/mL) were pre-warmed at 37 °C. The transformation vector contains a gene that provides resistance to kanamycin and allows for selection of positive transformed *E. coli*. Moreover, the vector also contains the *lac* operon (*lac* promoter and the *lacZ* gene) which allows for what is known as blue/white screening. The *lacZ* gene encodes for an enzyme, β-galactosidase, that can use X-Gal as a substrate to generate a blue pigment. If the insertion of the gRNA has been effective, the *lac* operon is not present and therefore the transformed bacteria cannot generate the blue pigment. These bacterial colonies will be white in the LA plates.

After the 1-hour incubation period, 50 µL and 100 µL of the transformed *E. coli* were plated onto the prewarmed LA medium. Finally, the plates were incubated at 37 °C overnight.

3.7.6. Colony Polymerase Chain Reaction

To check the correct transformation of *E. coli* with the transformation vector containing the gRNA, different white colonies were selected. A procedure known as Colony Polymerase Chain Reaction (Colony PCR) is commonly used to detect the presence of plasmids and inserts (the gRNA in this case) into transformed bacteria. The method relies on the use of a polymerase that will amplify a specific nucleotide sequence when provided with primers that target said sequence. To check the correct ligation of the gRNA inside the pDIRECT_25F vector, 4 different pairs or primers were used (Consult Appendix 3 for the primer sequences). Two forward primers and two reverse primers. One pair of primers was targeting a region in the pDIRECT_25F vector (pDIR25F_FW and NB463), which had the *lac* operon present inside it. This meant that if the gRNA was inserted, the resulting fragment should be shorter when comparing amplified regions of the ligated and non-ligated transformation vector. In the case of the ligated vector, the expected fragment product should be of 550 bp. The other set of primers had the same reverse primer as the previous pair, but the forward primer was the sense gRNA. In this case, the polymerase should only be able to amplify a product of 428 bp if the gRNA was inserted into the transformation vector.

To perform the colony PCR, tubes containing 5 µL of nuclease-free water were prepared per analyzed colony. Each colony was touched using a sterile pipette tip that was then immersed into the tube containing water. The tip was then dropped into a tube that had liquid LB media supplemented with kanamycin (50 µg/ml) and the tubes were incubated overnight at 37 °C in an incubator with shaking at 180 rpm.

The PCR mix composition consisted of DreamTaq PCR Master Mix (2X), Reverse primer (10 uM), Forward primer (10 µM), water containing transformed colonies, and nuclease-free water. (The PCR program can be found on Appendix 4).

The PCR products were then loaded into an agarose gel (2%; stained with GelRed® before casting) that was run at 60 V for 55 minutes. The GeneRuler Low Range (Thermo Scientific) was used as the DNA ladder. The gel was then visualized using a transilluminator, to confirm the presence or not and to compare the different size of the expected bands.

4. Results

4.1.Sterilization efficiency of different treatments

In all comparable experiments, tillers used for *in vitro* tillering were contaminated more than the tillers used for calli induction. The *in vitro* tillering tillers were contaminated more by fungi than in the case of the calli induction tillers, while the latter ones were contaminated more by bacteria than the *in vitro* tillering tillers, except for the treatment of line 3776 without cycloheximide.

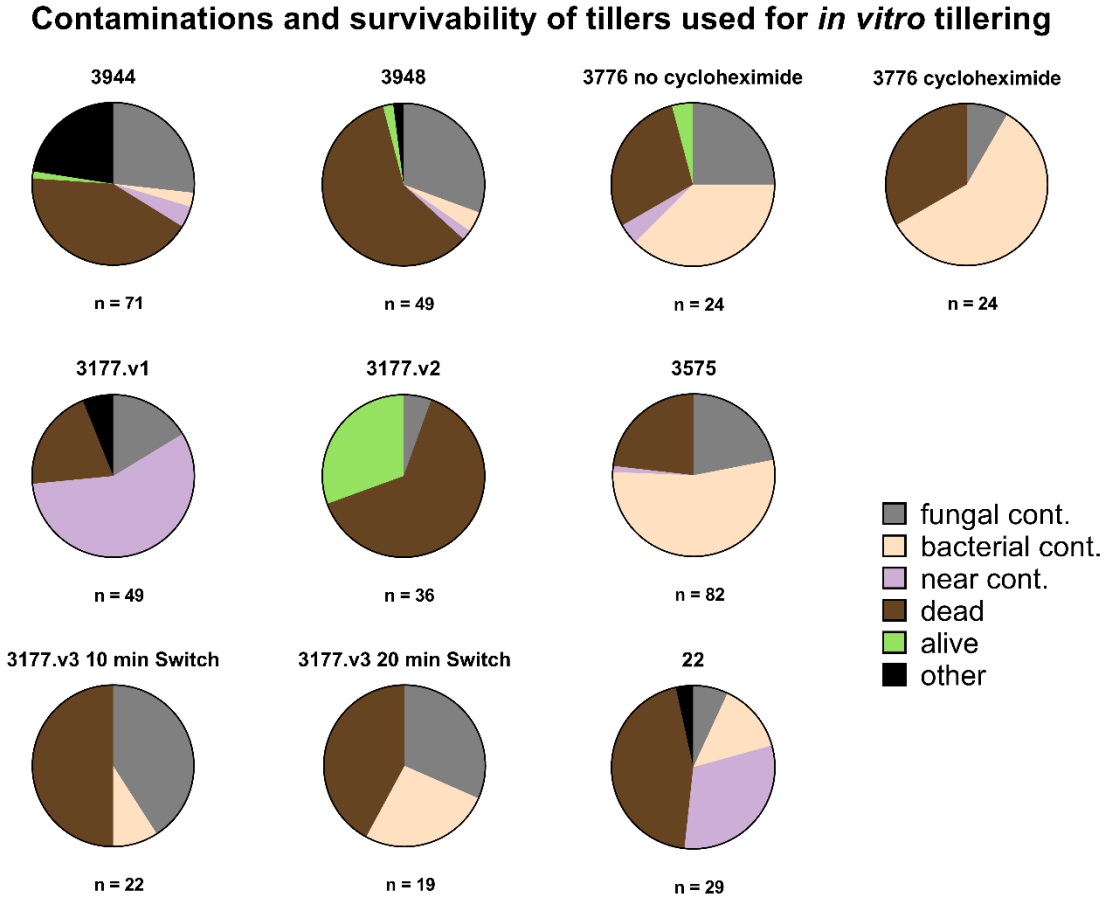

Figure 1. Pie charts representing contaminations and survivability of tillers used for *in vitro* tillering. The figure shows the results for ten experiments using different lines and/or treatments (3944, 3948, 3776 with and without cycloheximide, 3177.v1, 3177.v2, 3575, 3177.v3 10 and 20 minutes Switch, and 22). Each color represents a different recorded parameter, as it can be seen in the legend ("fungal cont." means fungal contamination; "bacterial cont." means bacterial contamination; "near cont." equals to secondary contamination; "other" represents tillers lost due to other events besides the previously mentioned) ("n =" represents the number of tillers used per experiment).

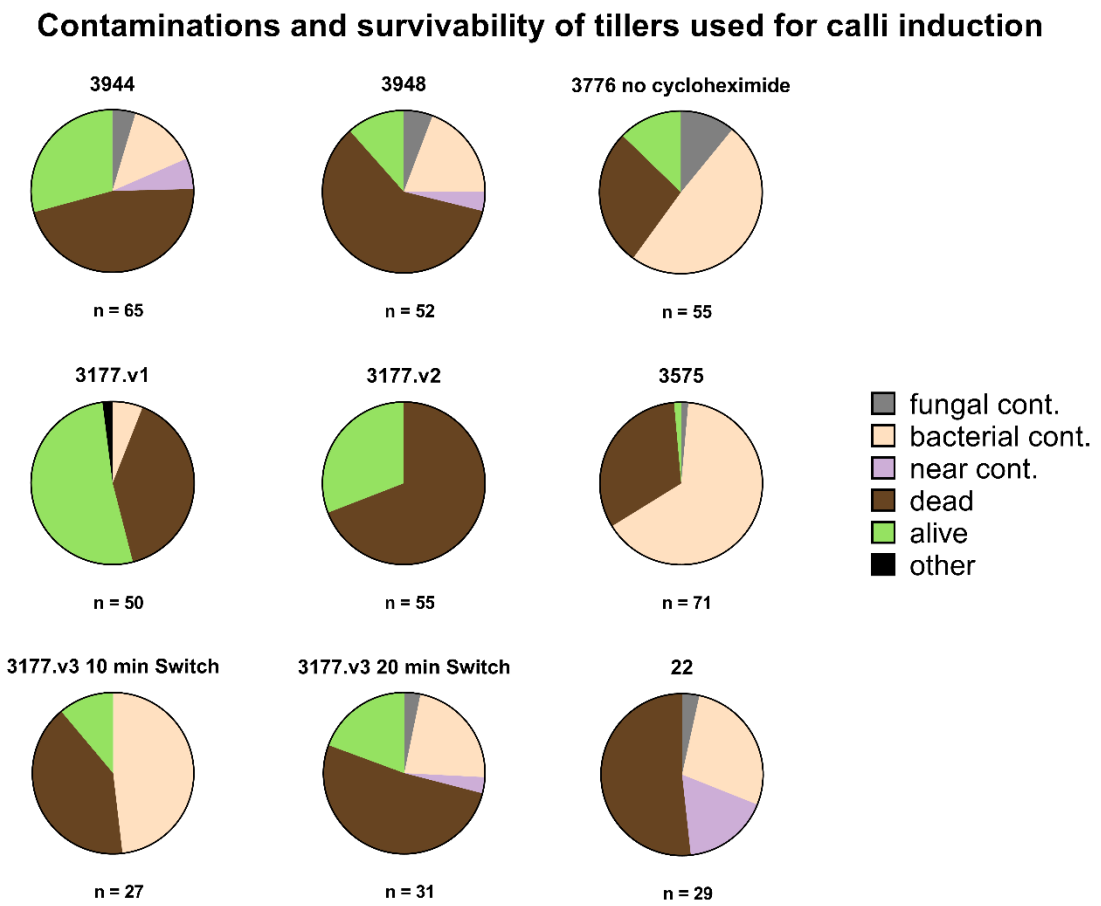

Figure 2. Pie charts representing contaminations and survivability of tillers used for calli induction. The figure shows the results for nine experiments using different lines and/or treatments (3944, 3948, 3776 without cycloheximide, 3177.v1, 3177.v2, 3575, 3177.v3 10 and 20 minutes Switch, and 22). Each color represents a different recorded parameter, as it can be seen in the legend ("fungal cont." means fungal contamination; "bacterial cont." means bacterial contamination; "near cont." equals to secondary contamination; "other" represents tillers lost due to other events besides the previously mentioned) ("n =" represents the number of tillers used per experiment).

4.1.1. Sterilization efficiency of tillers used for *in vitro* tillering and for calli induction

In Figure 1, a compilation of the percentages of contaminated, dead and alive tillers used for the *in vitro* tillering can be seen. Figure 2 shows similar data but related to the tillers used for calli induction. For tillers used for *in vitro tillering*, fungal contaminations ("fungal cont." in Figures 1 and 2) include filamentous fungi or yeast contaminations, and fungal/bacterial co-contaminations. For tillers used for *in vitro* tillering, bacterial contaminations ("bacterial cont.") include bacterial contaminations and fungal/bacterial co-contaminations.

Generally, the contamination of *in vitro* tillering tillers ranged from 6 to 85% (lowest for treatment 3177.v2, highest for treatment 3575), while for the calli induction tillers it ranged from 0 to 66% (lowest 3177.v2, highest 3575). For the tillering tillers, the fungal contamination (in grey in Figures 1 and 2) ranged from 6 to 43% (3177.v2; 3177.v3 10 min Switch) and from 3 to 60% (3944; 3575) for the bacterial contamination (in beige in Figures 1 and 2)In the case of the calli induction tillers, the fungal contamination ranged from 0 to 11% (lowest 3177.v1, 3177.v2 and 3177.v3 10 min SWITCH, highest 3776 no cycloheximide) and the bacterial contamination from 6 to 65% (3177.v1; 3575). Bacterial and fungal co-contamination were observed 8 times for line 3575, from which one was a combination of a bacteria and two different fungi, and only once in line 3177 with treatment v3 10 min Switch (data not shown). Secondary contamination (meaning a contamination derived

from other plant tissue sources located close to the tiller) ("near cont." in Figures 1 and 2, purple color) was high for the calli induction tillers of line 22 (17%) and for the *in vitro* tillers of treatments 3177.v1 (57%) and 22 (31%). The death rate ("dead" in Figures 1 and 2, brown color) of all treatments for the tillering tillers ranged from 20 to 70% (3177.v1; 3177.v2), and from 27 to 70% (3776 no cycloheximide; 3177.v2) for the calli induction tillers.

Lines treated with 20 minutes of fungicide Switch (3177.v2, 3177.3 20 min Switch, 3575, 3776) and without cycloheximide (3776 no cycloheximide) had fungal contamination for the tillering tillers ranging from 6 to 31% (lowest 3177.v2, highest 3177.v3 20 min), bacterial contamination from 0 to 60% (3177.v2; 3575) and dead tillers from 26 to 70% (3575; 3177.v2). The same lines had fungal contamination for the calli induction tillers ranging from 0 to 10% (3177.v2; 3776), bacterial contamination from 0 to 65% (3177.v2; 3575) and dead tillers from 27 to 69% (3776 no cycloheximide; 3177.v2).

Lines without any fungicidal treatment (22, 3177.v1, 3944, 3948) for the tillering tillers had fungal contamination ranging from 6 to 31% (22; 3948), bacterial contamination ranging from 0 to 13% (3177.v1; 22), and dead tillers ranging from 20 to 59% (3177.v1; 3948). Without fungicidal treatment, the calli induction tillers had fungal contamination ranging from 0 to 6% (3177.v1; 3948), bacterial contamination ranging from 6 to 28% (3177.v1; 22), and dead tillers ranging from 40 to 60% (3177.v1; 3948).

The v3 treatment of line 3177 resulted in none of the tillers for regeneration surviving, neither with 10 nor 20 minutes with fungicide.

Fewer fungal contaminations were observed for the cycloheximide treatment of tillers from line 3776 used for *in vitro* tillering than for the ones not treated with cycloheximide.

4.1.2. Contaminations in tillers used for *in vitro* tillering

Peaks of contamination appearing after the sterilization of tillers used for *in vitro* tillering can be observed in Figure 3 (fungal contamination) and Figure 4 (bacterial contamination).

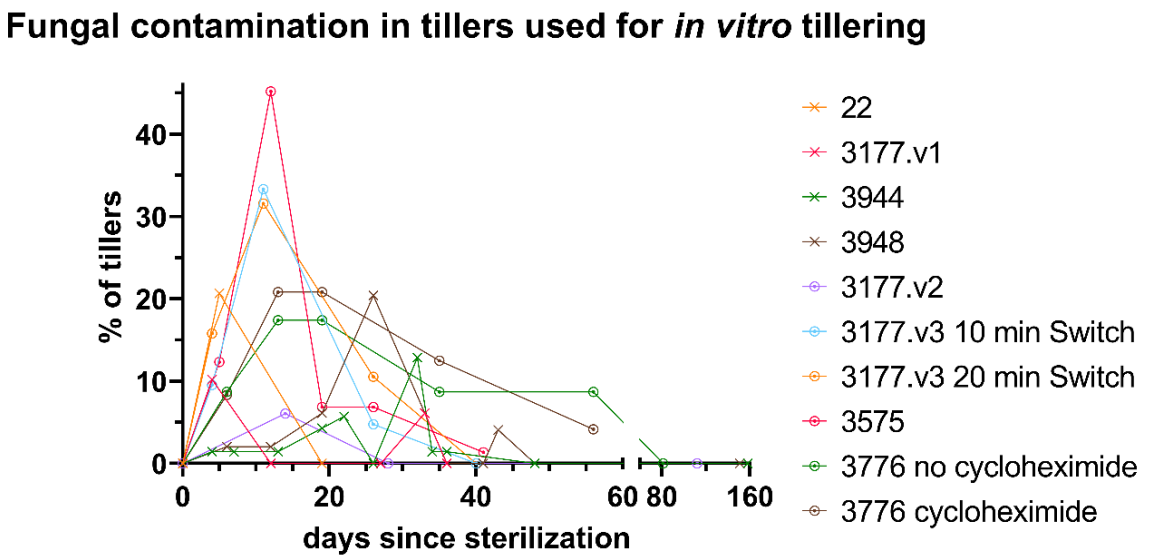

Figure 3. Fungal contamination of tillers used for *in vitro* tillering. The figure shows the percentage of contaminated tillers of ten different experiments (each combination of color and symbol represents a different experiment). The "⁎" symbol represents experiments without fungicide, while the "ɵ" symbol represents experiments with fungicide (Switch). The plant lines shown in the legend appear in the order in which they were sterilized. The plotted sample size of each experiment is the same one that can be seen in Figure 1.

In Figure 3, fungal contamination peaks near the 5th, 12th, 26th, 32nd and 43rd days after sterilization were observed. The first fungal contamination being on day 4 and the final being on day 56. Two lines (22 and 3177.v1) had a peak near the 5th day, with the percentage of tillers with fungal contamination being 21 and 10%, respectively. Six lines (3944, 3948, 3177.v2, 3177.v3 10 and 20 min Switch, 3575) had a peak near the 5th day, with the percentage of tillers with fungal contamination ranging from 6 to 45% (lowest 3177.v2, highest 3575). Line 3944 had a peak on the 12th day, with the percentage of tillers with fungal contamination being 6%. Line 3948 had a peak on the 26th day, with the percentage of tillers with fungal contamination being 20%. Two lines (3177.v1 and 3944) had a peak near the 32nd day, with the percentage of tillers with fungal contamination of 6 and 13%. Treatment 3948 had a peak on the 43rd day, with 4% fungal contamination. On day 4, four lines (3177.v1, 3944, 3177.v3 10 and 20 min Switch) had fungal contaminations, and on day 56, lines 3776 with and without cycloheximide had fungal contaminations.

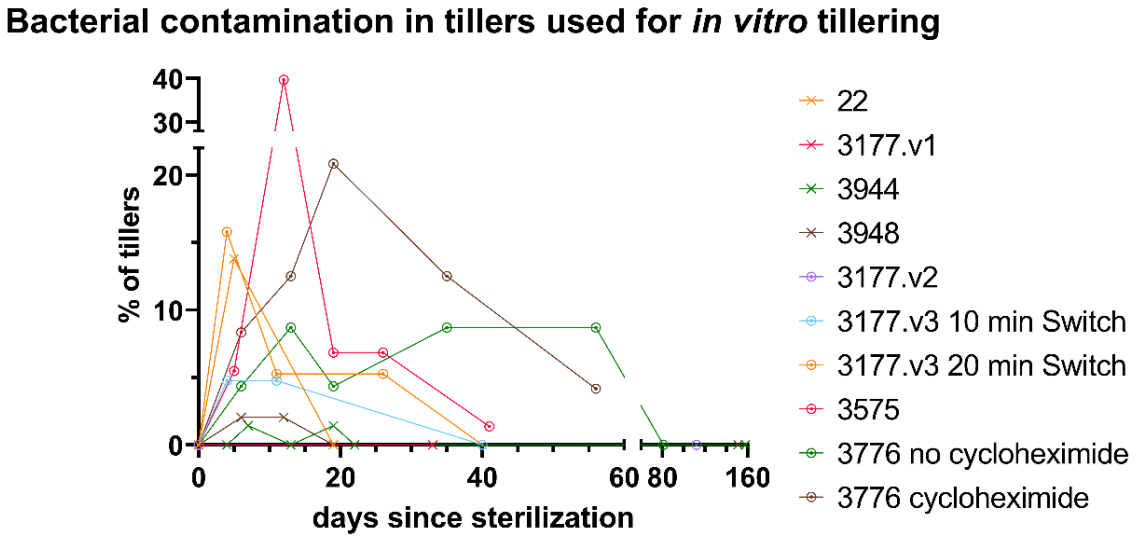

Figure 4. Bacterial contamination of tillers used for *in vitro* tillering. The figure shows the percentage of contaminated tillers of ten different experiments (each combination of color and symbol represents a different experiment). The "⁎" symbol represents experiments without fungicide, while the "ɵ" symbol represents experiments with fungicide (Switch). The plant lines shown in the legend appear in the order in which they were sterilized. The plotted sample size of each experiment is the same one that can be seen in Figure 1.

In Figure 4, bacterial contamination peaks near the 5th, 12th and 19th days after sterilization were observed; the first bacterial contamination being on day 4 and the final being on day 56 after the sterilization. Five lines (22, 3944, 3948, 3177.v3 10 and 20 min Switch) had a peak near the 5th day, with the percentage of tillers with bacterial contamination ranging from 1.4 to 16% (lowest 3944, highest 3177.v3 20 min Switch). Two lines (3177.v1 and 3776 no cycloheximide) had a peak near the 12th day, with the percentage of tillers with bacterial contamination of 40 and 9%. Two lines (3944 and 3776 no cycloheximide) had a peak on the 19th day, with the percentage of tillers with bacterial contamination of 1.4 and 21%. On day 4, the same line subjected to two different treatments (3177.v3 10 and 20 min Switch) had bacterial contamination, and on day 56, treatments 3776 with and without cycloheximide had bacterial contaminations. In the case of line 3177, for the first treatment (3177.v1) no bacterial contamination in the tillers used for calli induction was observed and the same was for the second treatment (3177.v2).

4.1.3. Contaminations in tillers used for calli induction

Peaks of contamination appearing after the sterilization of tillers used for calli induction can be observed in Figure 5 (fungal contamination) and Figure 6 (bacterial contamination).

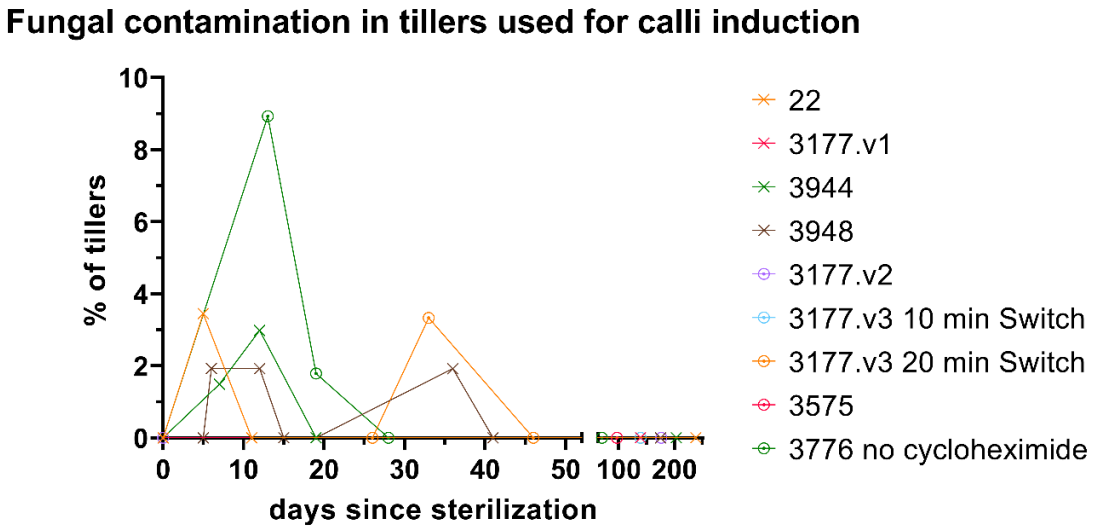

Figure 5. Fungal contamination of tillers used for calli induction. The figure shows the percentage of contaminated tillers of nine different experiments (each combination of color and symbol represents a different experiment). The "×" symbol represents experiments without fungicide, while the "ø" symbol represents experiments with fungicide (Switch). The plant lines shown in the legend appear in the order in which they were sterilized. The plotted sample size of each experiment is the same one that can be seen in Figure 2.

In Figure 5, peaks of fungal contamination in calli induced tillers were generally observed near day 10 and 35, the first fungal contamination being on day 5 and the final being on day 36 after the sterilization. Four lines (22, 3944, 3948, 3776 no cycloheximide) had a peak near day 9 with fungal contamination percentage ranging from 2 to 9% (lowest 3948, highest 3776 no cycloheximide). Two lines (3948 and 3177.v3 20 min Switch) had a peak near day 33 with fungal contamination percentages of 2 and 3%. On day 5, treatment 22 had fungal contamination which was also present on day 36 for treatment 3948.

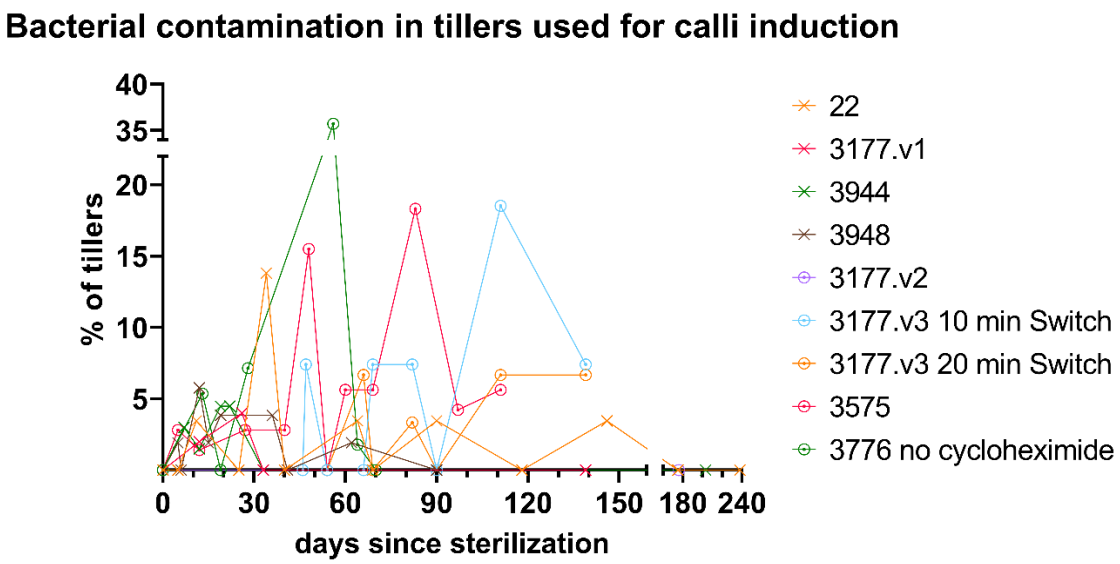

Figure 6. Bacterial contamination of tillers used for calli induction. The figure shows the percentage of contaminated tillers of nine different experiments (each combination of color and symbol represents a different experiment). The "⁎" symbol represents experiments without fungicide, while the "ɵ" symbol represents experiments with fungicide (Switch). The plant lines shown in the legend appear in the order in which they were sterilized. The plotted sample size of each experiment is the same one that can be seen in Figure 2.

In Figure 6, bacterial contamination peaks were observed near day 13, 48, 66, 83, 111 and 139, first bacterial contamination being on day 5 and final being on day 146 after sterilization. The graph shows several datapoints for bacterial contaminations from lines that have been treated with Switch (lines with the "ɵ" symbol). The bacterial contamination percentage peaked at 36, 18 and 19% for lines 3776 no cycloheximide, 3575 and 3177.v3 10 min SWITCH, respectively. Except for line 22, the bacterial contamination stopped appearing for the lines not treated with Switch (lines with the "⁎" symbol in Figure 6) earlier, at day 62, than in the case of the Switch treated lines on day 111 to 139.

4.2.Tillering efficiency

The rate of tillering, i.e. growing more tillers out of one tiller, was measured for only three lines. For the comparison to be accurate, data could be collected only for lines which had uncontaminated tillers for more than 2 months since the sterilization day. This data can be seen in Figure 7.

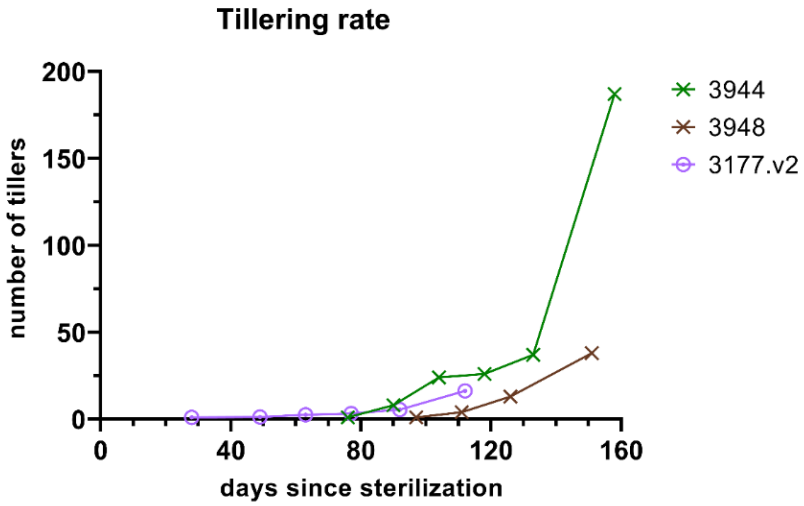

Figure 7. Tillering rate of 3 different lines. The figure shows the number of tillers observed on different timepoint (days in the X-axis). The "✕" symbol represents experiments without fungicide, while the "⊖" symbol represents an experiment with fungicide (Switch). The number of tillers observed in the Y-axis was normalized (i.e. the number of observed tillers was divided by the number of tillers before tillering started).

The results of normalized tillering rate show that line 3177 started to tiller early compared to other lines, over 2.5 times faster in comparison to line 3944 and over 3.4 times faster than line 3948. While it started to tiller earlier, it appears to tiller at a lower rate at the start of tillering. Line 3994 started to tiller 21 days sooner than line 3948. Despite the difference in start timepoint of tillering, it seems that at the start of tillering both 3944 and 3948 have the same rate of tillering.

4.3.Calli induction

To understand the possible differences between the callus induction rate of the different lines, a comparison of percentages of tillers from which calli was induced was needed. The comparison between lines with enough data is displayed in Figure 8.

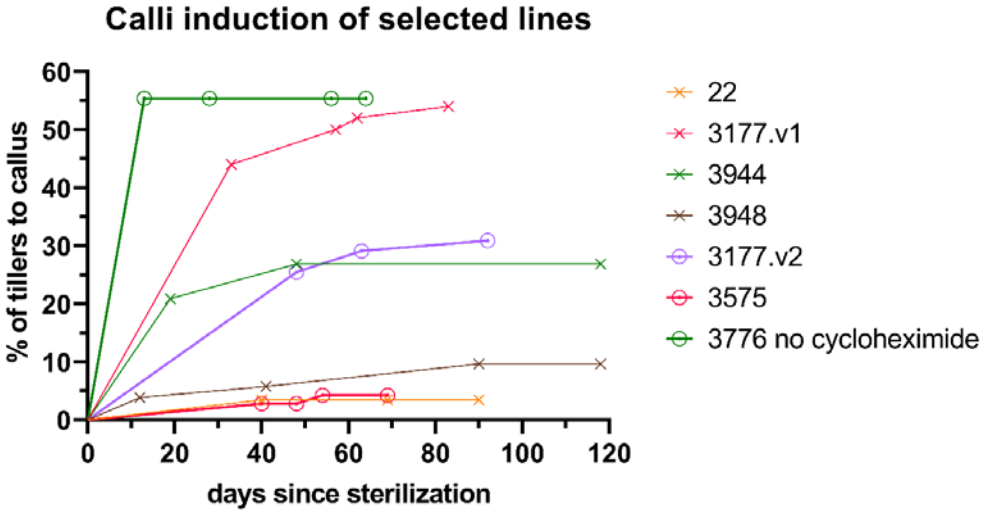

Figure 8. Calli induction of selected lines. The figure represents the percentage of induced calli (contaminated and uncontaminated) from seven experiments observed on each specific timepoint (days on the X-axis; each combination of color and symbol represents a different experiment). The "✕" symbol represents experiments without fungicide, while the "⊖" symbol represents experiments with fungicide (Switch). The plant lines shown in the legend appear in the order in which they were sterilized. The plotted sample size of each experiment is the same one that can be seen in Figure 2.

For the selected lines, the amount of calli induced by the end of data recording was varying in a range of 3.4 to 55% (lowest 22, highest 3776). Calli induction does not seem to be affected when using 20 minutes of Switch treatment (lines with the "ɵ"), as the range of calli percentage ranges from 4.2 to 55% for treatments with 20 minutes of SWITCH, while without any fungicide the range is 3.4 to 54%. Around day 40, the rate of calli induction is clearly reduced for almost all lines. According to the early rate of calli induction and the percentage of calli induced, the best line seems to be 3776, as it achieves a plateau phase by the 10th day after sterilization.
Additionally, as calli induction is an important step in transformation of plants, there must be a reliable way to generate a great amount of calli quickly. That is why the effect on calli induction rate of treatment details is compared in Figure 9.

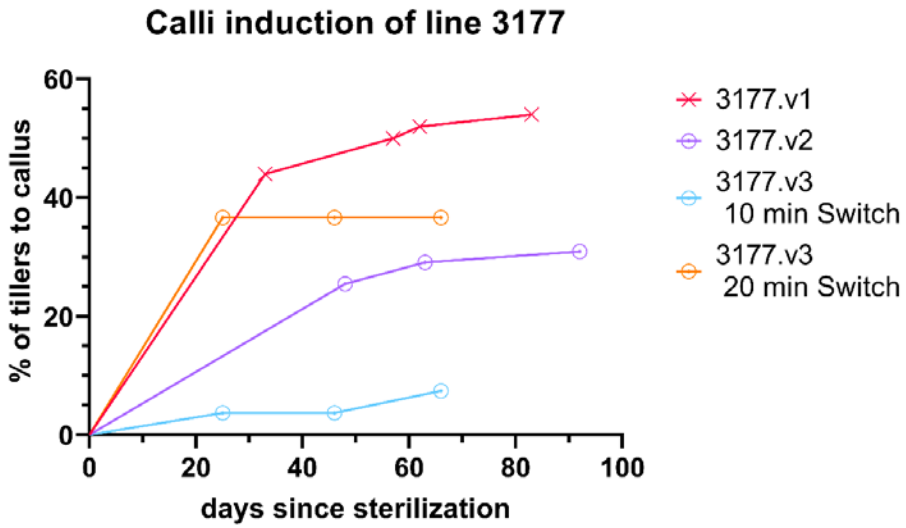

Figure 9. Calli induction of 4 different experiments using line 3177. The figure represents the percentage of induced calli (contaminated and uncontaminated) of four experiments observed on each specific timepoint (days on the X-axis; each color represents a different experiment). The "ɤ" symbol represents an experiment without fungicide, while the "ɵ" symbol represents experiments with fungicide (Switch). The plant lines shown in the legend appear in the order in which they were sterilized. For experiments 3177.v1, 3177.v2, and 3177.v3 10 and 20 min Switch, the number of tillers was 50, 55, 27, and 31, respectively.

In Figure 9, the percentage of callus generated from different treatments using line 3177 can be seen. Interestingly, the treatment without fungicide (red line) had the highest amount of calli observed for treatments applied to line 3177. This difference was by the end of experiment 18% higher than the percentage of callus of the v3 20 min Switch treatment (orange line), which was the best proliferating of the fungicide treatments (all points with the "ɵ"). A low amount (7.4%) of calli induction was observed for the 10 minutes Switch treatment (blue line), while for the two treatments with 20 minutes of Switch (purple and orange lines), the percentage of calli were similar. All treatments seem to have the same range of days (0 to 25) when most of the calli are induced.

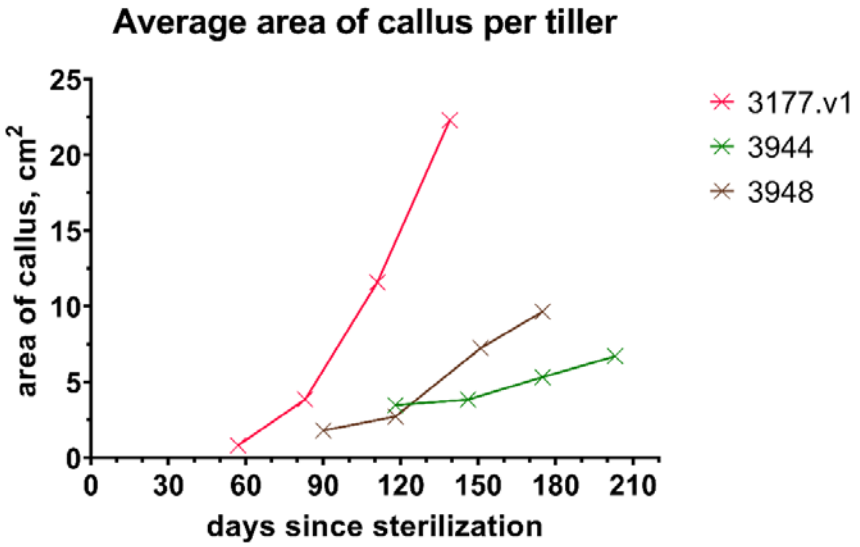

Figure 10. Average area of callus per tiller. The figure shows the area of callus observed on four different timepoints. For lines 3177.v1, 3944 and 3948, the number of tillers was 50, 67 and 52, respectively.

The amount of calli generated per cultured tillers can be seen in Figure 10. Line 3177 surpasses lines 3944 and 3948 in average area of callus induced per tiller per time. Line 3944's calli proliferates the slowest out of the three lines.

A representation of the development of tillers used for *in vitro* tillering and for calli induction can be found in Appendix 5.

4.4.Assembly of a single guide RNA into the pDIRECT_25F vector

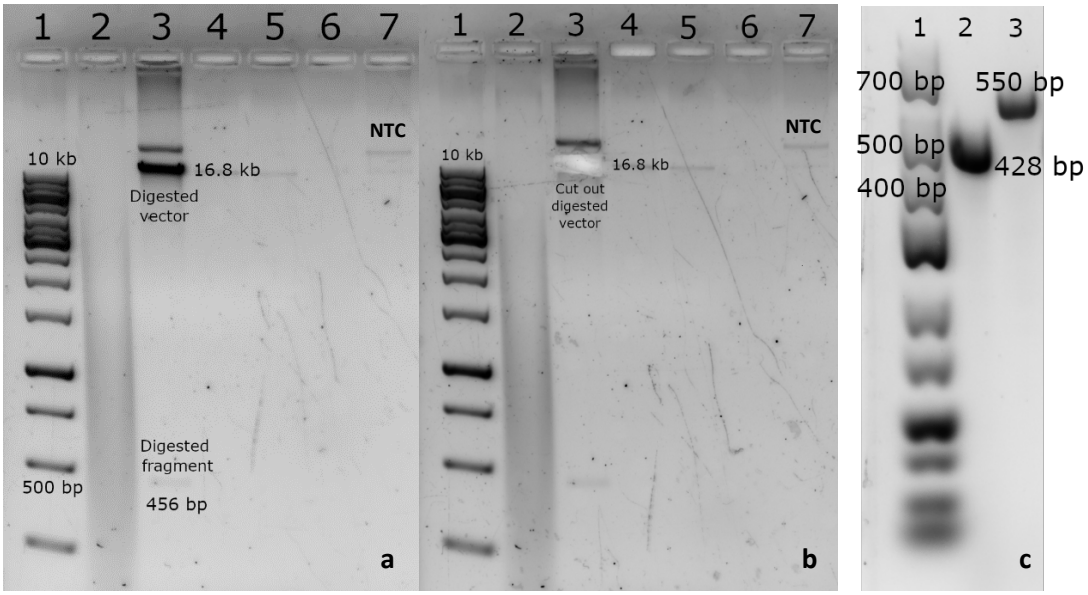

Figure 11. Images of transilluminated gels. In all gel pictures the first lane shows the DNA ladder used as reference (1 kb ruler in images "a" and "b", Low Range ladder in image "c"). First, images "a" and "b" show the product of the enzymatic digestion of the pDIRECT_25F vector (third lane). The first labeled band in this lane corresponds to the digested vector (16.8 kb) and the second labeled band shows the digested fragment (456 bp). On both photos ("a" and "b"), the seventh lane contains the unrestricted plasmid (negative control, NTC). Picture "b" shows the piece of gel that was cut out for further purification. Second, image "c" shows the result of performing an electrophoretic check of the colony PCR product. The second lane shows the product of using the gRNA as forward primer (428 bp), and the third lane shows the result of using primers flanking the lac operon encoding region in the pDIRECT_25F vector (550 bp).

As seen in Figure 12, dividing the Golden Gate process into two separate steps allowed the successful enzymatic digestion of the vector (Figure 12a and 12b) and the later insertion of the gRNA into it (Figure 12c). In Figure 12a, the expected band of 456 bp resulting from the enzymatic activity of *AarI* was visible, which indicated that the vector was indeed digested. Figure 12b shows the cutout digested plasmid (16.8 kb fragment). The presence of a 428 bp band in Figure 12C shows that the gRNA (targeting *Lolium perenne* L. gene *VIN3*) was ligated into the pDIRECT_25F plasmid. This product could only be amplified by the polymerase if the gRNA had been successfully inserted into the vector. The existence of the 550 bp in the gel (Figure 12c, column 3) corroborates the positive ligation of the gRNA, considering that the length of this band is shorter than what was expected from the non-ligated vector (961 bp product) as previously mentioned in the Material and Methods section. Nonetheless, the plasmid should be sequenced before transforming *A. tumefaciens*. After that, the calli can be co-cultivated with agrobacteria.

5. Discussion

To establish a method for *in vitro* tillering and for induction of calli from tillers, an efficient way to sterilize the plant tissue should be established. In our hands, the tillers for calli induction were contaminated less than the tillers used for their asexual reproduction (*in vitro* tillering), probably because most of the root of calli induction tillers was cut away, which is one of the major sources of fungal contamination. This could be supported by the fact that roots are in the soil and therefore in contact with many microorganisms, with whom plants have symbiotic relationships [28, 29]. Usually, smaller tillers or the ones that had developed less roots were selected for calli induction. There were also fungi growing out the cut area and it could be that some spores were not killed by sterilization, but it could also be that endophytic fungi, very common in grasses [30], were damaging the plant tissue.

As calli induction tillers were not contaminated as much with fungi, the bacterial contamination was more easily to happen and to be noticed. Fungal contamination spreads quickly and densely, so it is hard to notice bacterial colonies in the mycelial mat. Moreover, it is known that fungi produce antimicrobial compounds [31] and also bacteria generate antifungals [32]. Despite that, fungal and bacterial co-contamination were observed for two different treatments (lines 3575 and the 10 minutes of Switch treatment of line 3177), which may show that not all fungi produce antimicrobial compounds and vice versa. Other reasons, among others, could be that the concentration of the expelled compound is not enough for stopping the fast proliferation of the antagonist organism or that the compounds produced do not have a wide-spectrum effect. Interestingly, it seems that with every successive treatment (e.g., line 3776), the percentage of bacterial contamination rises. This may be because of decreasing fungal contaminations, which appeared fast and hide the possible bacterial co-contamination, and less death of plant tissue in general. Fewer deaths mean discarding of fewer tillers that may have had hiding bacteria or their spores.

The contamination also tends to spread to clean tillers or calli, this was called in the current thesis “secondary contamination”. Lines most affected by this were 22 and 3177.v1. One reason is the fact that the initial density of tillers per plate after sterilization was high. Reducing this density and closely following the tissues right after the sterilization procedures should help.

Surprisingly, the fungicide (Switch) does not seem to lower the amount of fungal contamination seen on *in vitro* tillering tillers nor calli induction tillers, as the values for Switch and no fungicide treatments groups were similar. This fungicide is of broad spectrum, used as a pesticide in different crops [33] and it may not affect the specific fungi present in perennial ryegrass.

The lower level of fungal contamination observed for the cycloheximide treatment of tillers from line 3776 used for tillering was probably due to the antifungal effect of this compound. Cycloheximide inhibits protein synthesis of eukaryotic cells; thus, it is used as a fungicide agent in crops [34]. The fact that the fungal contamination of tillering tillers using the fungicide was even lower than the observed for calli induction tillers, suggests that cycloheximide can be a better alternative than Swift. More data regarding the use of cycloheximide is needed to make a definitive conclusion.

The bacterial contamination in tillers used for calli induction was observed after five months from the sterilization, while the last contaminated tiller (with either fungi or bacteria) used for tillering was detected two months after the sterilization day. This indicates that the calli cultures need to be monitored more often and for a longer period than the culture of *in vitro* tillering tillers. Even

though the reason why the bacterial contamination in calli induction tillers remains for a longer period is unknown, being aware of this fact increases the chances of preserving uncontaminated cultures.

As expected, variations among the different lines were observed in terms of the tillering and calli induction rates. This relates to the previously mentioned genotype dependency of ryegrass in terms of *in vitro* culture [35].This relates to the previously mentioned genotype dependency of ryegrass in terms of *in vitro* culture [35]. Moreover, a difference between the calli induction and tillering ability was observed also in the treatments of the same lines. Line 3177 presented a higher tillering rate than for calli induction. For line 3944, the opposite was observed: the calli induction rate of this line was higher than the same line's rate of tillering. It was particularly interesting to observe the low tillering rate of line 3177, since this genotype grows vigorously in pots. This shows that *in vitro* growth of a line may not reflect its natural growth, since *in vitro* culture conditions are different. In addition, it is interesting that the rate of calli induction in the case of line 3177 treated with fungicide Switch was lower than in the case of the same line not treated with it. A possible interpretation is that the fungicide was affecting the process of inducing calli. Calli - and their induction - are sensitive to any change in their cultivation conditions [17].

Experience seemed to be a key factor for successful sterilization and subsequent establishment of the *in vitro* culture of tillers and calli. Performing the sterilization method over time had an impact in the survivability of the tilers and calli. There was a clear decrease in the death of tillers used for tillering and calli induction by the end of the thesis (lines 3575 and 3776), when compared to the initial experiments (line 22, which had no alive calli nor tillers).

During this thesis the design of one gRNA for the knockout of *VIN3* (*VERNALIZATION INSENSITIVE 3*), a gene related to tolerance to frost, was carried out. The gRNA sequence was inserted into a binary vector. The next steps are to transform agrobacteria with the obtained construct and proceed to co-cultivation of perennial grass calli.

Kokkuvõte

Karjamaa-raihein (*Lolium perenne* L.) tähtsa söödataimena peab suutma vastu pidada kliimamuutusest tingitud teistsugustele ilmastikuoludele. Tavaliselt kasutatakse kultuurtaimede puhul klassikalisi aretustehnikaid, et suurendada nende abiootiliste stresside taluvust. Raiheina puhul on see raskendatud, kuna see paljuneb vaid mittesuguluses olevate isenditega. Genoomi täppismuutmise tehnoloogiate abil oleks võimalik tekitada niisuguseid karjamaa-raiheina taimi, mis suudaks hakkama saada põua ja külmaga. Kõige rohkem geneetiliselt transformeeritakse taimi *Agrobacterium tumefaciens*'iga, kusjuures CRISPR-Cas9 tehnoloogia kasutamisel viiakse CRISPR-i kompleks taimerakkudesse mainitud agrobakteriga. Transformeeritud taimi on võimalik tekitada CRISPR-Cas9 kompleksi sisestamisel meristeemi (täpsemalt võsu tipmise meristeemi), mida on võimalik tekitada kiirelt ja palju kalluse indutseerimisega.

Antud lõputöö eesmärgiks oli luua steriilsed võrsete ja kalluse *in vitro* kultuurid mitmele karjamaa-raiheina genotüübile, kasutades algmaterjaliks mullas kasvatatud raiheina võrseid. Kasutatud genotüüpe rakendatakse rahvusvahelises projektis (EditGrass4Food) seoses CRISPR-vahendatud *Lolium perenne* L. genoomi täppismuutmisega, mille tulemuseks oleks raiheina taimed, mis oleksid vastupidavamad Põhja-Euroopa külmadele ja põudadele.

Erinevate steriliseerimismeetodite katsetamise tulemusel saadi nelja genotüübi võrsete ja viie genotüübi kalluse steriilsed *in vitro* kultuurid. Lisaks suudeti tekitada transformatsioonivektor, mis sisaldab CRISPR-Cas9 kompleksit koos disainitud giid RNA järjestusega. Mainitud vektorit on lähiajal võimalik hakata kasutama täppismuudetud genoomiga karjamaa-raiheina tekitamiseks.

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Appendices

Appendix 1 – Lines used for *in vitro* culture

Line ID	Type	Country of origin	Cultivar name	Drought sensitivity
3177	ecotype	Latvia		tolerant
3944	cultivar	Denmark	Kerem	moderate-sensitive
3948	cultivar	Netherlands	Magella	moderate
22	cultivar	Russia	Leningradskij 809	tolerant
3575	ecotype	Lithuania		sensitive
3776	ecotype	Slovakia		sensitive

Appendix 2 – List of oligos and primers

Guide RNA oligonucleotides sequences

Oligonucleotide name	Nucleotide sequence (5'-3')	Overhang
Sense guide RNA	CATGGTCCATCGCGTACAGA	ACTT
Antisense guide RNA	TCTGTACGCGATGGACCATG	AAAC

Primers used for Colony PCR

Primer name	Nucleotide sequence (5'-3')
pDIR25F_FW	TCACGATTGGGGAGAGCAAC
NB463	CGAACGGATAAACCTTTTCACG

Appendix 3 – Map of the pDIRECT_25F transformation vector

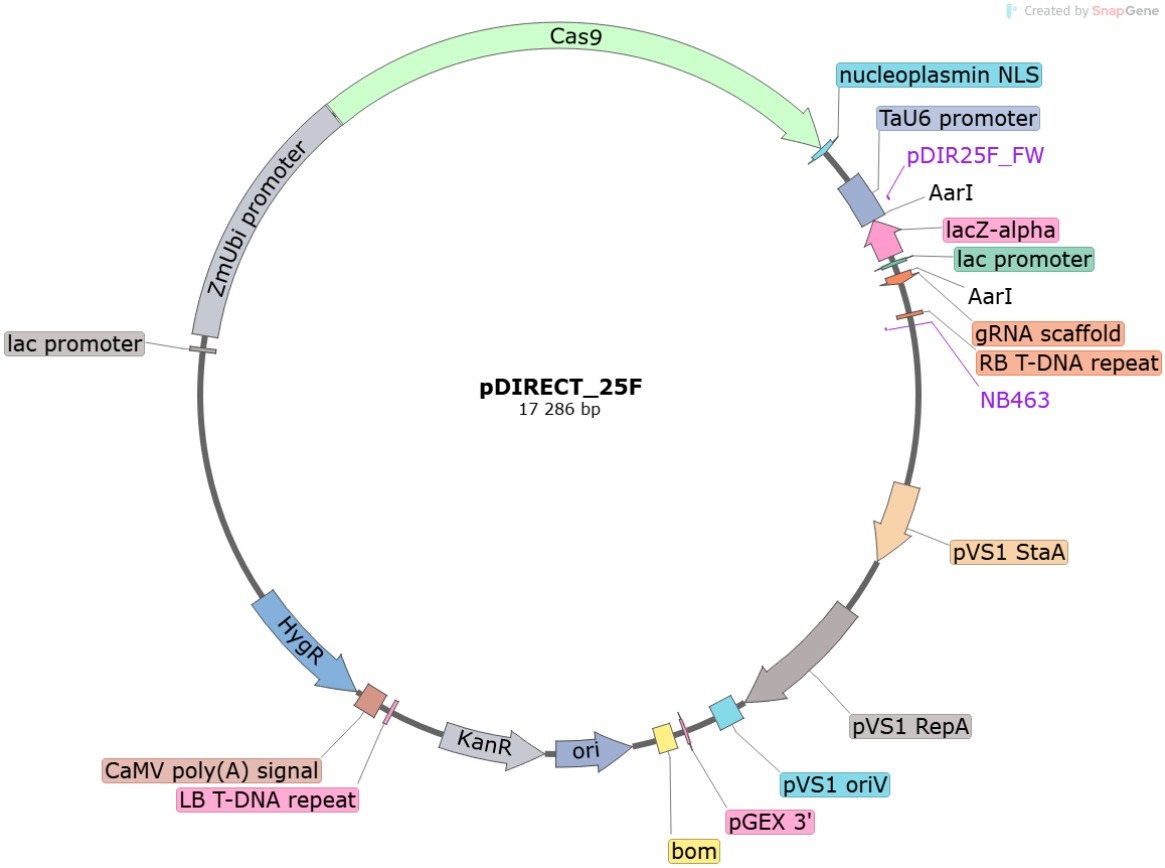
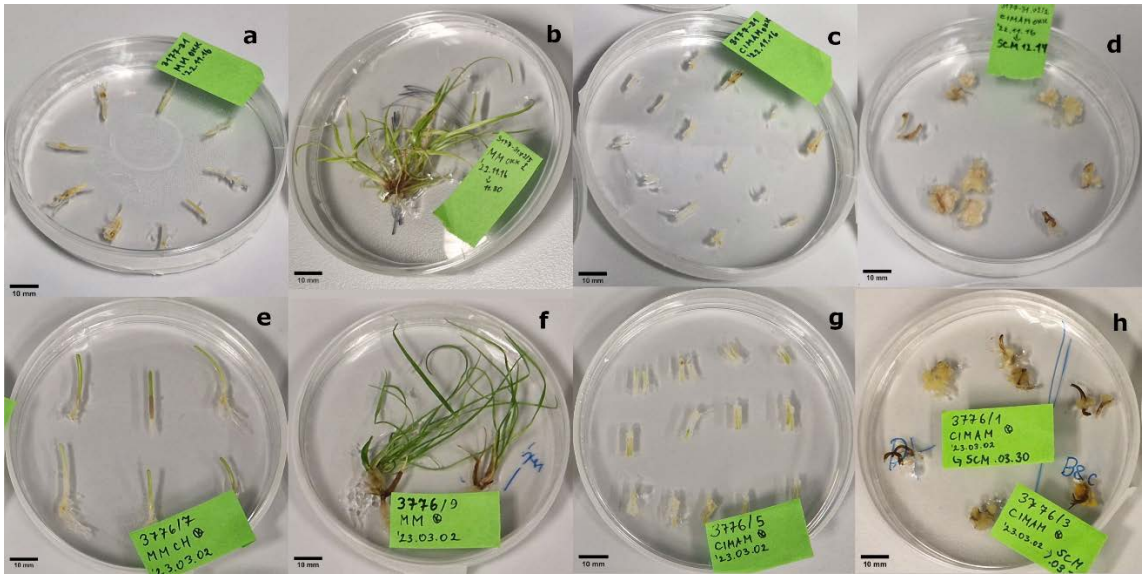


Image generated with SnapGene

Appendix 4 – Colony PCR program

Step	Description	Cycles
Initial denaturation	95 °C for 3 minutes	1
Denaturation	95 °C for 30 seconds	35
Annealing	53 °C for 30 seconds	
Extension	72 °C for 1 minute	
Final extension	72 °C for 5 minutes	1

Appendix 5 – Different stages of the in vitro culture experiments.



Appendix 5 shows how tillers look throughout the different stages of the previously described sterilization, tillering and calli induction experiments. Appendix 5a and 5e show tillers destined for *in vitro* tillering on the day of sterilization (top one being always line 3177 and bottom 3776). Appendix 5b and 11f depict the state of some of these tillers one month after sterilization. Figure 11c and 11g show tillers destined for calli induction on the day of sterilization, and the last two figures (Figure 11d and 11h) display already formed calli after one month from sterilization.