Master thesis.
Production of rubber from dandelion

-a proof of concept for a new method of cultivation

Lotta Oscarsson

Supervised by:
Leif Bülow
Division for Pure and Applied Biochemistry
Lunds Tekniska Högskola
&
Li-hua Zhu
Department of Plant Breeding
Sveriges Lantbruksuniversitet

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Abstract

Natural rubber is a biopolymer that is invaluable in our society. It is used in medical devices, tires, engines and many other consumer products, and so far no synthetic substance has been able to compete with its properties. Today, all natural rubber comes from the rubber tree, mainly grown in southeast Asia. Due to current threats to the rubber industry, finding an alternative source for natural rubber that can be grown in the Northern Hemisphere is vital. The Russian dandelion could be that option. However, previous experience tells us that there are great difficulties in growing the Russian dandelion in the field, so another method for cultivation has to be developed. In this project, dandelions have been cultivated in bioreactors with great success, indicating that this might be a method for the future. Protocols for sterile growth and for genetic transformation of dandelions have also been developed.

Table of contents

1. Introduction

1.1 Characteristics of natural rubber

Rubber is a material that surrounds us constantly in our everyday lives. It is found in more than 40 000 different products including tires, medical devices, gloves and many engineering and consumer products (van Beilen & Poirier, 2007). It has become an important part of our society, and many sectors, among them transportation and health care, would be paralyzed without it. This is mainly due to that natural rubber, today almost exclusively harvested from the sap of rubber trees, has unique properties. Its resilience, resistance to abrasion and impact, and efficient heat dispersion makes it ideal for use in for example airplane tires and truck tires that are exposed to enormous stress. The elasticity and heat and cold tolerance makes it perfect for medical uses, since it, for example, can be heat sterilized. So far, no synthetic rubber has been developed that can compete with natural rubber in these areas. Synthetic rubber is petroleum based, a non-renewable resource that is running out and growing more and more expensive (van Beilen & Poirier, 2007).

Natural rubber is a biopolymer found in the tree sap of rubber trees. It consists of 1,4 *cis*-linked chain of isoprene units $(C_5H_8)_n$ (Fig. 1), each with a molecular weight of 68 Da. This structure is complemented by minor components in the latex such as lipids, proteins, carbohydrates and minerals that together with the structure and high molecular weight of the molecule gives the rubber its special properties (van Beilen & Poirier, 2007).

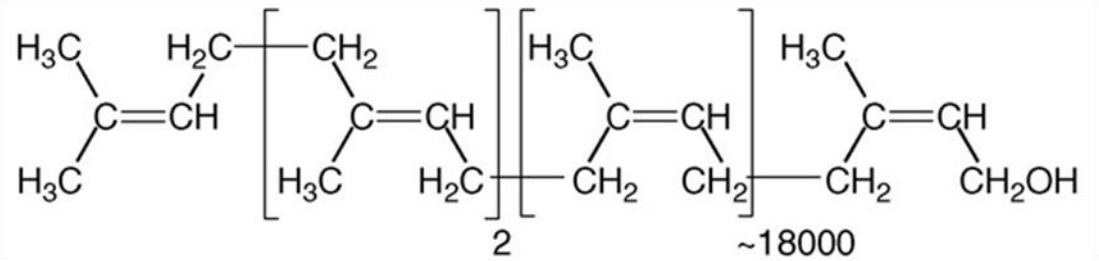

Figure 1. Molecular structure of natural rubber from *Hevea brasiliensis*. The complete molecule consists of isoprene units $(C_5H_8)_n$ linked together in a 1,4-cis configuration where *n* is usually approximately 18 000 in *H. brasiliensis.* Average molecular weight of rubber from *H. brasiliensis* is 1 310 kDa and 2 180 kDa from *Taraxacum kok-saghys*. (van Beilen & Poirier, 2007)

Many plant species produce low amounts of rubber in their sap (van Beilen & Poirier, 2007), possibly as a defence system against pathogens (Fricke, et al., 2013) but today only one is used for commercial production: the rubber tree *Hevea brasiliensis*. Other species have been investigated as alternatives, and mainly two have been of commercial interest: guayule, a shrub that grows mainly in semi-arid regions in southern USA and Mexico (Yulex, 2013), and the Russian dandelion *Taraxacum kok-saghys* that originates from Kazakhstan (Josefsson, 1953). Looking from a Swedish perspective, the latter seems to be a realistic option for rubber production in our own climate.

1.2 Threats to the current rubber industry

Today the production of natural rubber faces several major problems. The *Hevea brasiliensis* can only with great difficulty be cultivated in its natural habitat in South America and the Amazon due to an infectious fungal disease called South American leaf blight (SALB) that causes the leaves to fall and kill the trees. Due to this disease, 93% of all rubber trees are now grown in southeast Asia. All of

these trees originate from the same handful of seeds that were brought from Brazil in 1876 by Dr. Henry Wickam ((Davis & Moore, 1997) (Lieberei, 2007) (van Beilen & Poirier, 2007)). The few seeds that germinated have since then been propagated by cuttings, resulting in large plantations with very limited genetic diversity (Davis & Moore, 1997). This makes breeding on these trees difficult, and results in a limited possibility of developing resistance to disease (van Beilen & Poirier, 2007). So far, the SALB has not spread, but it is more or less just a matter of time (Davis & Moore, 1997) (Lieberei, 2007) (Rivano, et al., 2013). If that happens, it will only be a matter of years before the entire rubber production of the world is knocked out!

Another problem is that the countries that produce rubber today, for example Thailand, Indonesia, Malaysia, India, Vietnam, China, and Cambodia are politically unstable. This makes the distribution uncertain, and many of them also have a growing need for the rubber within the country as they are more and more industrialized (Davis & Moore, 1997) (van Beilen & Poirier, 2007). That leaves less and less rubber to be exported to countries further north. Also, the more lucrative oil palm competes with the rubber tree for land. All in all, the access to natural rubber in the Northern Hemisphere is decreasing, while the need for it is increasing since it has also been realized that synthetic rubber cannot compete when it comes to properties such as temperature stability, resilience, strength and resistance to abrasion and impact (Davis & Moore, 1997).

The need to find an alternative source of natural rubber is great and the benefits, both from an economical and environmental point of view, of having rubber production further north can be substantial. To achieve this, the *T. kok-saghys* could be part of the solution. It does not only contain a fair amount of rubber, the rubber is also hypoallergenic since it in contrast to the *Hevea* rubber does not contain the proteins that cause rubber allergy (van Beilen & Poirier, 2007). The *T. kok-saghys* also contains substantial amounts of inulin, a polysaccharide which might be used as a raw material for bio fuel or ethanol production (Josefsson, 1953), making it even more attractive to grow.

1.3 Cultivation and breeding of *Taraxacum* species

In the search for new rubber producing plants during the world wars, the Russian dandelion *Taraxacum kok-saghys* was found to be an interesting option (Josefsson, 1953). Much research was conducted on this plant mainly during and just after the Second World War, and though it was almost completely forgotten for many decades, research has recently been resumed (Schmidt, et al., 2010). When *T. kok-saghys* has been in focus, other close relatives also gain attention. The infamous weed dandelion *Taraxacum officinale* (also called *T. vulgare*) comes to mind and makes one wonder how there could be any difficulties cultivating dandelions. Another relative is the *Taraxacum brevicorniculatum* that is similar to the *T. kok-saghys* and is often found present in *T. kok-saghys* plantations. There are many more varieties within the *Taraxacum* family (Naturhistoriska Riksmuseet, 1997), but these are left unexplored within the frameworks of this project. However, since the relatives do not contain very much rubber (Josefsson, 1953) (Ramos, 2014), the *T. kok-saghys* is still the family member that will attract the most focus.

1.3.1 Growth and morphology of *Taraxacum* species

Dandelions are perennial herbs that belong to the family *Asteraceae*, tribe *Compositae* and the genus *Taraxacum* and grow mainly in the temperate regions of the Northern Hemisphere. The leaves grow in a rosette formation, and their jaggedness varies between species from smooth to the tooth-shaped appearance that has given them their name (“dent de lion” is French for “tooth of the lion”).

They have a taproot and through the upward transportation system, the xylem, running from root to shoot, there is a milky sap called latex. Yellow flowers are gathered in a capitulum on top of a hollow stalk (Naturhistoriska Riksmuseet, 1997). There are many subspecies within the *Taraxacum* family, some with fundamental differences. For example, the number of chromosomes varies between subspecies, so while the *T. officinalis* and the *T. brevicorniculatum* are triploids (Luo & Cardina, 2012), the *T. kok-saghys* is diploid (Josefsson, 1953). The diploid species are obligate sexual, meaning they need sexual reproduction, while many polyploids are facultative apomicts, meaning they most of the time reproduce asexually through apomixis, but also can reproduce sexually (Mártonfiová, 2011). In sexual reproduction a specific type of nucellus cell, called a mother cell, develops into an egg cell and needs to be fertilised by sperm cells (pollen). In *Taraxacum* apomixis, the egg develops through mitosis from another cell in the nucellus than the mother cell, and then develops into a zygote without fertilization (van Dijk, et al., 2003). Seeds produced by apomixis are genetically identical to the mother plant (Luo & Cardina, 2012). This makes dandelions difficult to systemize since many subspecies, referred to as apomictic subspecies, are similar in appearance with only minor mutational differences.

Apart from rubber, another interesting component of the roots of the Russian dandelion is inulin. It is a polysaccharide that is present in high concentrations in the roots, between 30-50% of the dry weight depending on the time in the growth season (Josefsson, 1953). This could be used as a raw material in for example bio fuel or ethanol production since it is easily fermentable into fructose by microorganisms. Other carbohydrates are also present in the root, between 45 and 30% of the dry weight, and the composition varies over the growth season. These could also be seen as a valuable by-product to the rubber. (Josefsson, 1953)

1.3.2 Previous experience of cultivating and breeding dandelions for rubber production

Large scale cultivation of the Russian dandelion has not been as easy as one might expect from a dandelion. While the common dandelion *Taraxacum officinale* grows fast and produces relatively large roots, its Russian relative proved to have weak seedlings which caused it to be out-competed by weeds (van Beilen & Poirier, 2007) (Josefsson, 1953). There were also difficulties collecting the seeds due to uneven flowering, weak stems and sensitivity to both dry and wet weather. The difficulties in harvesting the root efficiently also made the entire cultivation extremely labour intensive (Josefsson, 1953). Trials were made by the Swedish Seed Association (Sveriges Utsädesförening) in the 1940s and -50s to cross the *T. officinale* with the *T. kok-saghys*. The scope was to improve the cultivation properties of the *T. kok-saghys* (including larger roots, quicker growth rate, better and more even setting of seed etc.), but the trials did not reach the desired results. One problem was the already mentioned difference in the number of chromosomes. (Josefsson, 1953).

Conventional breeding was also conducted by the Swedish Seed Association in Svalöv, Skåne, between 1944 and 1952 (Josefsson, 1953) with the focus on increasing the rubber content of the root. The continuous selection also gave a slight increase in root size since the latex vessels were denser in the smaller root branches than in the taproot, which is why branched roots had higher rubber content than smaller unbranched roots. (Josefsson, 1953).

In 1952, after seven years of breeding, the average rubber content had increased to 15% in the bred populations (compared to about 6–7% of the wild type). The average rubber content of the best selection lines were 23%, which was three times more than the wild type starting material, while some plants even reached rubber contents as high as 30% (Josefsson, 1953). Though these stocks

were lost after the research was given up in 1953, these results show clearly that it is possible to increase the rubber content, even with classical plant breeding methods, to such an extent that it can be commercially viable to again realize it in research and production.

1.3.3 Modern options to breeding

Molecular techniques allow for studies of factors that affect the rubber synthesis both in *Hevea brasiliensis* and subsequently in *T. kok-saghys* and its close relative *Taraxacum brevicorniculatum.* Some of the results obtained in *Hevea* have been compared to other rubber producing plants such as the *Taraxacums* to get a broader picture of the rubber synthesis and its pathways. Many of the molecular pathways and proteins involved in the synthesis are now known (Post, et al., 2014) (Schmidt, et al., 2010) (Hillebrand, et al., 2012) (Wahler, et al., 2009), and it has also been found that rubber from *T. kok-saghys* contain less protein than rubber from *H. brasiliensis*, which would cause less allergic reactions in, for example, medical uses compared to *Hevea* (van Beilen & Poirier, 2007). The modern molecular techniques also make it possible to change the gene expression of the plant to suit the requirements and needs, without the amount of conventional plant breeding that was necessary in the 1940s. Currently, a few ideas for possible ways of increasing the rubber yield by using gene technology are considered and evaluated (Ramos, 2014) (Post, et al., 2014).

One of the most common and most stable ways to achieve the changes in gene expression is by using *Agrobacterium* mediated genetic transformations (Taiz & Zeiger, 2010). This technique utilizes the mechanism of a naturally occurring type of soil bacteria called *Agrobacterium tumefaciens* which can insert genes carried by the bacteria into the genome of wounded plant cells. The wild type *A. tumefaciens* carry onco-genes (cancer genes) which, when inserted into the cell, cause overproduction of growth hormones which cause uncontrolled cell growth, which in turn cause tumour-like structures on the plant. Other inserted genes force the plant cell to produce substances like opines, that only the bacteria itself can use as an energy source (Taiz & Zeiger, 2010). Today, new strains of *A. tumefaciens* can be designed to carry any selected gene instead of the original onco-genes while keeping the insertion mechanism intact. Thus, it is possible to insert new genes into plants in an efficient and controlled manner (Taiz & Zeiger, 2010). From the few modified plant cells, new whole plants can be regenerated that carry the new gene. This method is efficient compared to conventional breeding, where the risk of transferring undesired traits to the new generation is just as high as transferring the desired traits. Conventional crossbreeding is also time consuming as back-crossing is needed to get homozygous lines.

1.3.4 Modern cultivation options

Since the rubber in the *Taraxacum kok-saghys* is found almost exclusively in the roots (Josefsson, 1953), one way of avoiding the difficulties with cultivation in the field is to simply cultivate only the roots in root cultures. To make the root culture more effective, the *rolB* gene can be inserted into the plant genome through *Agrobacterium* mediated transformation, causing increased root growth while maintaining the stability of the plant. The *rolB* originates from another soil bacterium, *Agrobacterium rhizogenes*, that cause the “hairy root disease” in infected plants (Georgiev, et al., 2007). This way, the root can be cultivated in a sterile environment with complete control of the growth conditions without having to add plant hormones. Once infected, the growth conditions of the plant must be optimized taking the plant requirements and rubber yield into consideration. Thus, the root volume and thus the amount of rubber can be increased dramatically, and it can easily be harvested and purified. This system has previously been used for production of several products such as cosmetics,

antibiotics and cancer medication (Georgiev, et al., 2007) and is presently used commercially by ROOTec bioactives GmbH, Basel, Switzerland (ROOTec, Bioactivities, 2013). It is thus an established method that could be an option for rubber production in Russian dandelions.

Another option could be to grow only the rubber producing laticifer cells in cell culture. This has been tried as proof of concept, but not in larger scale (Post, et al., 2014).

1.4 Extraction of rubber

Finding an efficient extraction method for the dandelion rubber was one of the major difficulties in the 1950s, and the failure was one of the reasons why the project was abandoned (Josefsson, 1953). Several methods were tried, but none provided the desired results concerning purity, yield and cost efficiency (Normander, 1953). However, there are presently three extraction methods that are used to different degrees and that might be interesting to evaluate for extraction of rubber from dandelions.

One method tried both in small scale in scientific applications, and in commercial scale, is centrifugation (van Beilen & Poirier, 2007) (Schmidt, et al., 2010) (Yulex, 2013) (Buranov & Elmuradov, 2010). Slight variations of the method are used, but the basic principle is the same in all cases. The rubber bearing latex is separated from the biomass of the rest of the root either by grinding with water (for example (Yulex, 2013)) or by cutting the root material into pieces, allowing the latex to flow into an extraction buffer (Buranov & Elmuradov, 2010). Van Beilen & Poirier (2007) suggests the grinding to be followed by vibrating screens and flotation tanks to remove the biomass, while no such steps are currently used by Yulex (2013) and is completely unnecessary when using the flow method since simple decantation will suffice to remove the biomass. The extract is subject to centrifugation, where the rubber phase, containing small rubber droplets stabilised by proteins, can be separated from the water phase and the solid phase containing for example cell debris. The solid phase consists of discarded biomass and can be used for bio fuel production. Schmidt et al. (2010) and Buranov & Elmuradov (2010) use extraction buffers for more efficient extraction, while Yulex (2013) and Van Beilen (2007) claim no such needs. Overall this is a simple approach that is not dependent on large amounts of chemicals or expensive equipment. It only requires a centrifuge and large amounts of water. This can however be efficiently circulated in the facility after the centrifugation. If there is a strong desire to salvage the inulin from the rest of the biomass, this can be done early in the process, just after grinding. Since inulin is easily dissolved in warm water, it can be separated from the rest of the material (Normander, 1953).

Another method that is commercially available is dry extraction (NovaBioRubber, 2013). In this case, the plant material is first air dried which coagulates the rubber. It is then ground thoroughly to produce coagulated rubber threads and finely ground plant material. The material is then passed over vibrating screens where the finely ground plant material is separated and blown off, leaving only the rubber threads. The rubber threads are then purified further by stirring in warm water and skimming off the rubber threads (USPTO, 2013).

An extraction method that is theoretically possible, but to my knowledge, yet untried for rubber is supercritical CO_2 extraction. It is today used for extraction of other non-polar lipophilic biological compounds such as caffeine and spices (Catchpole, et al., 2012) and could also work for other similar compounds, such as rubber. The principle for this kind of extraction is that carbon dioxide under very high pressure (supercritical conditions) will be dissolved in the solution and act as a solvent for the

non-polar lipophilic compounds. The carbon dioxide can then easily be removed from the rest of the solution by lowering the pressure. As the pressure drops, the CO_2 would return to the gas phase and leave the solution, bringing the rubber along. The rubber would then be precipitated as the pressure drops further. This method provides a clean product with no traces of solvent. No other chemicals than the CO_2 is used, and it can easily be recycled within the extraction facility. The pre-treatment of milling and dissolving in water would probably still be necessary, and it is not yet clear if the method would work for high molecular weight compounds (Catchpole, et al., 2012).

2. Material and methods

2.1 Plant material and cultivation conditions

Seeds from *Taraxacum brevicorniculatum* were kindly provided by the Fraunhofer Institute in Münster, Germany. Some seeds were planted very shallowly (<2 cm) (Josefsson, 1953) directly in pots with sterile soil mixed with perlite, and placed in a light chamber (16 h photoperiod, 25°C, approximately 48% relative humidity) to enable studies of morphology and, in the longer perspective, seed production. The soil was kept moist, and when the seedlings were large enough, they were replanted to give them more space. At regular intervals, measurements were taken to enable morphological studies. These data are presented in Appendix 1. To evaluate factors that initiate seed production, both long days (20h) and short days (6h) were tried.

The seeds were sterilized by washing in 70% ethanol for 6 min, followed by washing with 5% bleach (Klorin) for 10 min. The seeds were then rinsed three times with sterile water before being directly transferred to sterile petri dishes with a germination medium (containing 20 g l^{-1} sucrose, 4.4 g l^{-1} MS supplemented with micro- and macro nutrients and 8 g l^{-1} agar, pH 5.8) (Post, et al., 2012). The plates were placed in a light chamber (16 h photoperiod, 25°C, approximately 48% RH) for 11 days. Germinated seeds without contamination were moved to root induction medium (20 g l^{-1} sucrose, 4,4 g l^{-1} MS supplemented with micro- and macro nutrients, 9 g l^{-1} agar and 2 g l^{-1} activated carbon, pH 5.8). The seedlings were allowed to grow between 4 and 9 weeks and the medium was changed every 1-2 months. These sterile plants were used for the *Agrobacterium* mediated transformation.

2.2 Transformation mediated by *Agrobacterium tumefaciens* and regeneration

For the transformation, *Agrobacterium tumefaciens* strain C58C1 (pGV3850) was used, generously provided by Li-Hua Zhu, Professor in Plant Breeding at SLU Alnarp. The strain harbours the binary vector pCMB-B:GUS seen in Fig. 2. It contains an *nptII* gene, which confers kanamycin resistance and enables selection of successful transformants, as well as the *gus* (a reporter gene not utilised in this project) and *rolB* (the gene of interest that induce root growth) genes both under the *rolB* promoter (Feyissa, et al., 2007).

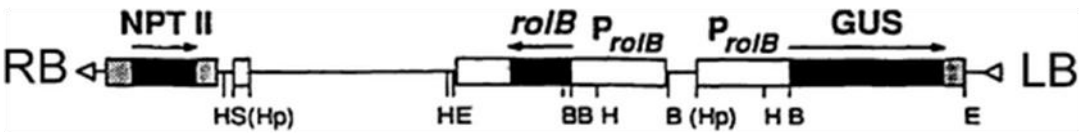

Figure 2. A schematic picture of the pCMB-B:GUS vector, displaying the position of the genes along with restriction sites (B: *BamHI*, E: *EcoRI*, H: *HindIII*, H: *HpaI*, S: *SalI*. Brackets indicate restriction sites that have been deleted due to cloning). (Feyissa, et al., 2007)

The transformation protocol used was adjusted from the protocol received from Ramos (2014), to improve the efficiency. Green and healthy leaves from sterile plants were carefully cut and put on sterile filter paper. Different ages of plants were used to evaluate if age affected the transformation efficiency. The abaxial sides of the leaves were wounded with a scalpel across the main nerve of each leaf. The leaves were then cut into pieces and put in a solution containing the *Agrobacterium* and incubated at room temperature in darkness for 20 minutes (Fig. 3A). The leaf pieces were then moved to sterile filter paper to wipe off as much of the Agrobacterium solution as possible, and moved to a co-culture medium (20 g l^{-1} sucrose, 4.4 g l^{-1} MS supplemented with micro- and macro nutrients and 8 g l^{-1} agar, pH 5.8) for three days (Fig. 3B).

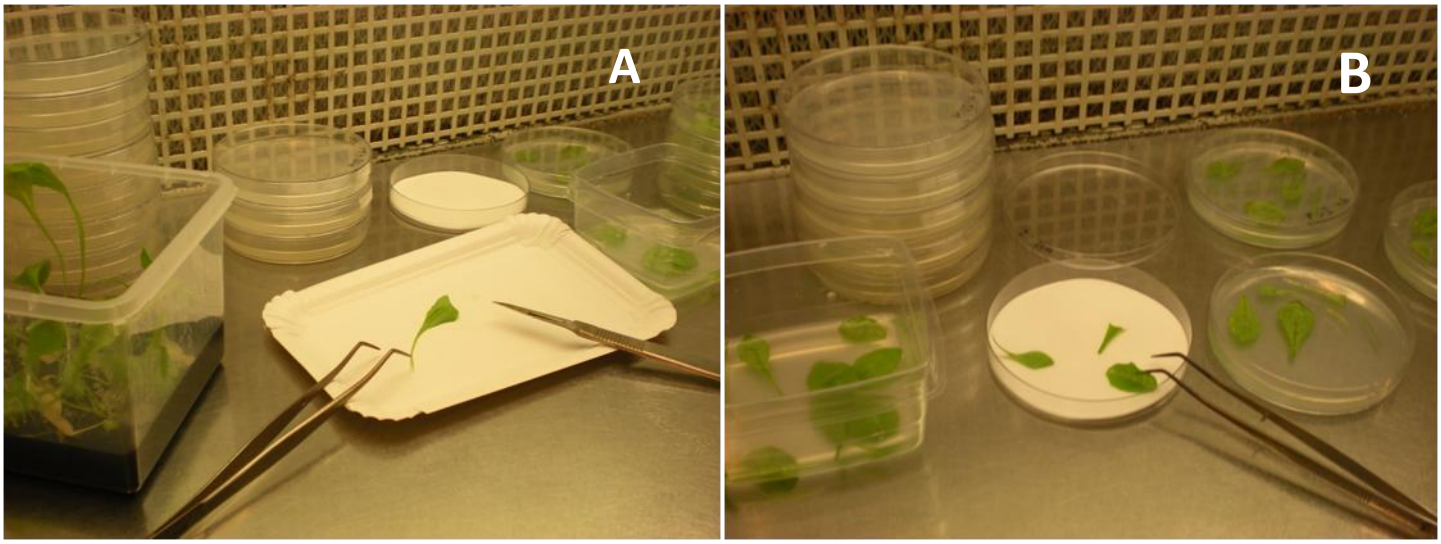

Figure 3. (A) The sterile leaves were cut from the plant and put on sterile paper. Using tweezers and a scalpel, the underside (abaxial side) of the leaves were wounded and the leaves were cut into pieces that were put into a solution containing the Agrobacterium. (B) After incubation with the *Agrobacterium* for 20 minutes, the leaf pieces were dried on sterile filter paper and left on petri plates with co-culture medium for three days.

The pieces were then moved to a callus inducing medium (20 g l^{-1} sucrose, 4.4 g l^{-1} MS with micro- and macro nutrients, 8 g l^{-1} agar, 1 mg l^{-1} BAP, 0.2 mg l^{-1} IAA, 50 mg l^{-1} kanamycin for plant selection, 150 mg l^{-1} ticarcillin and 150 mg l^{-1} cefotaxime to prevent growth of Agrobacterium, pH 5.8) until callus appeared. During that time, the medium was changed every week and blackened leaf pieces were removed. The callus were moved to shoot induction medium (20 g l^{-1} sucrose, 4.4 g l^{-1} MS with micro- and macro nutrients, 8 g l^{-1} agar, 2 mg l^{-1} zeatin, 0.1 mg l^{-1} IAA, 0.05 mg l^{-1} GA_3, 50 mg l^{-1} kanamycin for plant selection, 250 mg l^{-1} cefotaxime to prevent growth of *Agrobacterium*, pH 4.8) until the shoots were approximately 5 cm high. During this time, the media were changed every week. The shoots were then moved to root induction media (20 g l^{-1} sucrose, 4.4 g l^{-1} MS with micro- and macro nutrients, 9 g l^{-1} agar, 50 mg l^{-1} kanamycin for plant selection, 250 mg l^{-1} cefotaxime to prevent growth of *Agrobacterium*, pH 5.8). The plants were during all this time kept in a light chamber with 16/8 h photoperiod at 33 mol $m^{-2}s^{-1}$ and the temperature of 23°C/18°C (day/night).

2.3 Molecular analysis using PCR

The genetic material of the transformed plants was extracted using a CTAB miniprep protocol (Zhu, 2011). Plant tissue, approximately 100 mg from each of 21 putative transformed lines and one wild type for negative control, was collected and frozen in liquid nitrogen followed by grinding to a fine powder. Buffer containing CTAB and 2-mercaptoethanol was added and incubated for 10 minutes at

65°C to disrupt cell membranes. Chloroform and isoamylalcohol were used to separate the proteins from DNA. The DNA was then precipitated with isopropanol and washed with a washing buffer. The pellet was dried followed by resuspension in a TE-buffer containing RNase to remove RNA contamination. After incubation in 37°C for 1 h, the DNA concentrations and purity of the samples were established using Nanodrop, and the samples were subsequently frozen.

For PCR, the samples were thawed on ice. A master solution (containing dyeing buffer, dNTP, 3'- and 5'-primers and Taq polymerase) was prepared and 9µl added to each PCR tube together with 1 µl DNA sample. In the PCR, a positive control consisting of the pure plasmid with the *rolB*-construct, a negative control consisting of DNA from wild type *T. brevicorniculatum*, and a blank where water had been added instead of a genetic sample, were included as well as the ten different samples from transformed plants. The experiment was run twice with two different sets of primers, once with *rolB* primers, and once with *nptII* primers. The annealing temperature differs slightly depending on primer, and in this case 60°C was used for *rolB* and 65°C was used for *nptII*. After running the PCR, the samples were run on a 1% agarose gel that allows differentiation of the DNA fragments according to size. Staining with ethidium bromide and viewing under UV-light made the fragments visible.

2.4 Cultivation in bioreactor

Two bioreactors from Plantform (Welander, et al., 2014) were assembled according to the instructions and autoclaved along with 500 ml of growth medium (30 g l^{-1} sucrose, 4.4 g l^{-1} MS with micro- and macro nutrients and 280 mg l^{-1} cefotaxime) for each reactor. Shoots that had, according to the PCR results, successfully incorporated the *rolB* gene were put in one reactor. In the other reactor, shoots that had been run in the PCR but proved negative for *rolB* were included. The roots of the seedlings were washed in sterile growth medium before being placed in the reactors, in order to remove remnants of root induction media (RIM) that were stuck to the roots.

The two bioreactors were connected with silicon tubes according to the instructions (Welander, et al., 2014) and connected to two pumps on timers. Pump number one regulates the level of the growth medium. By pumping air into an inner chamber underneath the basket containing the seedlings, the surface of the liquid rises in the whole reactor, thus ensuring that the nutrients reach the seedlings. When the pump is turned off, the air in the inner chamber will eventually leave the chamber and the level of liquid will fall. Pump number one is on for six minutes at a time, twice a day. Pump number two regulates the aeration of the reactors and is on for four minutes at a time, 20 times a day.

2.5 Extraction of rubber and determination of rubber content

For these experiments, the cleaned taproots of the soil-grown plants were used. After evaluation of available methods, two approaches were tried, one according to Buranov and Elmuradov (2010) and one according to Schmidt et al. (2010). The two methods were very similar in many respects. They both build on the principle of extracting the latex of the root without grinding, but while Schmidt et al. (2010) used a syringe or pipette to move the latex from the root to the extraction buffer, Buranov and Elmuradov (2010) cut the roots into pieces and allowed the latex to flow from the pieces into the buffer during shaking. The extraction buffers used also differ: Schmidt et al. (2010) used a Tris-based buffer (100 mM Tris-Cl, 350 mM sorbitol, 10 mM NaCl, 5 mM MgCl2, 5 mM DTT, pH 8.9) while Buranov & Elmuradov (2010) used an ammonia-based buffer (0.1% Na_2SO_3 and 0.2% NH_3, pH 10). It is important to use a buffer with a slightly basic pH to prevent the rubber from coagulating during the

extraction. The extracts containing the latex were then centrifuged for 15 minutes at 12 000g (Schmidt, et al., 2010) or 18 000g (Buranov & Elmuradov, 2010), respectively. Since the density of rubber is lower than the density of water (0.92 g ml^{-1} for rubber and 0.998 g ml^{-1} for water (Sigma Aldrich, 2014)) it is expected that the centrifugal forces will separate the two phases and that the rubber will float on top of the water phase. The rubber should then be easy to remove in its liquid form using a pipette, or coagulated by lowering the pH and removed in a more solid form using tweezers.

After extraction the root pieces were dried in an oven at 50°C, and pieces from pre and post treatment as well as the extract, were subjected to analysis using Fourier Transform Infrared (FTIR) spectrums. FTIR is a form of spectrophotometer that can detect the specific vibrations in individual bindings in a molecule. Molecules of different structure have different bindings which will give different patterns of vibrations in the spectrum, making it possible to detect specific substances in complex samples.

To have something to compare to, a cis-polyisoprene reference material was ordered from Sigma Aldrich. Its solubility in both the Tris-buffer and the ammonia-based buffer was tried as well as the organic solvent hexane.

3. Results

3.1 Germination and growth of wild type plants

The seeds planted in soil started to germinate after 4 days, and small seedlings were visible after 7 days. The plants grew well though they were very sensitive to both drought and over watering. On some plants, the leaves turned spotted with yellow for no apparent reason. No fungus contamination could be seen, and not all plants were affected, so one theory is that it was due to over watering. The discoloured leaves could be removed without seeming to affect the growth of the rest of the plant. After 30-40 days, the plants had reached their maximum rosette diameter, and further growth did not increase the size of the plants, though the edges of the leaves on some plants turned more jagged as they grew older. For morphological data, see Appendix 1. After growing for 7 months in "summer conditions" (16 h daylight, 25°C), no flowering had yet occurred. Longer days (20 h daylight) for four weeks did not induce flowering. Vernalisation for 7 weeks (6 h daylight, 15°C), however, seemed to be what was required. 17 days after restoring "summer conditions" after the short day treatment, the first plant started to produce flowers. A total of 59 flowers were produced the first 4 weeks of flowering, divided on 18 pots.

Roots were harvested after 22 weeks and had average root weight of 30 g ww and 7.5 g dw per pot (4-6 plants). The roots consisted of a small taproot and many root threads throughout the entire pots. Larger taproots would have been expected if the plants were planted with more space (Josefsson, 1953).

The original seed sterilization protocol received from Ramos (2014) did not work well (after three weeks, only 3.1% of the seeds had germinated) but trials to modify the protocol resulted in a method that gave no fungus contamination and still had a high germination frequency (87.5% on the 8^{th} day). After being moved to RIM, all seedlings grew well and no fungus contamination was observed.

3.2 Agrobacterium mediated transformation and regeneration

When the transformation protocol was successfully developed, transformations were performed in two major batches one week apart. In each batch, sterile plants of ages between 4-9 weeks after sowing were included to evaluate if tissue age affected the efficiency of the transformation. This resulted in a total of 40 petri plates with leaf explants, similar to the ones seen in Fig. 4. The first three days after transformation, the leaf explants were put on co-culture medium (called MSI), and were later moved to a callus inducing medium (CIM). A short time after the transformation some explants started to turn brown or black. These were gradually removed every week when the explants were moved to fresh medium.

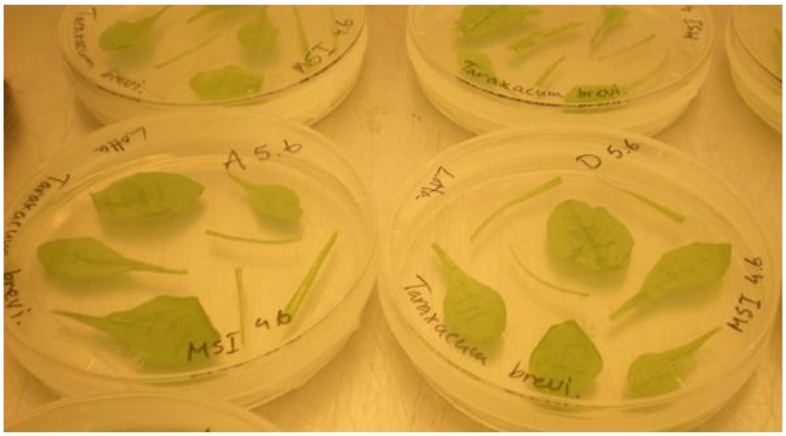

Figure 4. Petri plates with co-culture medium and newly transformed leaf explants.

After 17 days on CIM, the first callus with shoots had appeared. Most of the callus and shoots were formed along the main nerve of the leaves, as can be seen in Fig. 5. The shoots were cut from the explants with a scalpel and moved to shoot induction medium (SIM) as in Fig. 6A. The formation of new shoots continued, and every week new shoots were cut from the explants and moved to SIM.

Figure 5. Explants on CIM that have started forming shoots, mainly along the main nerves of the leaves. Calli without shoots can also be seen in some of the wounds. The pictures are taken 25 days after transformation.

The last explants that were judged unlikely to produce more shoots were discarded approximately 50 days after the first transformation. The shoots were left on SIM for a few weeks. The medium was changed every seven days, and the shoots were also given more and more space as they grew larger (Fig. 6B). Some calli with shoots were very large, and these were divided into more than one shoot, forming two or more shoots of the same genetical line. When the shoots were large enough they were moved to RIM, Fig. 6C. Where they were allowed to grow until they were ready for the bioreactor.

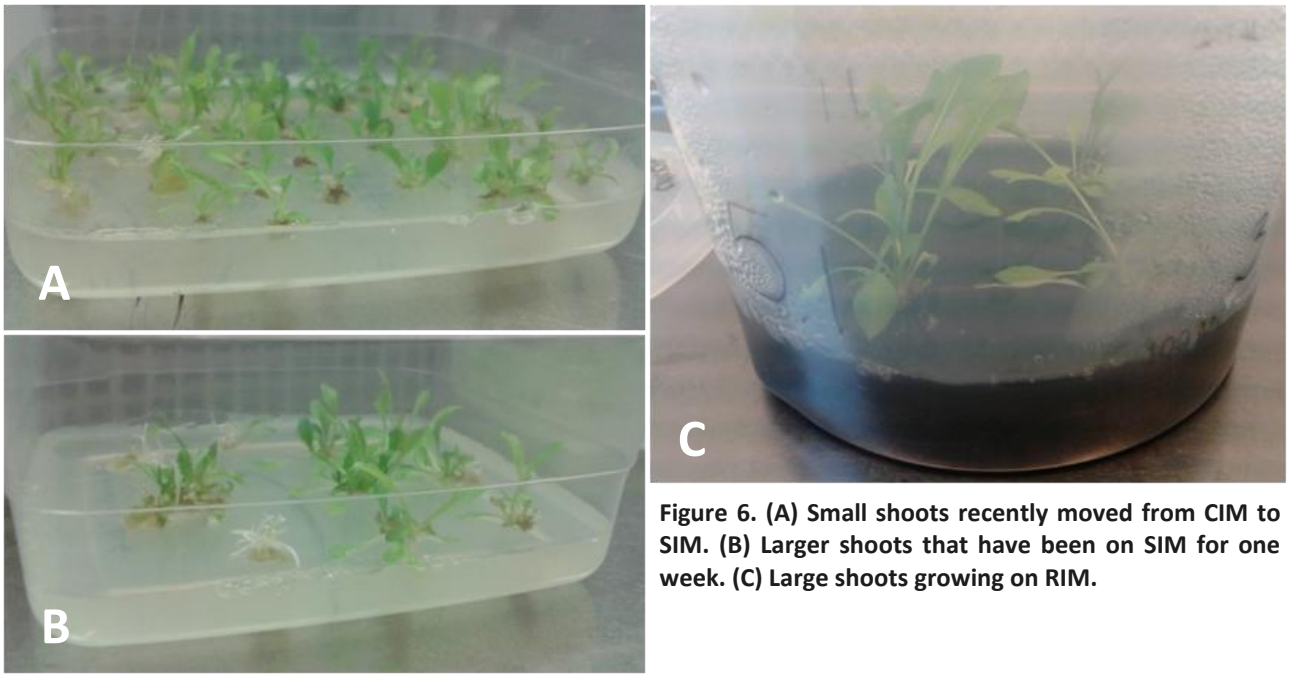

Figure 6. (A) Small shoots recently moved from CIM to SIM. (B) Larger shoots that have been on SIM for one week. (C) Large shoots growing on RIM.

Plants of different ages were used in the transformation to evaluate the age-effect on transformation efficiency. Every week, the numbers of shoots from each age of explants that were moved from CIM to SIM were counted and have been compiled in Fig. 7. In the batch transformed 12.8 the number of successful transformations steadily decreased with decreased age of the plant. In the batch transformed 19.8, the efficiency seemed to be significantly higher with the explants from 8-week old plants than with other ages. Though these results are not conclusive, they indicate that using 8-week old plants would provide the highest transformation efficiency.

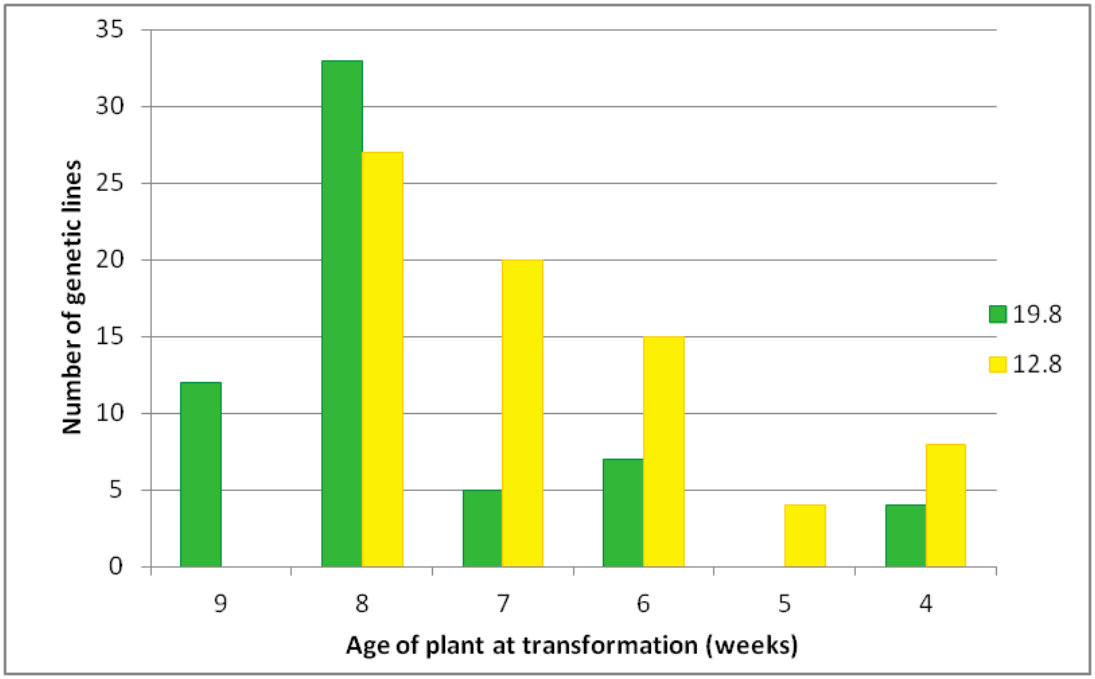

Figure 7. The number of genetic lines sprung from explants of different ages. Green bars represent the batch transformed 19.8 and yellow bars represent the batch transformed 12.8. Observe that no 9-week old plants were used 12.8, and no 5-week old plants were used 19.8.

3.3 Molecular analysis using PCR

In the picture of the agarose gels in Fig. 8A and B, the PCR results of the first ten samples are shown. Clear bands that match the size of the fragment from the positive control are seen in some of the samples. It seems likely that all samples except sample 5 have been successfully transformed and have acquired the *nptII*-gene (Fig. 8A). However, only sample 1, 4, 6 and 7 seems to successfully have acquired the *rolB*-gene (Fig. 8B).

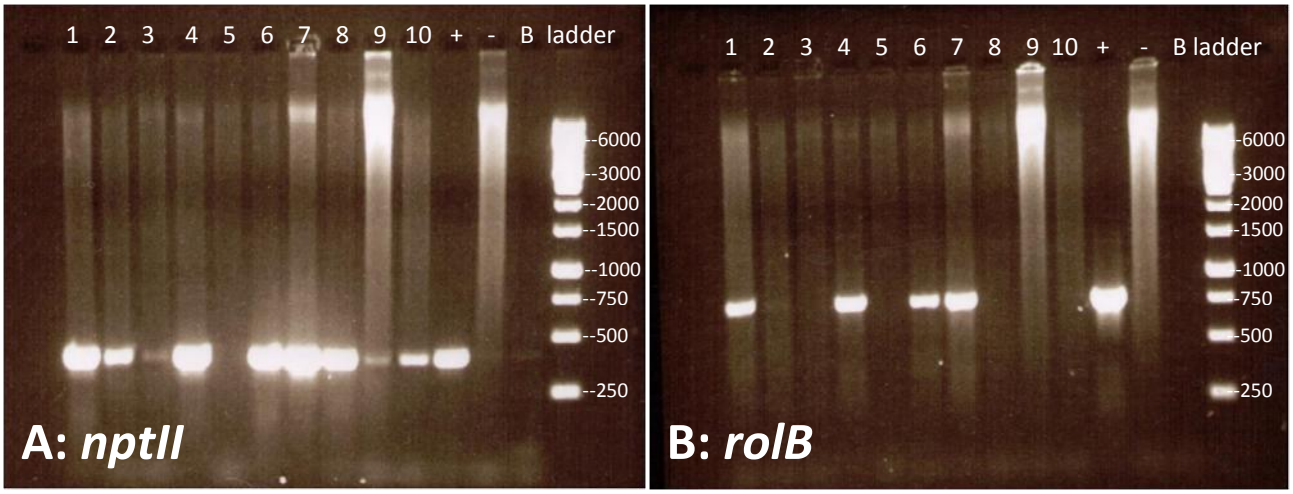

Figure 8. Pictures of the agarose gels of PCR-products using *nptII* primers (A) and *rolB* primers (B). Samples were loaded on the gel in the order presented at the top: 1-10 are the samples of 10 different transformed genetic lines, + is the positive control, - is the negative control, B is a blank using water as template, and lastly the molecular ladder with the molecular sizes presented in number of bp.

3.4 Cultivation in bioreactor

After cultivation in bioreactor for 4 weeks, the reactors were disconnected and the seedlings moved to new solid RIM medium. The *rolB* negative seedlings grew well in the reactor and produced long thin roots and increased their rosette diameter. The reactor with the *rolB* positive shoots was contaminated with *Agrobacterium* and thus grew less and hardly produced any roots, since the shoots struggled to survive at all in competition with the bacteria. On the roots of one of the *rolB* positive seedlings, however, some of the "hairiness" that was expected for the transformants could be seen. The root hairs were absent in all of the control seedlings. Pictures of the roots can be found in Appendix 2.

The roots of the seedlings from the bioreactors were compared to roots from WT seedlings grown on solid RIM, and the *rolB* negative seedlings in the bioreactor showed a similar development as the roots grown on solid medium. They were both long and thin with some branching, but with clear taproot morphology. The roots from the bioreactor, however, were both longer and sturdier than the ones grown on solid medium. The WT seedlings were grown from seeds and were 15 weeks old, and were compared to the seedlings in the reactors which were transformed 14.5 or 15.5 weeks ago.

3.5 Extraction of rubber and determination of rubber content

In the initial preliminary trial, the method of Buranov and Elmuradov (2010) was used with the protein casein added to the buffer with the purpose of stabilizing the rubber particles (Buranov & Elmuradov, 2010). In this trial, the "creamy white layer" that was expected to be visible after centrifugation was absent. When adding acid and thus lowering the pH, however, a white or yellowish turbidity appeared. This was believed to be coagulated rubber. When the samples again

were centrifuged, the coagulum was expected to float on top of the water phase, but instead it sedimented. The sediment was collected on pre-weighed filter papers and dried. An estimation of the total amount of coagulum produced in this trial was 40 mg g^{-1} dry root with no way of knowing anything about the purity of the sample, or indeed if it actually was rubber or some other substance, perhaps proteins, that coagulated.

Roots that had not been exposed to the extraction process, roots after the extraction and the collected coagulum from the preliminary trial were dried and analysed using Fourier Transform Infrared (FTIR) spectrums. These spectrums are shown in Fig. 9 along with the spectrum of dried clean filter paper for comparison. For more detailed pictures, see Appendix 3 where the spectrums are divided into more than one picture.

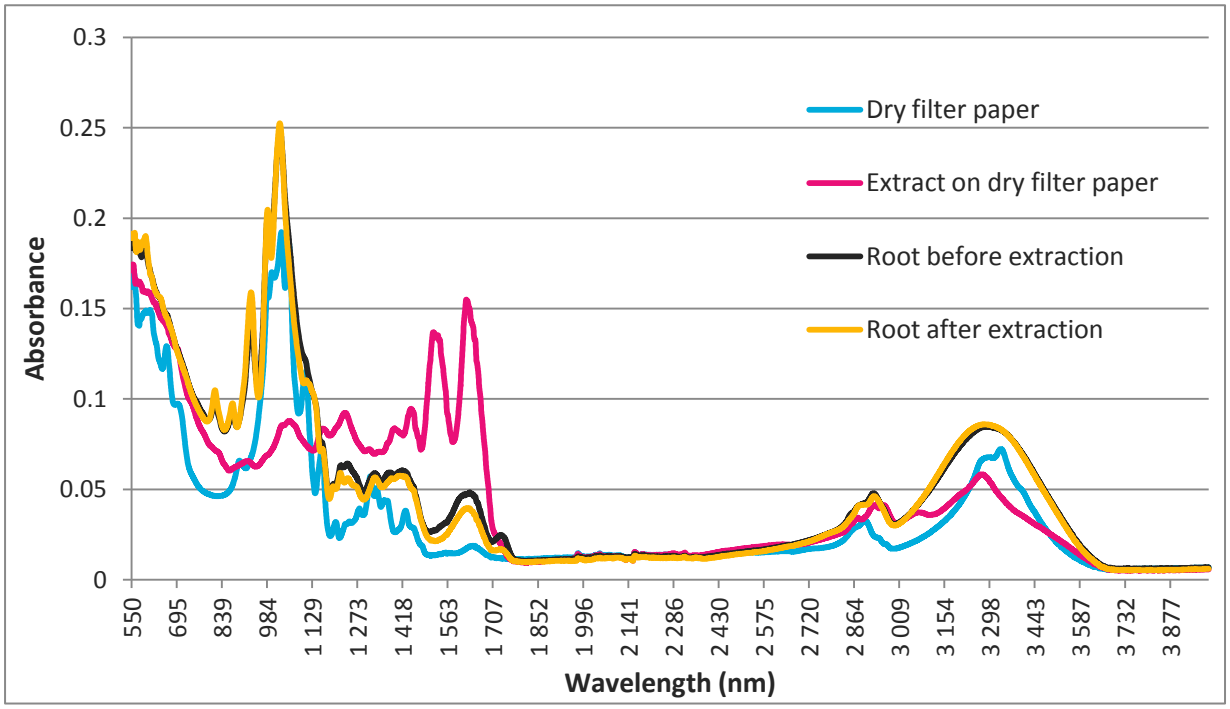

Figure 9. Spectrums from the Fourier Transform Infrared analysis of the initial trial. The root before and after extraction was analysed, as well as the coagulated extract with the clean filter paper on which it was gathered as background. For a more detailed view of the spectrums, see Appendix 2.

In the following trials, neither of the methods used, (Buranov and Elmuradov 2010 and Schmidt et al. 2010), resulted in any available rubber or visible coagulum. This is probably due to the limit of detection. According to Christian Schulze Gronover (professor at the Fraunhofer Institute in Münster, Germany), the wild type *Taraxacum brevicorniculatum* strain that have been used in this project usually does not contain more than 1.5 mg rubber/g dry root weight. Since I have not had access to very much root material and the water content of the roots was approximately 70%, the amount of rubber that could be extracted has been in the order of magnitude of 2.5 mg per experiment, which is too little to detect and analyze. It is also too little to proceed and be able to run either the dry extraction process (NovaBioRubber, 2013) or the critical CO_2 extraction (Catchpole, et al., 2012) mentioned in the introduction.

4. Discussion

4.1 Germination and growth of wild type plants

The germination speed of seeds was faster for soil-grown plants than for sterile seeds. This was probably due to the sterilization process that was tough on the seeds. However, the used protocol had higher germination frequency than the protocol initially received from Ramos (2014) (results not shown), but still showed no fungus contamination.

The plants in soil also grew quicker and became larger than their sterile in vitro counterparts. The sterile plants were weaker and more sensitive since they grew in a closed environment in their plastic container where the air humidity was higher. Therefore, they did not develop the protective layer of wax that usually coats the leaves. Interestingly, the edges of the leaves of the sterile plants remained smooth while most of the soil-grown plants developed the jaggedness so typical for the dandelion species.

Surprisingly, after growing for seven months in a climate chamber at 20°C and 16h of light per day the soil-grown plants still had not flowered. Induction with longer days (20h of light) was tried for more than three weeks to mimic summer season, without any results. It has been suggested that the *Taraxacum brevicorniculatum* is a biannual and requires a winter season to flower. This is supported by Flora of China (www.efloras.org, 2014) that claims the *T. brevicorniculatum* is in fact a hybrid with *Taraxacum kok-saghys,* which is a perennial (Plants For A Future, 2014), as one of the parental taxa. Since some *T. kok-saghys* do not flower until their second year of growth (Josefsson, 1953), the same might apply for *T. brevicorniculatum*. The winter season usually means a change of mainly two parameters, temperature and light, with colder weather and shorter days. *Taraxacum kok-saghys* have previously been grown in greenhouses over the winter season where the temperature have been kept at approximately 15°C (Josefsson, 1953), which would not be low enough to induce a cold response, and flowered the following spring. It is, therefore, likely that it is the short days that are required. To evaluate this theory, the photoperiod in the climate chamber was set to 6 h daylight, and the temperature lowered to 15°C.

4.2 Agrobacterium mediated transformation and regeneration

Transformation was initiated based on the protocol from Ramos (2014) without success. Modifications were thus made regarding different factors affecting the transformation efficiency, such as *Agrobacterium* incubation time and the way of wounding. The incubation time was lowered from 30 to 20 minutes, since the longer time was too tough on the explants apparently due to the violent bacterial growth. The co-cultivation time was lowered from 6-7 days to 3 days, since the *Agrobacterium* grew so violently and almost devoured the explants. Different ways of wounding were also tried, and the most efficient turned out to be wounds across the main nerve of the leaf, preferably using the lower part and stalk of the leaf rather than the wider upper part. Cutting the leaves into smaller pieces and having smaller but more wounds was also tried and resulted in explants that stayed green longer, but produced fewer calli. Not all explants produced callus, and some explants produced many, which is likely due to the physiological or developmental differences or position effects of individual leaves. Also, the batch transformed on 12.8 produced more shoots (74 altogether) than the batch transformed on the 19.8 (only 61), even though they both started with 20 petri plates of explants in co-culture and CIM.

Some explants stayed green for a very long time, while others blackened quickly. Some produced calli and shoots in spite of turning black. Overall the stem pieces remained greener than the top leaf pieces, but there were large differences between different stem pieces as well. The size of the explants did not affect their endurance in any clear way, since some small explants were healthy for a long time while others blackened almost immediately. The same thing applied to large pieces, though really large pieces often consisted of the top part of the leaf which seemed to be weaker. The leaf tip is the eldest part of the leaf, while leaf petiole is relatively more juvenile (Taiz & Zeiger, 2010) and thus easier to regenerate. The leaf tip might, therefore, release more senescence related signals, causing the leaf to grow more weakly under stress condition than the more juvenile material.

When a plant is infected by a pathogen, in this case the *Agrobacterium*, it can locally induce cell death in what is called necrotic lesions. This isolates the infection since the pathogen will not have any nourishment from the plant, and prevents the whole plant from being infected. Another possibility is that under stressful conditions, the plant tries to save as much energy as possible while losing as little nutrients as possible. Metabolites will then be broken down at the fringe of the plant and sent toward the center (Taiz & Zeiger, 2010). In this case, that results in the metabolites accumulating in the stem of the leaf while leaving the rim. Perhaps some explants were more successful in absorbing nutrients from the culturing medium and thus were less stressed. These might have stayed greener longer if they did not experience the stress responses as severely.

4.3 Molecular analysis using PCR

The discrepancy between the number of successful transformants depending on the set of primers (compare Fig. 8A and 8B) is probably dependent on the genes' location on the construct. Since the *nptII* gene is located first in the gene construct (Fig. 2), some plants might have only received the first piece, while some have received the whole construct which includes *rolB*. Since the *rolB* is the target gene to be inserted in this case, only the 7 out of 21 samples containing this gene will be the ones considered as successful transformations of the *rolB* gene.

Some smear can be seen in the lanes in Fig. 8, which could be a sign of overloading of the gel. The DNA samples were not diluted before loading into the PCR, which might result in a larger quantity of genetic material than necessary. Another possible explanation to the trailing is that the primers have bound in other places in the DNA than the desired position, thus possibly amplifying unwanted sequences of greater size than the *rolB*. Unspecific binding in several places could result in sequences of many different lengths, which could result in the trailing visible especially in sample 9 and the negative control in Fig. 8. Since the size of the band is so great, it is also possible that it is genomic DNA, which could also a probable explanation to the residues visible in the wells of the gel.

In Fig. 8A, faint lines corresponding to the amplified sequence can be seen both in the negative control and in the blank samples. This could be due to leakage from another well when loading the gel, resulting in small amounts of sample ending up in the wrong well.

4.4 Cultivation in bioreactor

Based on the results from the *rolB* negative seedlings, it seems that growing *T. brevicorniculatum* in bioreactors is possible. It is however very important to avoid bacterium contamination for the plants to grow and roots to develop properly. In this experiment, the hairy roots that were desired were only displayed on one of the *rolB* positive seedlings due to the contamination by *Agrobacterium*. The presence of the hairy roots is still a confirmation that this proof of concept could be a possible

alternative for growth of rubber producing dandelion species. It is a relatively simple and cheap method that, once in place, needs minimal work and takes less space than many growth alternatives. The root production also seemed to be larger for the *rolB* negative seedlings grown in the bioreactor compared to seedlings grown on solid medium, indicating this could be an option for growth even without the *rolB* effects.

4.5 Extraction of rubber and determination of rubber content

More detailed pictures and discussion can be found in Appendix 3. A few things can, however, be deduced at a first glance when looking at the spectrums in Fig. 9. First, the spectrums from the root samples before and after extraction roots are very similar. They only differ notably in two places, the peaks between 1220-1430 nm and the peak between 1490-1750 nm. In both those cases, the absorbance is lower for the root after extraction, which might imply that something has disappeared from the root during the treatment. Second, that the peak between 2980-3635 nm in those two samples is probably water since this is where water usually absorbs light. The root pieces had been dried in an oven at 50°C for two days before the FTIR measurement, but apparently still contain some water. The major water peak at 3280 nm is visible in the other two samples as well, but to a lesser extent. In the spectrum from the dry filter paper, another peak at 3330 nm can be seen which, even if it was present in the root samples, would have been completely hidden by the water peak. Third, very notably, is the peak at 1520 nm present in the spectrum for Extract on dry filter paper that does not seem to have a counterpart in any of the other spectrums.

According to Chen et al. (2013), there are different peaks to be expected in the FTIR spectrum depending on the structural form of the polyisoprene. The peaks in an FTIR spectrum are caused by absorption of energy by individual bonds within molecules, causing vibrations. Different types of bonds, e.g. single or double bonds, have different stretching vibrations and *cis*- and *trans*-configurations have different scissoring vibrations. Since *Taraxacum brevicorniculatum* is expected to contain *cis*-1,4-polyisoprene (van Beilen & Poirier, 2007), it could be expected to find peaks at 837, 1036, 1140, 1311, 1375, 1663 and 3035 nm (Chen, et al., 2013). However, only a few of those peaks can be seen while some peaks corresponding to other polyisoprene configurations (1,2-, 3,4- and t*rans*-1,4) are also visible. For a visualisation of this and further discussion, see Appendix 3 and figure A3.4.

The uncertainties in FTIR spectra are great since some peaks might interfere with others and large peaks might hide smaller ones (as in the example of water above). There might also be other substances in these complex samples that have peaks in roughly the same wavelengths. A clear pattern with many of the peaks corresponding to the same substance is required to make a reasonably certain prediction of the presence or absence of said substance. In this case, that clear pattern is missing. It is thus not possible to conclude from the spectra in Fig. 9, whether there is or is not natural rubber present in the sample or of the possible structure of that rubber, since some peaks that should be there are, but others that should be there are not.

Even though it cannot be concluded whether the coagel from the trial was rubber or not, something did coagulate when the pH was lowered, just as was expected for the rubber. The same reaction is however expected from proteins that denature at low pH. It cannot be excluded that it was polyisoprene in some form, even though it sedimented, against what was expected from the rubber. The sedimentation could be due to the presence of casein (ρ_{casein}=1.25 g cm^{-3} (Sigma Aldrich, 2014)) in

the buffer, which was supposed to bind to the rubber and stabilize the rubber particles. If it did, the casein might have increased the density of the particles enough to cause the total density to be higher than the density of the water based buffer (ρ_{water}=1 g cm^{-3}). Other unknown impurities that might have been caught in or around the particles could probably cause similar effects. It is also possible that some other substance present in the sap of the root reacted to the lowering of the pH, and caused the turbidity that was visible. Though what that substance would be, I do not know. If this setup would be performed again, it might be interesting to run an SDS-PAGE analysis on the extract to evaluate its contents.

5. Conclusions

Within this project, protocols for both sterilization of seeds, transformation and regeneration have been developed and has worked well. It has also been established that it is possible to cultivate *Taraxacum brevicorniculatum* in bioreactors, though the effect of the transformation with *rolB* is difficult to evaluate due to *Agrobacterium* contamination. There are, however, results that show indications of how the roots of plants transformed with *rolB* could look, and those results are promising for a new trial.

No final conclusions can be drawn from the results concerning the rubber content or type of rubber present. More root material is most likely needed for further extraction trials, and the analysis needs to be complemented with additional methods.

6. Acknowledgements

Many thanks to Christian Schulze Gronover that allowed me to come visit the Fraunhofer Institute in Münster, Germany, and lab technician Gianina Ramos from the same institute that taught me everything about transforming and regenerating *Taraxacum* species. A great many thanks to all colleagues at Pure and Applied Biochemistry in Lund, especially PhD Nélida Eriksson, for helpful advice and sharing of knowledge. I am also extremely grateful for all the good advice and invaluable help given by Annelie Ahlman and her colleagues at SLU in Alnarp.

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7.1 Picture sources

Cover: Dandelion from http://fearlessparent.org/eat-weeds/ (accessed 2014-09-30),
Lund University logo from http://www.stimesi.rl.ac.uk/locations/LUND.cfm (accessed 2014-09-30)
SLU logo from https://internt.slu.se/sv/stod-och-service/kommunikation/grafisk-profil/logotyp/hamta-slus-logotyp/ (accessed 2015-01-19).

Figure 1. Molecular structure of natural rubber. (Van Beilen and Poirier 2007)

Figure 2. A schematic picture of the pCMB-B:GUS-vector. (Feyissa, et al., 2007)

Figure 3, 4, 5 and 6. *Taraxacum brevicorniculatum* in various stages of transformation and regeneration. My own photographs.

Figure 8. Pictures of agarose gels and PCR products. Scanned from pictures printed from the UV measurement.

Figure 9. FTIR spectrum.

Appendix 1: Figure A1.1. *Taraxacum brevicorniculatum* plants growing on soil. My own photographs.
Figure A1.2. Flowering *T. brevicorniculatum* growing on soil. My own photographs.

Appendix 2: Figure A2.1. Biorector with *rolB* negative seedlings. My own photograph.
Figure A2.2. Roots of seedlings grown in bioreactor and solid medium. My own photographs.
Figure A2.3. Taproot from *rolB* negative and hairy root from *rolB* positive seedling. My own photographs.

Appendix 3: FTIR spectrums.

Appendix 1:
Growth and morphology of soil-grown *T. brevicorniculatum*

Signs of germination were visible on day 4, but quantitative parameters could not be measured until day 7 (Table A1.1). The total number of seeds planted was not counted, so germination frequency could not be calculated. The cotyledon and rosette diameter were measured at the widest place (cotyledon was judged to turn into rosette when there were three or more leaves). The leaf width was measured at the widest place of representative leaves and averaged. When the seedlings grew large enough, the space in the pots became limited and it was difficult to separate the individual plants. Thus it was also difficult to measure the rosette diameter and number of leaves per seedling. However, the size of the leaves stabilized at about this time, indicating that the plants had reached their adult phase. See also Fig. A1.1 for growth development.

Table A1.1. Morphology and developmental data for soil-grown plants seen in days after planting. All values are approximates.

	Number of seeds germinated	Average cotyledon/rosette diameter (mm)	Average leaf width (mm)	Number of leaves per seedling
Day 7	154	10	4	2
Day 9	157	19	6	2
Day 11	168	24	8	2-3
Day 14	168	30	11	3-4
Day 17	168	50	15	5
Day 22	168	70	20	5-6
Day 24	168	90	25	6
Day 29	168	120	25	7-9
Day 31	168	-	25	-
Day 35	168	-	25	-

The plants remained approximately the same size during their adult phase while growing in summer conditions, with the only change occurring that the edges of the leaves of some of the plants turned jagged. Vernalisation (6h daylight, 15°C for 7 weeks) made most of the leaves wilt, but when summer conditions (18h daylight, 25°C) were restored new leaf formation started. The edges of the new leaves were very jagged on most plants. 17 days after terminating the vernalisation the first plant started to produce flowers, see development in Fig. A1.2. After a few days, the reason for the difficulties with seed collection in the 1950's (Josefsson, 1953) becomes evident. By day 22 after terminating the vernalisation, there are flowers in all stages of maturity: from buds to faded flowers. An appropriate time to harvest the seeds is therefore difficult to find. By day 31, most flowers are faded and the seeds are starting to ripen, though some are still flowering and a few buds still have not bloomed.

Figure A1.1. Development of soil-grown *Taraxacum brevicorniculatum* days after sowing.

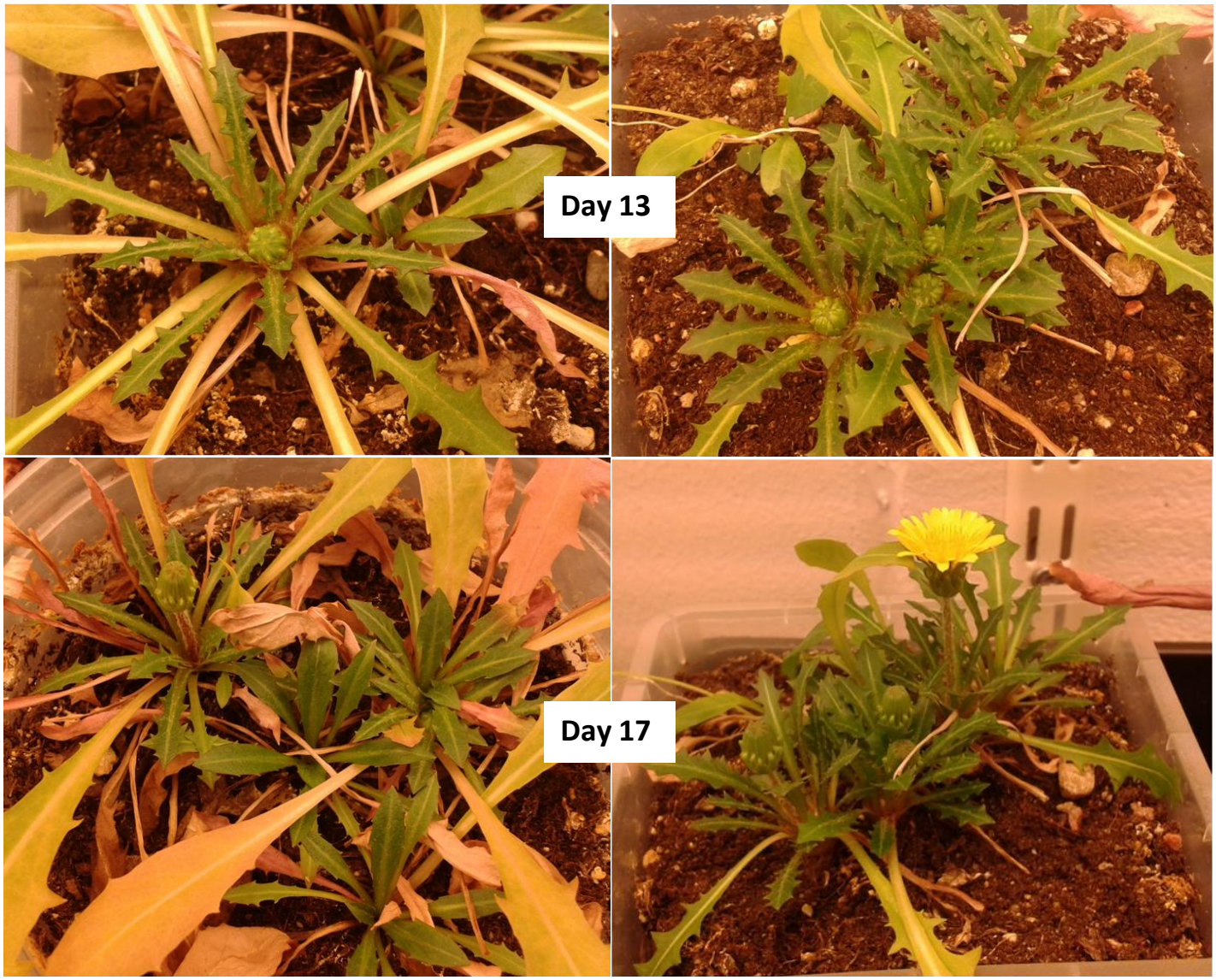

Figure A1.2. Flower development in days after termination of winter conditions.

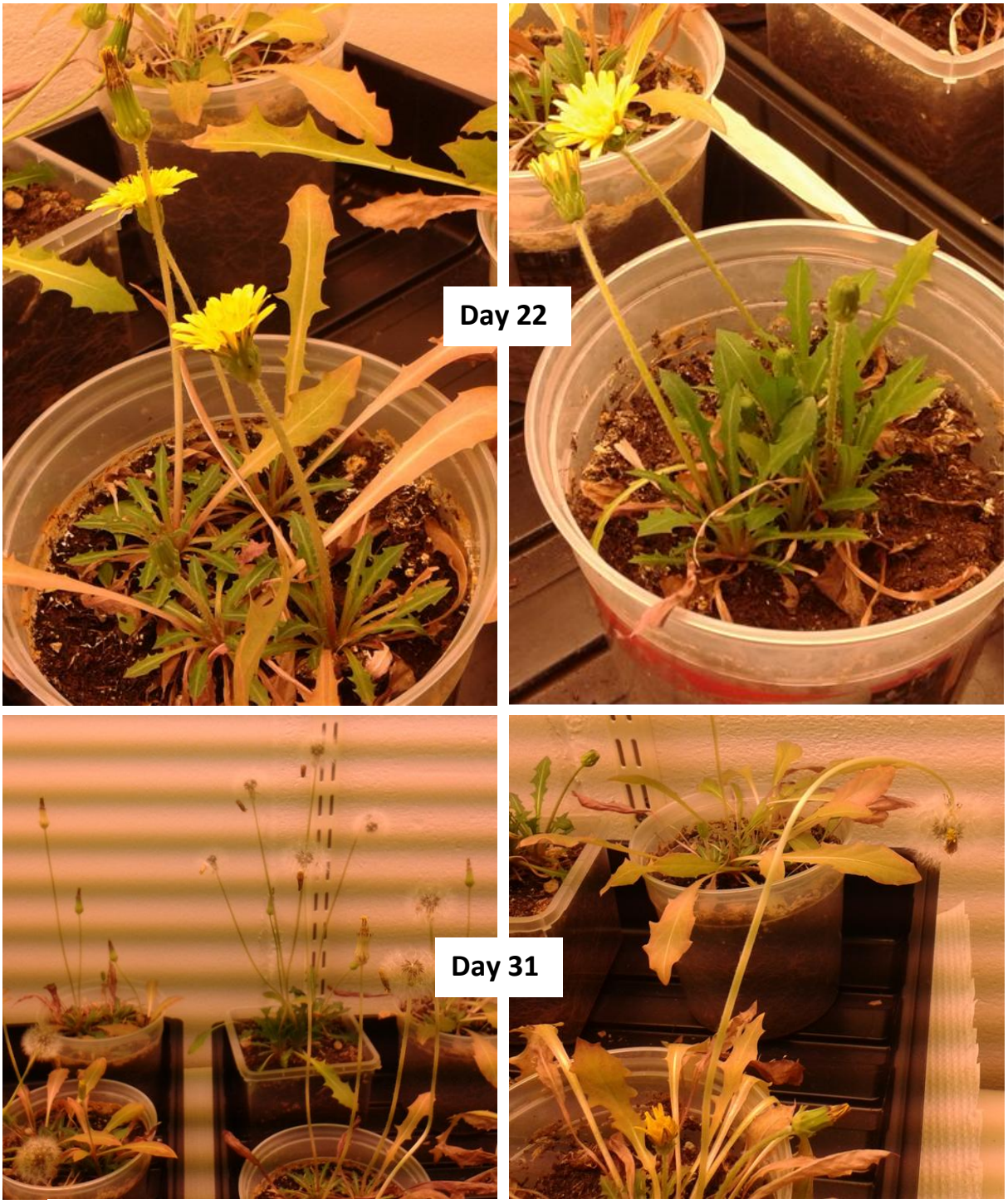

Figure A1.2 continued. Flower development in days after termination of winter conditions.

Appendix 2:
Root morphology of seedlings grown on solid medium and in bioreactor

The control bioreactor with *rolB* negative seedlings after 4 weeks of growing in the reactor can be seen in Fig. A2.1. The seedlings are healthy and have grown happily in the reactor. Roots from some of these seedlings are shown in Fig. A2.2, along with roots from a WT seedling grown on solid medium for comparison. A close up picture of one of the *rolB* negative roots can be seen in Fig. A2.3, where the taproot with branches is seen. For comparison, a picture of the *rolB* positive seedling that produced roots is included to show what would have been the expected look of those roots if the reactor had not been contaminated with *Agrobacterium*.

Figure A2.1. The control bioreactor with *rolB* negative seedlings after 4 weeks of growth in the reactor.

Figure A2.2. Roots from seedlings grown in control bioreactor and roots from a WT seedling grown on solid RIM.

Figure A2.3. Branched taproot of the *rolB* negative seedlings and hairy root of one *rolB* positive seedling.

Appendix 3:
FTIR spectrums

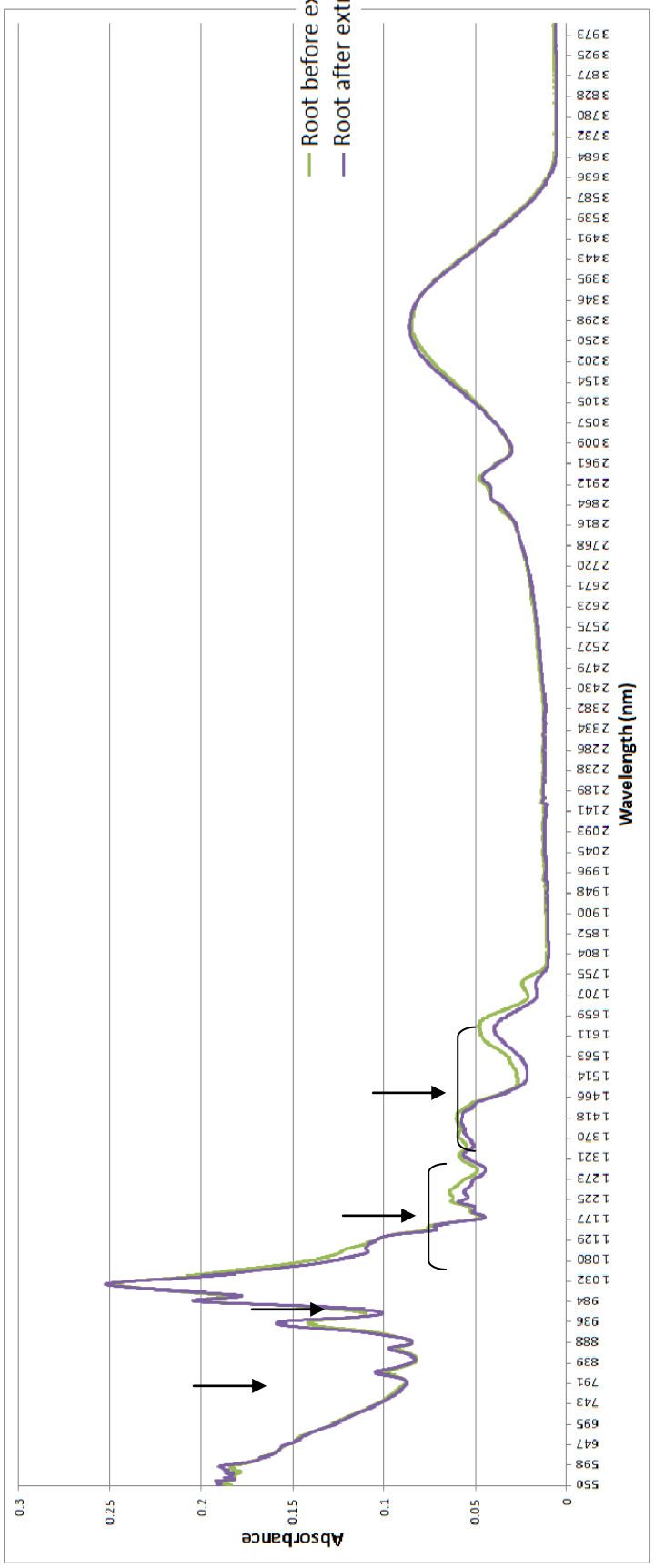

Figure A3.1. FTIR spectra for roots before and after extraction. As already stated, they only differ notably in a few places (marked in the figure with black arrows): the peak at 930 nm, the shoulder at 1100 nm, the peaks between 1220-1430 nm and the peak between 1490-1750 nm. In the last three of those places, the absorbance is lower for the root after extraction, which might imply that something has disappeared from the root during the extraction. The peak at 930nm, however, is higher for the root after the extraction than for the one before. With the same logic that implies that the concentration of a substance that absorbs light at this wavelength has increased. This might be related to the fact that the extraction buffer contained casein. If some of the casein has been absorbed by the root or stuck on the root surface, this peak might be a "protein peak" enhanced by the extra casein.

The peaks between 2980-3635 nm in those two samples are probably water since this is the wavelength where water usually absorbs light. The root pieces had been dried in an oven at 50°C for two days before the FTIR measurement, but apparently some water still remains.

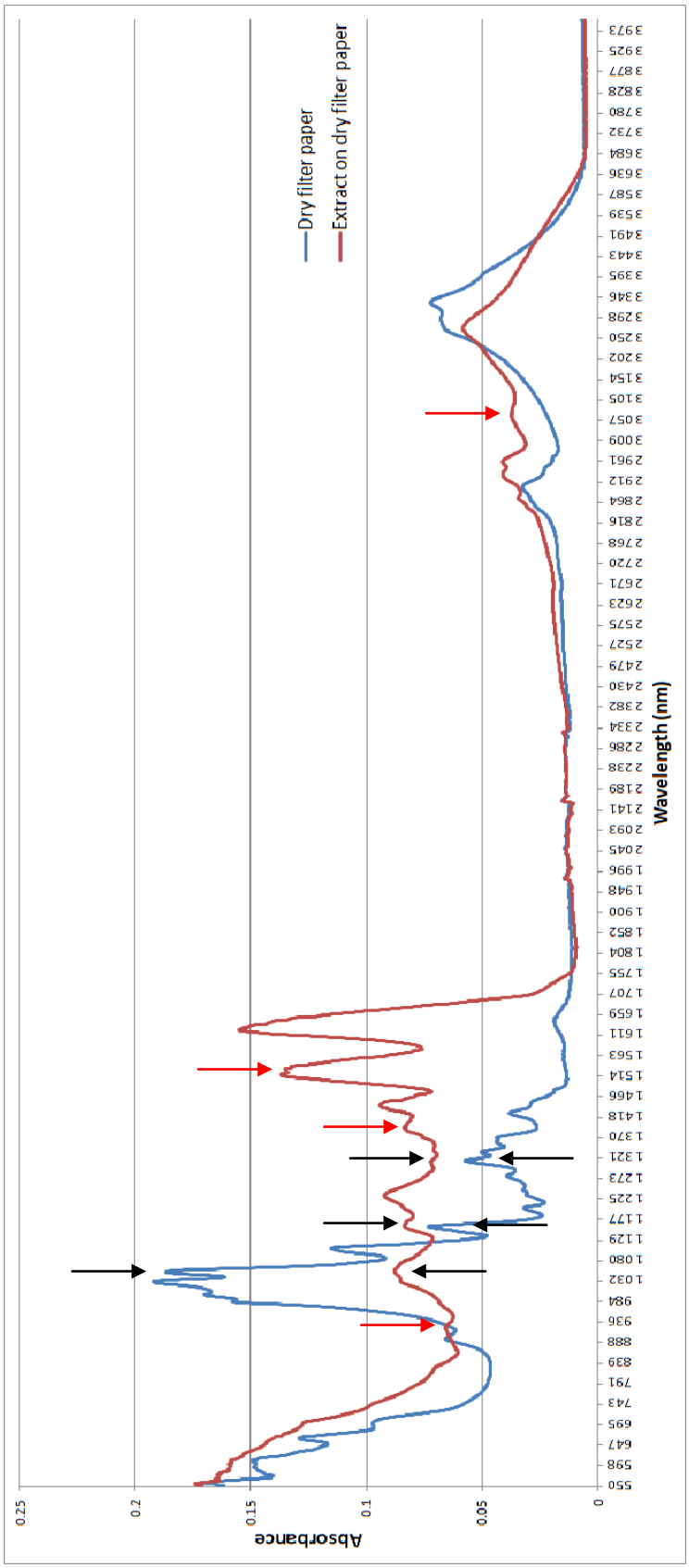

Figure A3.2. FTIR spectrums of the clean, dry filter paper, and of the filter paper with extract.

The major water peak at 3280 nm is visible in these samples just as in the root samples, but to a lesser extent. In the spectrum from the dry filter paper, an additional peak at 3330 nm can be seen which, even if it was present in the root samples, would have been completely hidden by the more prominent water peak.

Over all, the dry filter paper can be assumed to be almost exclusively cellulose, which might explain why some peaks from the paper are reoccurring in the root samples, since plant material to a high degree consists of cellulose. What is interesting, is therefore the peaks that differ between the paper and the extract. Though the absorbance measured for the two samples differ in many cases, many peaks in one sample can be found that corresponds to a peak in the other, even if the heights of the peaks are different (look for example at the double peak at 1030-1050 nm, the peaks at 1160 nm and 1310 nm marked with black arrows). Some peaks that are not at exactly the same wavelength still might have something in common. They might show up slightly different due to interference with other substances that might cause the vibrations to change slightly. What changes are caused by interference and what actually are different peaks is difficult to tell.

What is clear, however, is that some peaks exist only in the extract and not in the paper (marked in the figure with red arrows). Hopefully, these correspond to substances that have disappeared from the root during treatment.

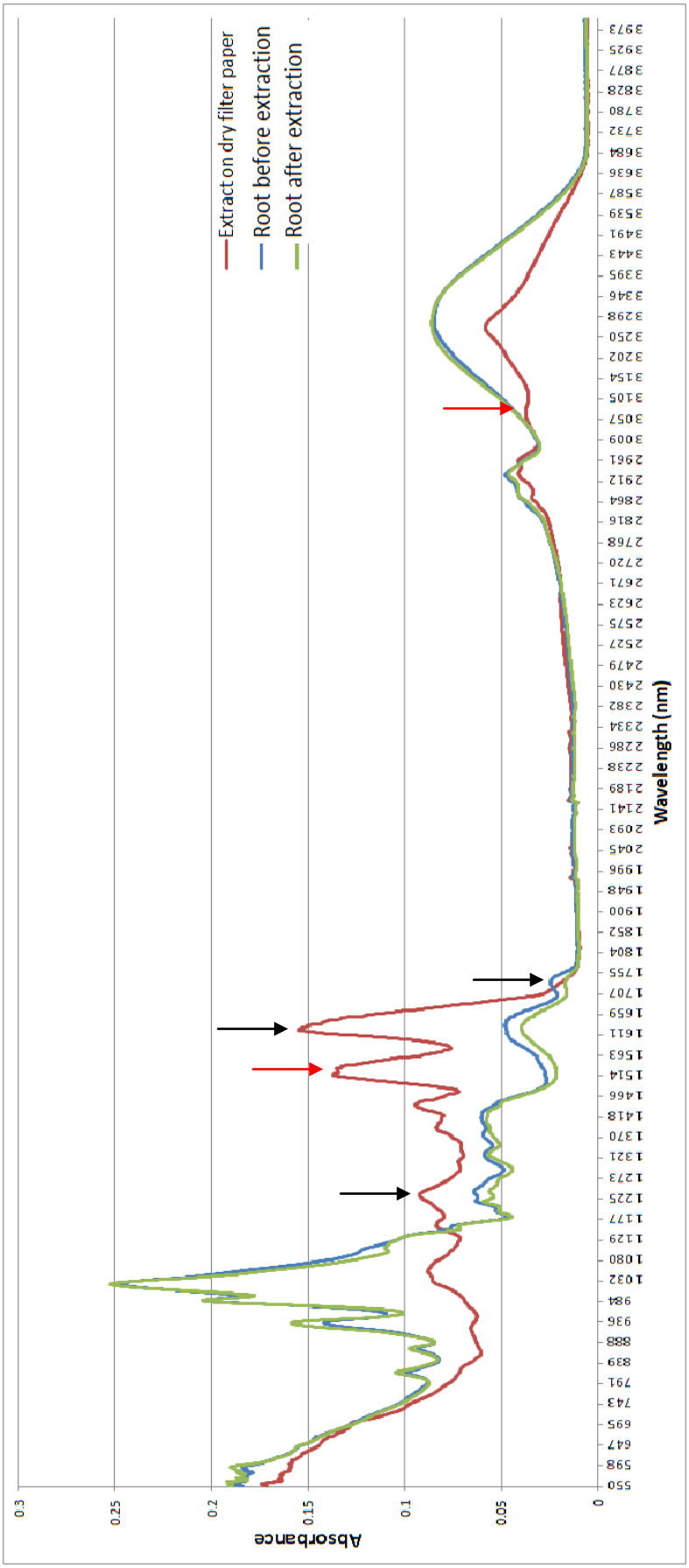

Figure A3.3. FTIR spectra of root after extraction, root before extraction and extract on filter paper.

Here, when the peak at 1020 nm in the root pieces is compared to the same wavelength for the clean filter paper, the peaks are similar, indicating that this peak might be cellulose. Since this peak is not so prominent in the extract, it might be due to that the cellulose was not dissolved by the extraction buffer, and thus the extract contains very little.

In the spectrum from the extract, the peaks at 1235 nm, 1630 nm as well as the shoulder at 1720 (marked with black arrows), are also represented in the root spectra, and corresponds to the places where the roots before and after extraction differ. This might indicate that these wavelengths correspond to a substance that has been extracted. The reason why the peaks are so much stronger in the extract spectrum could be that their relative concentration is higher when there are less other substances around to interfere with their absorbance.

Worth noticing is the small peak at 3070 nm in the extract. It is not possible to know if that has a corresponding peak in the roots, since it is completely covered by the massive water peak in those two spectra. Also, the peak at 1520 nm, that does not have a counterpart at all in the root pieces, just as it did not have a counterpart in the filter paper. Both these peaks are marked with red arrows in the figure.

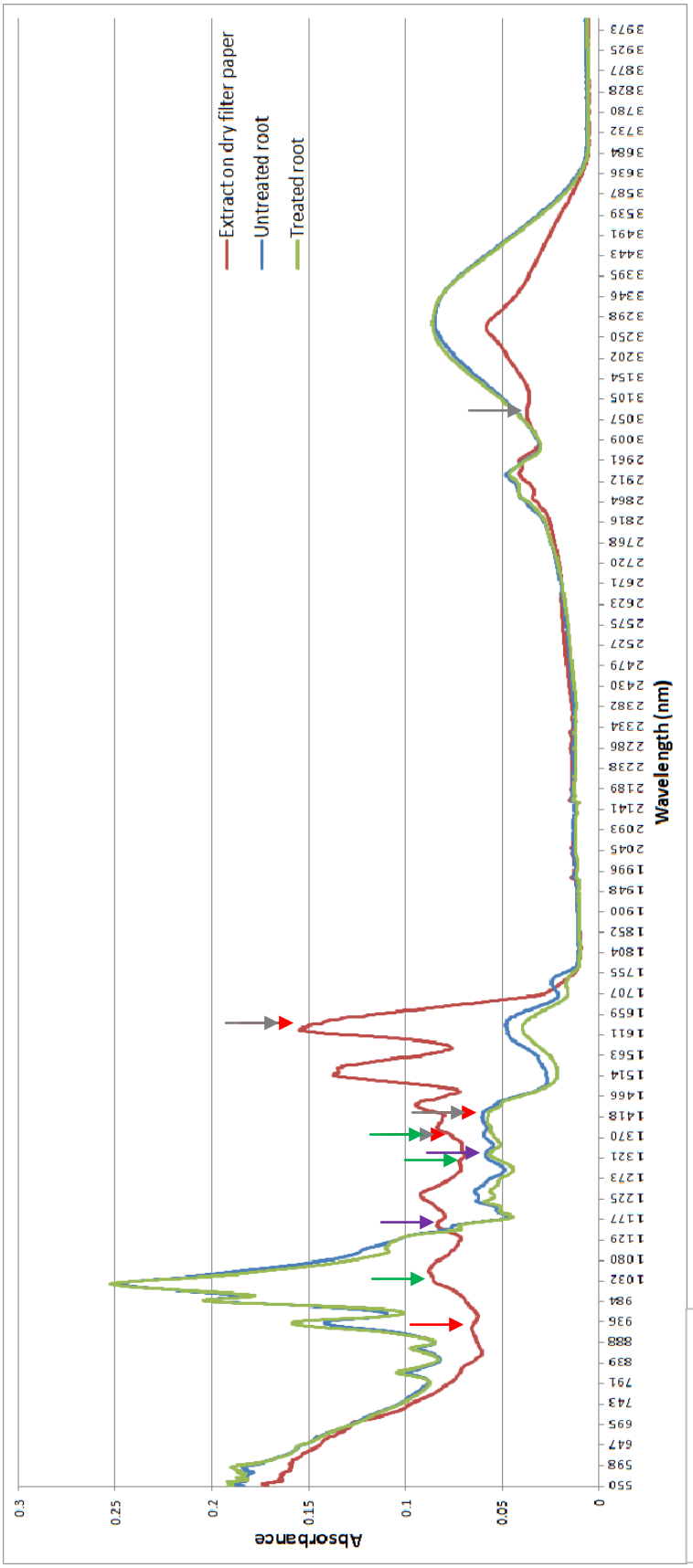

Figure A3.4. FTIR spectra of treated root, untreated root and extract with markings for which peaks correlates with peaks for different types of isoprene. The box below shows which arrows correlate to which structure of polyisoprene (Chen, et al., 2013) **and the brackets show the wavelengths of the peaks.**

Though it was expected that the samples would contain some kind of polyisoprene, probably of the configuration *cis*-1,4 (van Beilen & Poirier, 2007)**, this cannot be confirmed by the results of these spectra. It seems that there are peaks present that correspond to all four of the different structures of polyisoprene. Not all of those peaks are necessarily polyisoprene since there might be other substances with absorbance at similar wavelengths. There are also many more wavelengths where absorbance is expected for polyisoprene that are not visible here. That might be due to that they are absent, or simply the signals are too weak to be seen in this molecular context. It is possible that signals seen in only one of these spectra are present in others as well, but might be hidden inside larger peaks. It is thus not possible to say if there is, or is not, polyisoprene in this sample or which configuration it has.**

→ **1,2 polyisoprene (910, 1375, 1413, 1644 nm)**

→ **3,4 polyisoprene (1375, 1413, 1644, 3070 nm)**

→ **Cis-1,4-polyisoprene (1036, 1311, 1375 nm)**

→ **Trans-1,4-polyisoprene (1150, 1325 nm)**