Taraxacum koksaghyz Rodin as an alternative source of natural rubber and inulin; agronomic advances and genetic linkage map.

Marina Arias Royo
Leioa 2016

Director de tesis: Enrique Ritter

eman ta zabal zazu

Universidad del País Vasco
Euskal Herriko Unibertsitatea

neiker tecnalia

eman ta zabal zazu

Universidad del País Vasco | Euskal Herriko Unibertsitatea

Department of Plant Biology and Ecology
(Faculty of Science and Technology)

PhD Thesis

Taraxacum koksaghyz Rodin as an alternative source of natural rubber and inulin; agronomic advances and genetic linkage map

Marina Arias Royo

Directed by Enrique Ritter (Neiker-Tecnalia)

Leioa 2016

eman ta zabal zazu

Universidad del País Vasco
Euskal Herriko Unibertsitatea

Department of Plant Biology and Ecology
(Faculty of Science and Technology)

PhD Thesis

Taraxacum koksaghyz Rodin as an alternative source of natural rubber and inulin; agronomic advances and genetic linkage map

Marina Arias Royo

Directed by Enrique Ritter (Neiker-Tecnalia)

neiker tecnalia

Leioa 2016

A mis padres,

a Sergio, Izaro y Marco.

"THE IMPORTANT THING IS TO NEVER STOP QUESTIONING."

(ALBERT EINSTEIN)

AGRADECIMIENTOS/ AKNOWLEDGMENTS

Esta tesis, que tanto me ha costado conseguir, no sólo ha visto la luz gracias a mi trabajo, sino a la colaboración de muchas personas y al apoyo incondicional de "Mi Gente", que me ha ayudado a mantener siempre viva la confianza en mí y en mis posibilidades.

En primer lugar me gustaría agradecer, a la Fundación Candido de Iturriaga y María de Dañobeitia, la concesión de la beca que me permitió realizar los dos primeros años de trabajo, y a Neiker –Tecnalia por la contratación durante los dos últimos años, además de por poner sus instalaciones a mi disposición.

Le agradezco al Dr. Enrique Ritter su ayuda como director de tesis y por las correcciones exprés que, a veces, ha tenido que realizar. Y especialmente por su colaboración en el último tramo de esta tesis, a pesar de su sobrecarga laboral. También le agradezco su ayuda y apoyo a la Dra. Mirem Karmele Duñabeitia por haber aceptado ser mi ponente y porque, aun habiendo coincidido en pocas ocasiones, siempre me ha recibido con buenas palabras y me ha prestado su ayuda y apoyo. Quisiera agradecer también al departamento de Biología Vegetal y Ecología por aceptar que desarrollara mi trabajo doctoral en él.

Quisiera agradecer al departamento de Biotecnología de Neiker-Tecnalia su buena acogida y a todos los compañeros con los que, durante cuatro años, compartí tantos buenos momentos. En especial a los becarios, por tenderme una mano siempre que la he necesitado y amenizar las horas de trabajo.

Gracias a todos mis compañeros de bus y coche, con los que tantas y tantas charlas he compartido: Uxue, a través de cuyos ojos da gusto ver el Mundo; Alex, quien me hizo un hueco cuando me vio necesitada; Montse, ¡qué grandes charlas!; Itzi, con quien compartí (y comparto) tantas experiencias vitales; Aitor, porque hacías mucho más ligera la vuelta a casa; Javi, Vane, Olatz, ¡qué buenos ratos en el bus!, y cómo olvidarme de Itxas, con su pequeño bólido…

Por su ayuda directa en el desarrollo del trabajo, tengo que agradecer enormemente su ayuda a María y Javi, por los grandes momentos compartidos en el campo y laboratorio; a Uxue, por dejarse la espalda conmigo limpiando raíces y por introducirme de forma sencilla y amena en el mundo de la genética; a Mónica, por enseñarme la base del trabajo para el desarrollo del mapa y por su colaboración, junto a Naroa, en la obtención de marcadores moleculares; a Itzi por enseñarme a hacer mis pinitos con el cultivo *in vitro;* a Elena Fernández por cuidar de las plantitas del invernadero durante el último año; a Isi por sus enseñanzas en el laboratorio y a Emma y Javi por ayudarme en momentos de dificultad con la parte genética; a Gorka por su colaboración con la parte teórica del ensayo de riego; a Bego por resolver mis dudas de cultivo *in vitro* y por cuidar las plantitas antes de que yo apareciera en escena; a Manu y Silvana por hacerme esas correcciones exprés y finalmente a Carlos, Endika, Sonia Suárez , Natxo , Elena Bagazgoitia y Carlitos por resolver mis dudas en momentos puntuales.

No me puedo olvidar de Maite, por amenizar las mañanas con sus charlas y sin cuyo trabajo de base, el mío habría ido mucho más lento. De la misma manera agradezco al personal de mantenimiento sus numerosos inventos que hicieron posible mi trabajo. ¡Ya quisieran muchos laboratorios disponer de un personal tan eficaz!

¡Cómo olvidarme del personal de campo!, siempre presente para ayudarme con ese duro trabajo y, lo que es más importante, acompañándome con su buen humor!

Noe, ¡qué habría sido de mí sin tu apoyo moral durante todo este tiempo! Gracias por haber compartido tantas experiencias dulces y amargas.

Tengo que dedicarle un apartado especial a seis compañeros que, a lo largo de este tiempo, se han convertido en buenos amigos. Además de todo lo dicho, agradezco a Itzi su apoyo con la estadística en esos momentos en los que me encontraba tan perdida; su ayuda desinteresada, incluso fuera del horario de trabajo; su apoyo moral y tantas y tantas conversaciones compartidas; a Javi, por su gran ayuda durante todo el desarrollo del trabajo práctico y posterior escritura, por su asesoramiento en tantos momentos de duda, por compartir tantos proyectos ilusionantes conmigo y por llevar la alegría y el buen rollo allá donde va; a Vane por su tan importante apoyo moral durante estos años; a Isi por ofrecerme su amena compañía en el laboratorio y poner esa nota de humor y “glamour” en un sitio que puede ser tan frio; a Emmita, por su ayuda técnica y moral, por su bondad y por las risas compartidas. Y a Uxue por hacer que la vida sea más fácil. Pero, sobre todo, a todos ellos les agradezco enormemente el haberme brindado su amistad.

I would also like to thank all the people in the European Project EU-PEARLS, especially to the Münster University staff for their great reception in the lab. I want to particularly thank Olivier and Christian for making me feel at home. And also Peter, for his great collaboration in the genetic map development.

I want to dedicate a special paragraph to Dr. Max E. Tate for teaching me that Science can be funny and that any person could have something interesting to say. For providing me the opportunity to share his so important teachings about science and life, and for still being by my side.

Gracias a mis amigos, porque vuestro apoyo emocional ha sido y es muy importante para mí; gracias a los conquenses, los fortuneros, los gallegos, murcianos, villalbinos, alcalaínos, bilbaínos, argentinos, madrileños, alcalaínos, australianos… etc. Sois muchos y fundamentales para mí.

Pero especialmente quisiera agradecer a mi familia el haber estado ahí, siempre presentes, en este largo camino que ha sido el desarrollo de la tesis. Por su apoyo, por creer en mis capacidades, por ver más allá de la dificultad presente y por reírse conmigo en los momentos difíciles. Gracias Paco, Eva, Tamara, Alfonso y Nerea por hacer que me sienta en casa. Gracias Mamá, porque sin tu ayuda y soporte emocional, este trabajo no habría sido posible. Nunca dudaste de que pudiera conseguirlo. No hay palabras para agradecértelo. Gracias Papá, por tu cariño y por confiar en mí y no dudar de mis capacidades. Gracias Lucas, Antón, Manu, Alba y Gabi porque sois para mí un soporte vital y me habéis ido acompañando a lo largo de todo este largo camino. Gracias Marta, Vir, Silvi, Boris, Jenny y Andrea, porque ya sois fundamentales en esta familia. Gracias Leire, Hugo y Alejandra por aportar tanta alegría a nuestras vidas. Gracias Sergio por haber recorrido a mi lado este camino, a veces complicado. Por no haberme dejado caer ante la adversidad, por tu apoyo y comprensión y por facilitarme largos ratos de trabajo. Y gracias Izaro y Marco, mis niños, por vuestra paciencia y por ser mi luz y mi alegría, por darme fuerzas para sentarme a escribir cada día y por celebrar mis éxitos de la forma más bonita y sincera.

Escribiendo esta sección me he dado cuenta de lo afortunada que soy por necesitar tanto espacio para agradecimientos. De todo corazón, GRACIAS.

INDEX

INDEX

ABBREVIATIONS

AFLP: Applied Fragment Length Polymorphism

CGPDB*: Compositae* Genome Project Data Base

COS: Conserved Orthologue Set

DNA: Desoxiribonucleic acid

EM: Expectation Maximization -DW: Dry weight

EST: Expressed Sequence Tag

EU-PEARLS: European Union-based Production and Exploitation of Alternative Rubber and Latex Sources.

GO: Gene Ontology

LDW: Leaf dry weight

LfN: Leaf number

LFW: Leaf fresh weight

Lg: Linkage group

LL: Leaf length

LOD: Logarithm of the odds

N: Number

NCBI: National Center for Biotechnology Information PC: Primer combination

QTL: Quantitative Trait Loci

R_0, R_1, R_2: irrigation regimes.

RDW: Root dry weight

RF: Recombination Frequence

RFW: Root fresh weight

SqR: Square root

SSR: Simple Sequence Repeats

STS: Sequence Tagged Site

TAE: Tris-Acetate-EDTA (Ethylenediaminetetraacetic acid)

TKS: *Taraxacum koksaghyz* Rodin.

Tukey's HSD: Tukey's honestly significant difference

ABSTRACT/ RESUMEN

1 ABSTRACT

Natural rubber (NR) is one of the most important polymers, for human life, produced by plants (Puskas *et al.,* 2006). Its use is known since 1600 B.C. by the ancient Mesoamericans (Hosler *et al.*, 1999). It was crucial for the industrial revolution (Vunovic X, 2009) and nowadays it is present in more than 40,000 consumer products including more than 400 medical devices, surgical gloves, and aircraft tires (van Beilen *et al*., 2007). Even wehen 12,500 species produce latex, only 1,800 contain rubber in it and only natural rubber of *Hevea brasiliensis* (99% of the world market) and rubber of *Parthenium argentatum* (1% of the world market) are produced commercially (Tanaka *et al*., 2005). The main production of *Hevea brasiliensis* rubber is in Asiatic hands (FAOSTAT, 2015), and none of it is produced in Europe. This centralization of the production (that could mean a lack of supply if frontiers are closed to this product as it happened in the WWII), the threat of some lethal diseases that can devastate vast plantation areas (Whaley WG, 1948), the increasing NR demand, the change of cultivated areas to the more economically profitable oil palm (FAOSTAT, 2013) and the increasing incidence of allergies to *Hevea brasiliensis* latex (Sell *et al.,* 2013), have encourage several research groups Worldwide to look for alternative natural rubber sources, since it cannot be replaced by synthetic rubber.

Guayule is a very good alternative for hypoallergenic latex extraction (Venkatachalam *et al.,* 2013) and it is already being commercialized by Yulex Corporation (Yulex, 2015). Even when it has a good rubber quality, it has some undesired characteristics that make it a difficult species to work with from a breeder´s point of view. The other promising alternative for natural rubber is *Taraxacum koksaghyz* Rodin (TKS). It has a great rubber quality and was already studied in the WWII as a rubber source, but its research was abandoned after the war was finished. Its rubber is contained in laticifers but it cannot be tapped as the rubber tree; it must be either pressed or homogenized in order to extract its latex (van Beilen and Poirier, 2007a). Because of a self-incompatibility system that prevents self-pollination TKS, breeding it is quite complicated (van Beilen *et al.*, 2007a). On the other hand it can be grown by tissue culture and it is relatively easy to transform, what makes it susceptible of becoming a model rubber-producing plant (van Beilen *et al.*, 2007 b). Because of the

very good rubber quality and the also very profitable subproduct inulin, we have started a deep study of the species as a good alternative as a natural rubber (NR) source. Improvement of the species must be done in agronomic and molecular aspects, since it is a wild species.

Our work was divided into three main blocks: 1. population study, 2. water needs and 3. genetic study.

1. TKS seeds were collected in 2008 in an expedition to Kazakhstan by Kirschner in order to analyze agronomical characteristics of selected, wild TKS populations. Nine populations and two planting dates were examined by our group. Root dry weight, rubber and inulin content were measured and evaluated statistically. Large variations for all traits were observed between single plants. Despite of this, a significant population effect for rubber and inulin contents was detected. No planting date effect was observed, but a significant interaction effect of population by planting date for inulin contents and root dry weight. Although yields are relatively low, three populations were found to be promising candidates for increased rubber and/or inulin production. In any case additional agronomic studies are required and particularly intensive breeding using mass selection and crossings, for converting TKS into a useful crop for rubber and inulin production.

2. The water needs of the species were studied with the aim of finding the best conditions for optimum rubber, inulin and biomass production. Three irrigation regimes, including two different dosages and a non irrigated control, were evaluated. Only a significant influence of the irrigation regime on percentage of TKS rubber contents was observed, while no significant effects on inulin contents, biomass production and other morphological traits were detected. Additional water supply for optimal growth seems not to be necessary for TKS optimum development under local weather conditions (Northern Spain fields), but further studies at dryer locations should be performed. A strong positive correlation was observed between root and leaf dry and fresh weights and a weakness to moderate negative correlation between percentages of rubber and inulin contents.

3. A limited amount of genomic resources exist for TKS and particularly no genetic linkage map is available in this species what is a essential tool for marker assisted breeding (MAS). We have constructed the first TKS genetic linkage map based

on AFLP, COS, SSR and EST-SSR markers. The integrated linkage map with eight linkage groups (LG), representing the eight chromosomes of Russian dandelion, has 185 individual AFLP markers from parent 1, 188 individual AFLP markers from parent 2, 75 common AFLP markers and 6 COS, 1 SSR and 63 EST-SSR loci. Blasting the EST-SSR sequences against known sequences from lettuce allowed a partial alignment of our TKS map with a lettuce map. Blast searches against plant gene databases revealed some homologies with useful genes for downstream applications in the future.

Our studies together with other carried out by our European project partners (EU-PEARLS, 2015) have settled a strong base for improvement of TKS production from an economical point of view. It has been now taken over by the European project DRIVE4EU (Dandelion Rubber and Inulin Valorization and Exploitation for Europe) which objective is to set up a new European chain for the production and processing of natural rubber that will enable the EU to become less dependent on the importation of natural rubber and at the same time to respond to the threat of a global rubber shortage.

1 RESUMEN

El caucho natural es uno de los polímeros, producidos por plantas, más importantes para la vida humana (Puskas *et al.* 2006). Se sabe de su uso por los antiguos mesoamericanos desde antes del 1600 a.C (Hosler *et al.*, 1999). Fue crucial para la revolución industrial (Vunovic X, 2009) y hoy en día está presente en más de 40.000 productos de consumo, incluyendo más de 400 artículos médicos, guantes de laboratorio y ruedas de avión (van Beilen *et al.*, 2007). Aunque unas 12.5000 especies producen latex, sólo 1.800 de éstas contienen caucho en su interior y sólo el caucho natural de *Hevea brasiliensis* (99% del total del caucho del mercado) y el caucho de *Parthenium argentatum* (1% del caucho del mercado) se produce comercialmente (Tanaka *et al.*, 2005). La mayor parte de la producción de *H. brasiliensis* está en manos asiáticas (FAOSTAT, 2015), y en Europa no se produce nada. Esta centralización de la producción (que podría suponer una falta de suministro si se cerraran las fronteras a la entrada de este producto, como sucedió en la II Guerra Mundial), las amenazas de algunas enfermedades letales para el cultivo, que pueden acabar con grandes áreas cultivadas en cortísimos períodos de tiempo (Whaley WG, 1948), el aumento de la demanda de caucho natural (FAOSTAT, 2015), el paulatino cambio de cultivo de *H. brasiliensis* a palmera de aceite (que resulta más rentable económicamente) (FAOSTAT, 2013) y la creciente incidencia de alergias al latex proveniente de *H. brasiliensis* (Sell *et al.,* 2013), han animado a varios grupos de investigación alrededor del Mundo a buscar fuentes alternativas de caucho natural, ya que éste no puede ser sustituído por caucho sintético.

Guayule es una muy buena alternativa como fuente de latex hipoalergénico (Venkatachalam *et al.,* 2013) que ya está siendo comercializado por Yulex Corporation (Yulex, 2015). Aún cuando tiene un caucho de buena calidad, tiene algunas características indeseables que le convierten en una especie con la que es difícil trabajar, desde el punto de vista de un mejorador. La otra gran promesa como fuente alternativa de caucho es *Taraxacum koksaghyz* Rodin (TKS). Contiene caucho de gran calidad y ya fue objeto de estudio durante la II Guerra Mundial como fuente alternativa, pero su estudio fue abandonado una vez que se reabrieron las fronteras al caucho. Su caucho está contenido en células laticíferas pero no puede ser sangrado como el árbol del

caucho, sino que debe ser aplastado u homogeneizado para su extracción (van Beilen and Poirier, 2007a). Debido a un sistema de auto incompatibilidad que previene su auto polinización, la mejora genética de TKS es bastante complicada (van Beilen *et al.*, 2007a) aunque, por otro lado, puede ser reproducido *in vitro* y es bastante fácil de transformar, lo que le hace susceptible de convertirse en una planta modelo de producción de caucho (van Beilen *et al.*, 2007 b). Por esta razón, por la gran calidad de su caucho y por su muy aprovechable subproducto, inulina, hemos comenzado un profundo estudio de la especie como buena alternativa para la obtención de caucho natural. Es necesario realizar mejora a nivel agronómico y molecular ya que aún es una especie silvestre.

Nuestro trabajo se dividió en tres bloques principales: 1. estudio poblacional, 2. evaluación de necesidades hídricas y 3. estudio genético.

1. En 2008 salió una expedición hacia Kazajastán encabezada por Kirschner, con el fin de conseguir semillas de TKS. Se pretendía analizar las características agronómicas de una selección de poblaciones de TKS. Nueve de estas poblaciones y dos fechas de siembra fueron analizadas por nuestro grupo. El peso seco de la raíz y contenido en caucho e inulina fueron medidos y evaluados estadísticamente. Se encontraron grandes variaciones para todas las variables, en plantas individuales. A parte de esto, se detectó un efecto significativo de población para caucho e inulina. No se encontró un efecto significativo para la fecha de siembra, pero sí para la interacción entre población y fecha de siembra, para contenidos de inulina y peso seco de la raíz. Aunque las cosechas son relativamente poco cuantiosas, tres poblaciones fueron candidatas prometedoras para el aumento de producción de inulina y/o caucho. En cualquier caso se requieren más estudios agronómicos y particularmente de cultivo intensivo usando selección masal y cruces, para convertir a TKS en un cultivo útil para la producción de caucho e inulina.

2. Las necesidades hídricas de la especie fueron estudiadas con el fin de encontrar las mejores condiciones para la obtención de la producción óptima de caucho, inulina y biomasa. Se estudiaron tres regímenes de riego, incluyendo dos dosis diferentes y un control sin riego. Sólo se apreció una influencia significativa del riego en los porcentajes de contenido en caucho, mientras que no se observaron efectos significativos sobre el contenido de inulina, producción de biomasa y otras variables

morfológicas. No parece necesario el suministro de agua para obtener un crecimiento óptimo de TKS bajo las condiciones climáticas locales (campos de cultivo en el norte de España), pero se deberían realizar estudios en campos de cultivo más secos.

Se observó una correlación positiva entre peso fresco y seco de raíz y hojas y una correlación negativa de ligera a moderada entre porcentajes del contenido de caucho e inulina.

3. Existe una cantidad limitada de recursos genómicos para TKS y, particularmente, no se dispone de un mapa genético de ligamiento de la especie, que es una herramienta fundamental para la mejora asistida por marcadores (MAS). En este trabajo hemos contruído un mapa genético de ligamiento basado en marcadores AFLP, COS, SSR y EST-SSR. El mapa integrado de ligamiento, con ocho grupos de ligamiento (LG), representa a los ocho cromosomas del diente de león ruso, tiene 185 marcadores AFLP individuales del parental 1, 188 marcadores AFLP del parental 2, 75 marcadores AFLP comunes y 6 locus COS, 1 SSR y 63 EST-SSR. Enfrentar las secuencias EST-SSR contra secuencias conocidas de lechuga permitió el alineamiento parcial de nuestro mapa de TKS con el de lechuga. La búsqueda de alineamiento con secuencias de bases de datos de genes de plantas reveló algunas homologías con genes útiles para futuras aplicaciones.

Nuestros estudios, junto con los llevados a cabo por nuestros compañeros de proyecto (EU-PEARLS, 2015), han establecido unas importantes bases para la mejora de la producción de TKS desde un punto de vista económico. El relevo ha sido cogido por el proyecto europeo DRIVE4EU (Valorización y Explotación de Cuacho e Inulina de Diente de León Ruso para Europa), cuyo objetivo es establecer una nueva cadena de producción y procesamiento de caucho natural que pueda dotar a la Unión Europea de mayor independencia de las importaciones de caucho natural y al mismo tiempo, poder responder a la amenaza de reducción de caucho a nivel global.

GENERAL INTRODUCTION

2. INTRODUCTION:

Polymers are natural or synthetic substances composed of very large molecules, called macromolecules that are multiples of simpler chemical units called monomers. Biopolymers are those produced by living organisms. Many materials in living organisms including proteins, polysaccharides, and nucleic acids, are polymers (Britannica online encyclopedia, 2015). Two of the most important biopolymers are rubber (a polymer of the hydrocarbon isoprene) and inulin (a mixture of oligo- and polysaccharides).

2.1 Rubber:

Natural rubber is one of the most important polymers, for human life, produce by plants. Its usage in thousand of products and hundreds of medical devices converts it in an essential raw material. It is obtained from latex, an aqueous emulsion present in the laticiferous vessels (ducts) or parenchymal (single) cells of rubber producing plants. (Puskas *et al.* 2006).

2.1.1 History:

Hosler *et al.* (1999) mentioned in their paper "Prehistoric Polymers: Rubber Processing in Ancient Mesoamerica" how ancient Mesoamerican peoples were already processing rubber by 1,600 B.C., predating development of the vulcanization process by 3,500 years. They wrote about several items made of rubber and explained how Mesoamericans used liquid rubber for medicines, painted with it, and used it in rituals. Some of the uses are captured in several old paintings (Figure 1 and Figure 2).

Figure 1: Rubber ball game players represented in the murals of Tepantitlaen Teotihuacan. Source: Hosler *et al.* (1999).

Figure 2: Aztec God Xiuhtecuhtli in the Borgia Codex offering up rubber balls (dates from XV century)

Source: Hosler *et al.* (1999).

Christopher Columbus was recognized by Maurice Morton in his book "Rubber Technology", as the first European to discover natural rubber, in the early 1490s, when he found natives in Haiti playing ball with an extract from a tree (Ciesielski A, 2000). Little by little the use of latex was expanded by white conquerors. F. Juan de Torquemada wrote in 1615 in his "De la Monarquía Indiana" about Indians and Spanish settlers wearing, shoes, clothing and hats made by dipping cloth into latex. But it still had some limitations: it became sticky in response to warm weather and it hardened and cracked with cold weather (Whaley WG, 1948). One century later, in 1734, Charles Marie de la Condamine went to South America in order to solve whether the Earth was an elongated or a flattened sphere. He landed just north of Guayaquil, in the West coast and went over the Andes by way of Esmeraldas. He could appreciate different uses of rubber utilization along his trip. For example in torches and candles. Charles Marie de la Condamine wasn´t a botanist and he didn´t realize the difference between the trees bearing rubber that he found in his trip (*Hevea or Siphonia* and *Castilla* trees) (Whaley WG, 1948). In the eighteenth century rubber reached Europe. Para (Belem) was the first Portuguese settlement in the far North of Brasil. Several pairs of royal boots were sent there in order to be waterfroof. In 1759 the government of Para presented a coat made of fabric covered with the new substance (Wolf *et al*., 1936).

The reason why *Hevea* succeeded in respect to *Castilla* rubber tree was the way their latex is transported along the tree and Ridley´s discovery. In *Hevea* tree there are connected latex tubes forming a network while they don´t form a connected system in

Castilla trees. This makes a big difference in the way the latex can be extracted. In *Hevea* trees latex flows continuously and it does not in *Castilla* trees (they can die by severe tapping). In the other hand Ridley discovered a method for harvesting *Hevea* plantations by continuous tapping. This discovery rapidly changed civilizations and social relations. Rubber became an essential material in the development of countries (Cook OF, 1941).

In 1839 Charles Goodyear invented the vulcanization process. Combining sulfur and heat he could harden rubber and keep its elasticity. The problem of having rubber changes when it was exposed to variation of temperature was solved. It didn´t crack in the winter and melt in the summer (Vunovic X, 2009). Another important push for the international industry of rubber was made by Dunlop in 1888, when he invented the pneumatic tire. Dunlop´s wheel was firstly used for bicycles and from 1890´s in the emerging car industry. Dunlop´s invention boosted rubber as a raw material of worldwide strategic importance (Ullán de la Rosa FJ, 2004).

The industrial revolution couldn´t be the same without rubber. From 1850 through 1912, the demand for rubber increased constantly by industrial Europe and the United States. Businessmen pushed entrepreneurs and traders to penetrate Amazonian rainforests searching for high-quality rubber trees to exploit (Vunovic X, 2009). The period between 1880 and 1920 was marked by the exploitation of rubber extraction in the Amazonia. During this period the Amazon had the monopoly of rubber (Ullán de la Rosa FJ, 2004). Along these years the price and volume of rubber was continuously increasing in Worlds market in a geometrical progression. It was due to the developing industry that was growing in Europe and EEUU and to the non stopping discovery of new applications of rubber. (Ullán de la Rosa FJ, 2004). In this atmosphere Brazil prohibited the export of rubber seeds or seedlings. But it was a very tantalizing treasure and H. A. Wickham smuggled 70.000 rubber seeds hidden in banana leaves and brought them to England in 1876. Out of the stolen seeds, 1900 seedlings germinated and survived and they were used to start the rubber plantations in Malaya late in the nineteenth century (Carraher ChE, 2003). It was the beginning of the end of Brazil as main rubber producer. In 12 years the Eastern plantations reached the peak of production in the Amazon and became the main World´s natural rubber supplier. It was more competitive in price. From these days until the First World War, the rubber collection from wild sources of tropical America declined tremendously (Whaley WG, 1948). During the war the supply of rubber was cut off. EEUU recognized, early in 1942, that the stock pile of natural rubber would be insufficient to meet the demands, encouraging the search for alternative rubber sources either natural or synthetic (Whaley WG, 1948). After the War the supplies of rubber by the Eastern plantations started again and the effort to look for new rubber sources decreased tremendously. The price of Eastern rubber was much more competitive than the one obtained from new rubber sources.

2.1.2 Natural Rubber (NR) and Synthetic Rubber (SR)

2.1.2.1 Natural Rubber

NR is considered to be one of the most versatile agricultural products. It is a strategic material, being utilized in the manufacturing of more than 40,000 consumer products including more than 400 medical devices, surgical gloves, and aircraft tires (van Beilen *et al*., 2007). It is a biopolymer consisting of isoprene units $(C_5H_8)_n$ linked together in a 1,4 *cis*-configuration (Figure 3).

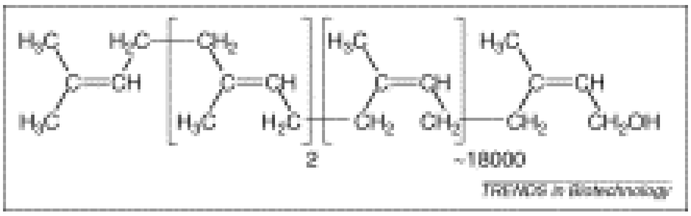

Figure 3: Chemical structure of natural rubber.
Source: van Beilen and Poirier (2007a).

The molecular weight of an isoprene monomer in natural rubber (C_5H_8) is 68 Da. At an average molecular weight of 1,200 kDa (*H. brasiliensis)*, n is approximately 18,000 (van Beilen and Poirier*,* 2007a). NR combines high strength with incredible resistance to fatigue. It has moderate resistance to environmental damage by heat, light and ozone which are one of its drawbacks. Natural rubber compounds are exceptional for their flexibility, good electrical insulation, low internal friction, and resistance to most inorganic acids, salts and alkali, even though it has poor resistance to petroleum products such as oil, gasoline and Naphtha (Vijayaram TR, 2009) (Table 1).

After six decades of industrial research, the produced synthetic rubber doesn´t meet the price-performance ratios that match those of natural rubber. Natural rubber still has a strategic importance. It cannot be replaced by synthetic alternatives in most of its applications (van Beilen J.B. *et al*., 2007).

NR PROPERTIES		
Molecular behaviour	Glass transition temperature (° C)	-70
	Melting temperature (° C)	25
	Hardness range (Shore A)	30-100
	Maximum tensile strength (at 70 F, psi)	4000
	Maximum elongation (at 70 F, %)	750
Advantages	Physical resistance	Excellent resilience
		Exc. tears trength
		Exc. abrasión resistance
		Exc. impact strength
		Exc. cut growth resistance
		Good compression set
	Environmental resistance	Exc. wáter resistance
		Good-exc. low temperature flexibility
		Fair-good oxidation resistance
	Chemical resistance	Good resistance to alcohols and oxygenated solvents
		Fair-good resistance to acids
Limits	Environmental resistance	Poor ozone resistance
		Poor sun light resistance
		Very little flame retardance
	Chemical resistance	Poor oil and gasoline resistance
		Poor resistance to (aliphatic and aromatic) hydrocarbon solvents

Table 1: NR properties
Source: (UNCTAD, 2015a).

As it was writen in 2006 by Bradley S. Bushman *et al.*, twenty botanical families, 900 genera, and 12,500 species produce latex, of which eight families, 300 genera, or 1800 species, produce rubber in their latex. Although approximately 2,000 plants synthesize poly (cis-1,4-isoprene), only natural rubber of *H. brasiliensis* (99% of the world market) and rubber of *Parthenium argentatum* (1% of the world market) are produced commercially (Tanaka *et al.*, 2005). Rubber is compartmentalized within sub cellular rubber particles, located in the cytosol of cells, whether these be specialized laticifers (pipe-like anastomized cells systems which produce latex), as in *H. brasiliensis* (D´Auzac J *et al.*, 1989, in Mooibroek *et al.*, 2000), Taraxacum koksaghyz R. (van Beilen and Poirier, 2007a) and *F. elastic* (Heinrich G, 1970, in. Mooibroek *et al.,* 2000), or generalized cells, such as the bark parenchyma of *P. argentatum* (Backhaus RA, 1986, in Mooibroek *et al.*, 2000).

Hevea latex is tapped by incision into the bark (Verheye W, 2010), Russian dandelion must be either pressed or homogenized in order to extract its latex (van Beilen and Poirier, 2007a) and guayule´s tissue must be throughly macerated to free the rubber particles (Campos-Lopez *et al.*, 1978 in Backhaus *et al.*,1983). It is one of the few rubber-containing plants that do not contain laticifers (Backhaus *et al.,* 1983).

The content of poly (cis-1,4-isoprene) in *Hevea* latex is approximately 30% and is harvested by a "tapping" method after the bark of the plants is cut diagonally. One tree yields 100 to 200 ml per 3 hours. The tapping can be carried out every 2-3 days and this results yielding up to 2,500 kg of natural rubber per year per ha. (Rose *et al.,* 2005). Trees are tapped for 25 –30 years, starting when the young tree is 5–7 years of age.

Latex can be coagulated with formic or acetic acid in order to obtain crude rubber or it can be concentrated in separators for latex products like surgeon's gloves, condoms, tubing, elastic thread, and foam-backed (Carraher Ch E, 2003). A 10% of the latex harvested from *Hevea* trees is manufactured into latex products (van Beilen *et al.,* 2007).

Microbial degradation of NR has been investigated for several years. There are numerous research articles describing how bacteria, as well as fungi, are capable of degrading rubber, being the mentioned biodegradation a slow process (Rose *et al.,* 2005).

2.1.2.2 Synthetic Rubber

Some years after the first pneumatic tire saw the light, around 1910, the motor car truly arrived and both, the use and price of natural rubber exploded (Vijayaram TR, 2009).

In 1826 several scientist tried to synthesize a SR, after After Michael Faraday´s definition of the molecular structure of NR, but the goal wasn´t achieved until the demand of rubber exceeded the production (beginning of 20th century). Fritz Hofmann was the first scientist in obtaining a polymer of SR, in 1909. One year later two scientists (Mathews and Strange in England and Harries in Germany) discovered independently the use of sodium for catalyzing the reaction to speed up polymerization. This discovery made possible the commercial production of SR. During World War I methyl rubber was made in Germany on a commercial scale (International rubber study group, 2015). Later in the 30´ SR was developed because of the uncertainty of the supply of natural rubber due to the shortages derived of the World War II (Vijayaram TR, 2009). In 1940´SR production was emphasized with the aim to complement NR to meet the World´s demand. By 1956 SR was covering 40% of total rubber consumption and by 1979, it was 70%. NR producers thought that NR was going to be totally replaced by SR. The United States of America cautioned producing countries against the expansion of NR production capacity. After this warning NR producing countries look for methods to improve the quality of their NR in order to compete with SR. It resulted in building better rubber processing factories. NR was finally not replaced by SR and nowadays is accepted that they´re both needed and that they can only complement each other. At present NR consumption supposes a 43.5 % of total World´s consumption (Umar *et al.*, 2011) (Figure 4).

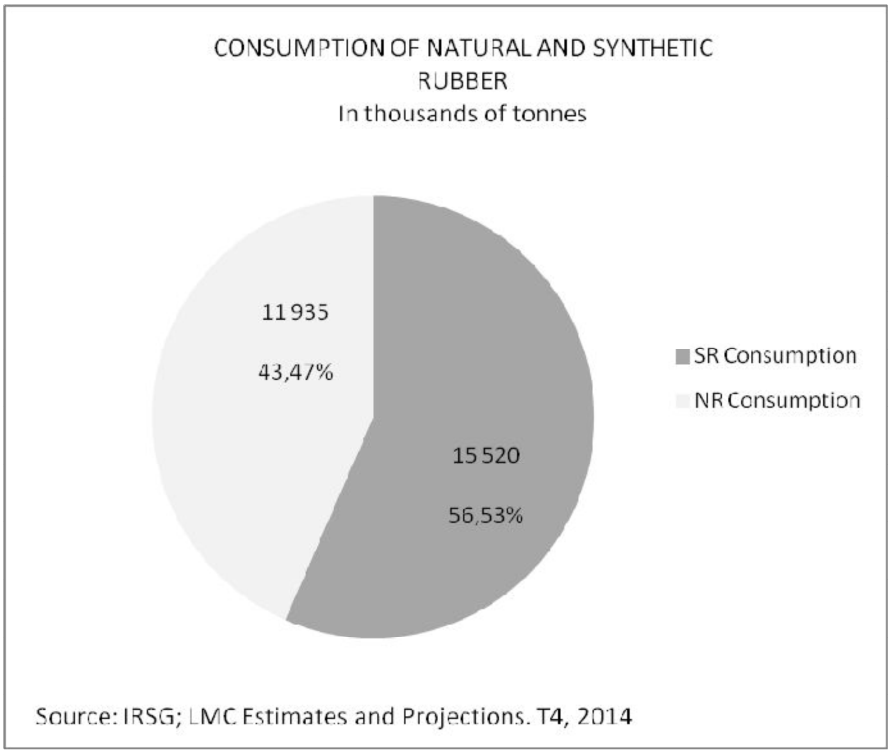

Figure 4: Global distribution of rubber consumption.
Legend: SR: Synthetic rubber; NR: Natural rubber.

SR is a white, crumbly plastic mass which is processed and vulcanized in the same manner as natural rubber (Vijayaram TR, 2009). There are around 200 types of SR. They are different in constituents and qualities, but they all involve a polymerization process (IRSG, 2015) or a polycondensation of unsaturated monomers. Some of the common properties of synthetic rubbers are better abrasion resistance, better heat and aging resistance, good insulation, flexibility at low temperatures, flame retardant and resistance to grease and oil (Vijayaram TR, 2009). The group of countries belonging to Asia and Oceania are the bigger producers and consumers of SR in the World, followed by the European Union and North America.

Biodegradation of vulcanized rubber material is also possible although it is even more difficult than the degradation of natural rubber due to the inter linkages of the poly (*cis*-1,4-isoprene) chains (Rose *et al*., 2005).

2.1.3 Economy

2.1.3.1 NR Production

Since 1914, more or less, the global rubber market is in Asiatic hands. Up to 92% is produced in Asia. Thailand is the largest producer at a World level (Figure 5). It occupies the second place in rubber plantation extension. The difference between plantation extension and yield is due to the higher production per plant compared to other countries. A higher productivity was raised in the Asiatic countries due to the improvement of the production techniques during more than 80 years, the usage of high production clone material and plant breeding programs (Pulido-Sierra *et al.*, 2012). Improvements of botanists through grafting and breeding have accomplished 1000 % more productive trees than the wild *Heveas* (Carraher ChE, 2003).

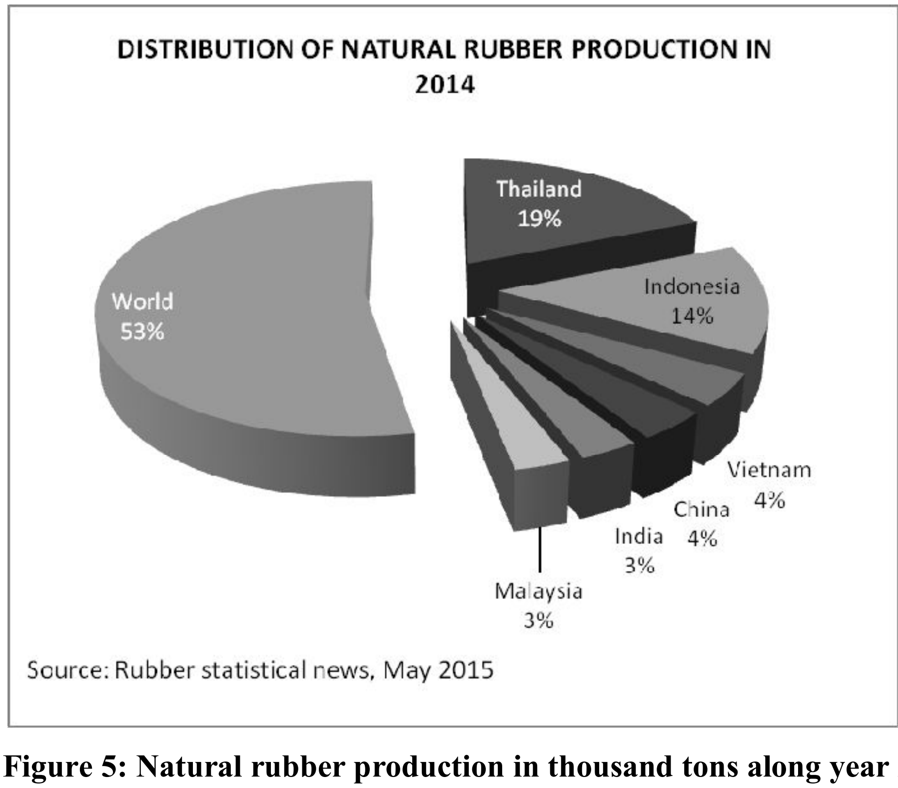

Figure 5: Natural rubber production in thousand tons along year 2014.

*The section "World" includes other countries that are not listed above.

The biggest harvested area of *Hevea brasiliensis* in the World is the Asiatic continent, followed from a distance by Africa and America (Figure 6).

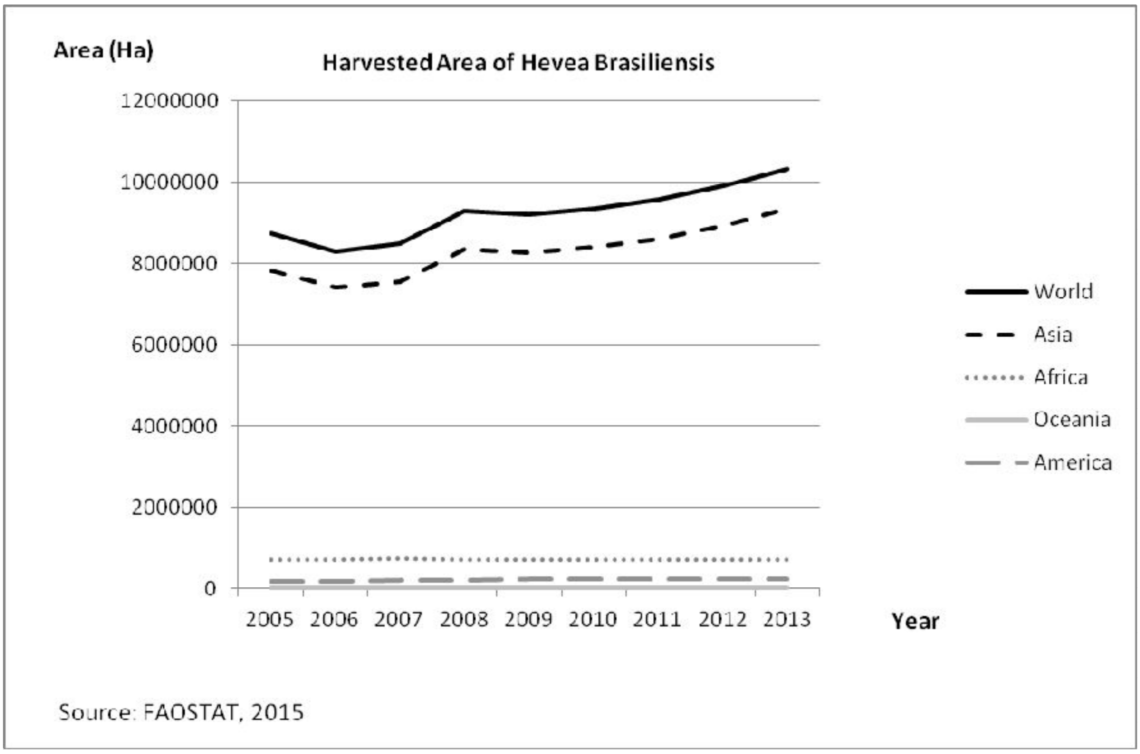

Figure 6: Evolution of *Hevea brasiliensis* harvested area per continent (tones) along the period going from 2005 to 2013.

*Europe hasn´t got any *H. brasiliensis* plantation.

India, with its more or less 687 thousand hectares under rubber plantation (which means a 7% of total rubber area plantations) occupies the first rank in terms of productivity (1,819 kg/Ha) for the Rubber Board, even though America is the first in a ranking per continents (Figure 7). Another emerging area, with less than 5% of the world's production is West Africa that offers an ideal development potential for the production of rubber with its favorable climatic conditions, large areas of available land and farm workers. African rubber, which stands at 0.4 million tones, is finding a natural outlet in the European market (Société Internationale de Plantations d´Hévéas, 2015).

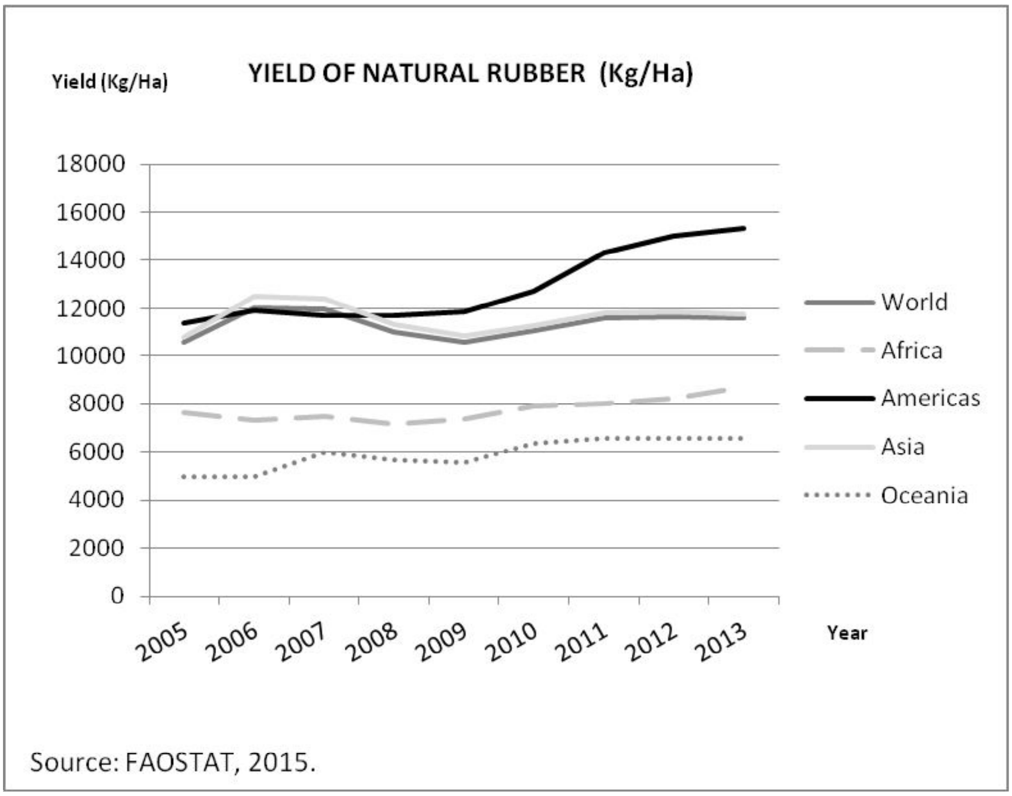

Figure 7: Natural rubber yielding per continent (in Kg/Ha) in the time period 2005-2013.

*Europe is not a producer.

Europe is not a producer because it doesn´t meet the needs for *Hevea brasiliensis* cropping. The ideal growing conditions are high temperature, altitude no more than 400 m and high humidity (NMCE, 2013).

2.1.3.2 NR consumption

NR consumption cannot be replaced by SR. It´s thoroughly used in around 50,000 products over the World (Prabhakaran KP, 2010). Qualities like low heating and ability to regain its original shape make it essential for its usage with heavy goods vehicles, agricultural vehicles and aircrafts and in civil engineering.

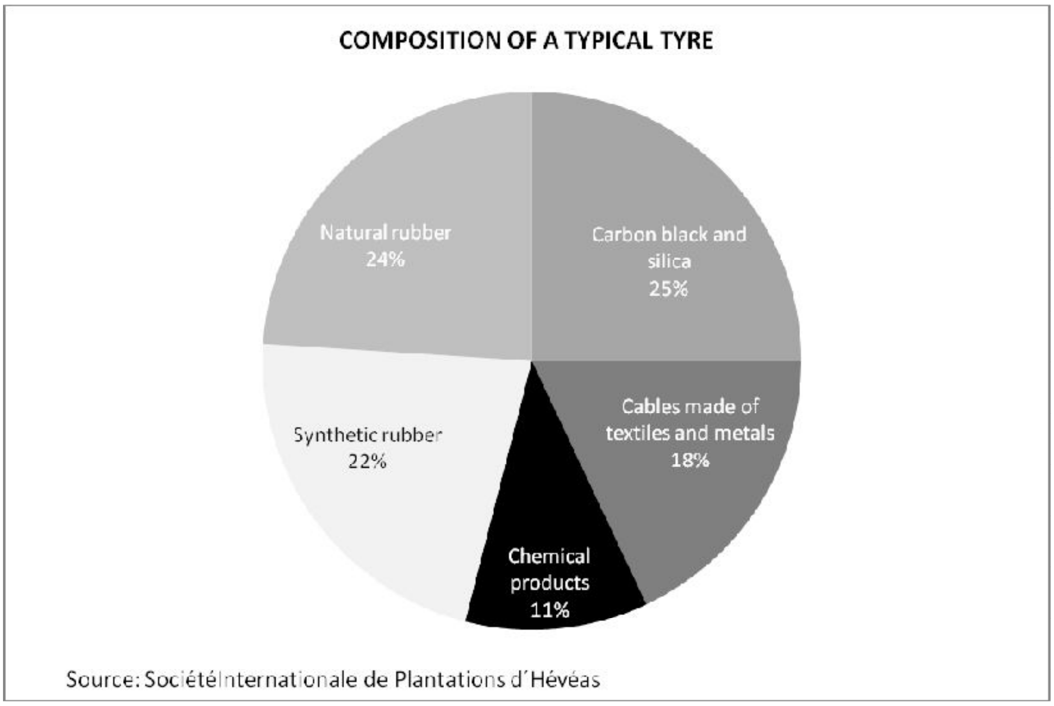

Figure 8: Composition of a typical tire.

65-70% of the global consumption of NR is done by the World´s tyre manufacturing industry (Bruins J, 2003) and 24% of a typical tyre composition is NR (Figure 8) so it has a privileged place in the World´s economy.

The economy is changing very fast in a global sense and emerging countries are occupying the first positions concerning NR consumption. 22% occurs in China, followed by Europe, India with 5% of total NR consumption (Figure 9). China has raised its consumption in 82% in the last nine years and continues increasing (Figure 10).

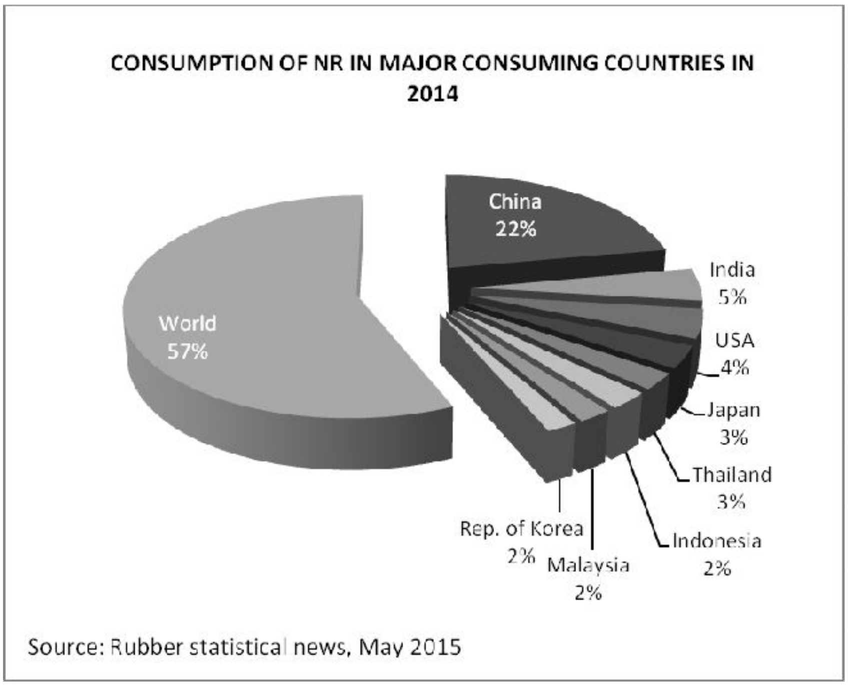

Figure 9: Natural rubber consumption (´000 tons) along year 2014.

*The section "World" includes other countries that are not listed above.

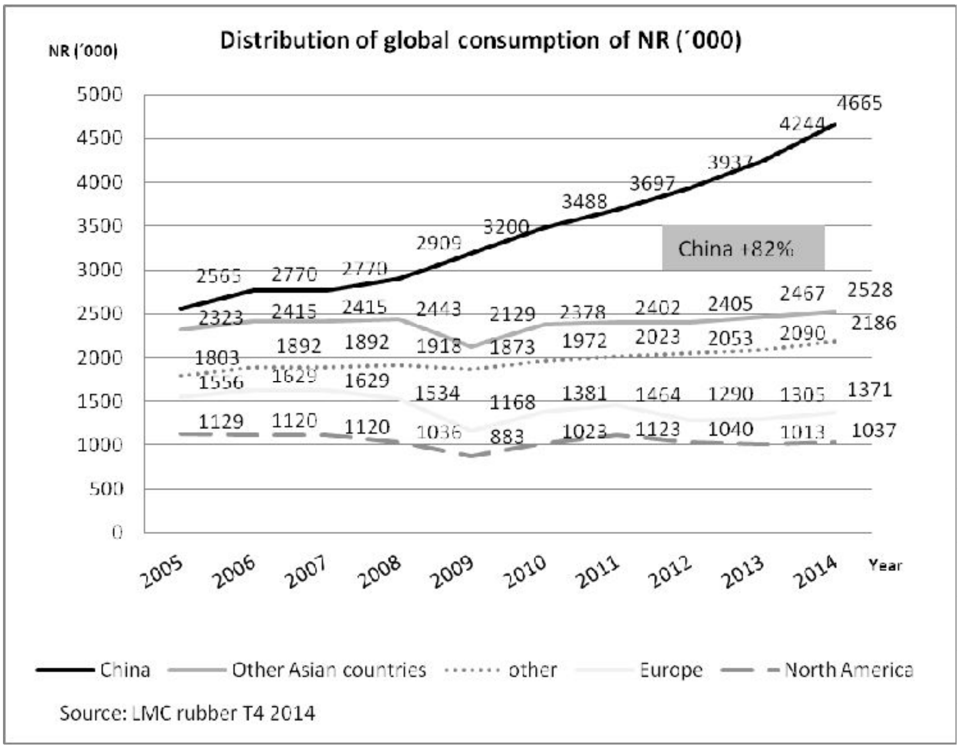

Figure 10: Evolution of global consumption of NR from 2005 to 2014.

As shown by the NMCE, there´s an important correlation (76%) between Rubber and crude oil prices. When the crude oil price goes cheaper, synthetic rubber, that needs it for its fabrication, also goes cheaper and it affects natural rubber demand. When crude oil price rises, so does synthetic rubber price and the natural rubber demand increases (Figure 11).

Correlation of Rubber and Crude Oil Prices

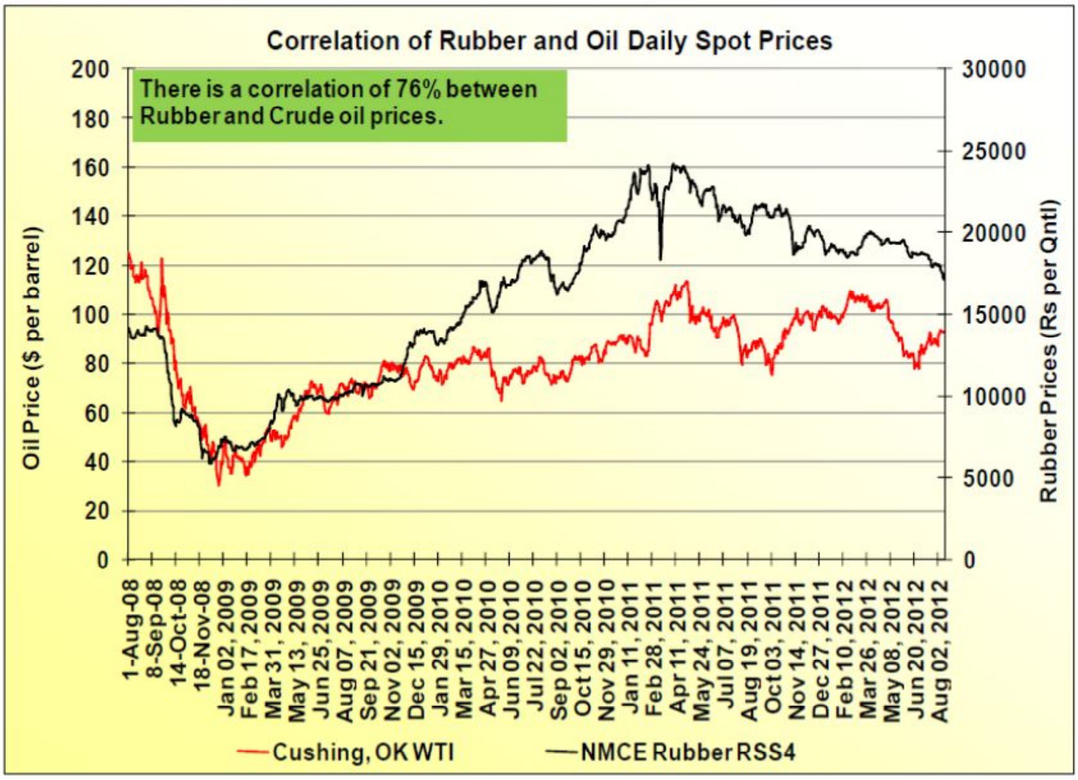

Figure 11: Correlation of Rubber and oil daily spot prices.

Legend: *West Texas Intermediate (WTI) is a grade used as a benchmark in oil pricing.

Source: NMCE (National Multy commodity Exchange). Natural Rubber Report 2012-2013

Global demand of NR is growing slowly, but the production (and exports) are moving higher in Thailand and Indonesia, what have supposed a 20% fall of the prices since the beginning of 2013. Due to the surplus of NR, falling prices are expected until the end of 2015 (Figure 12).

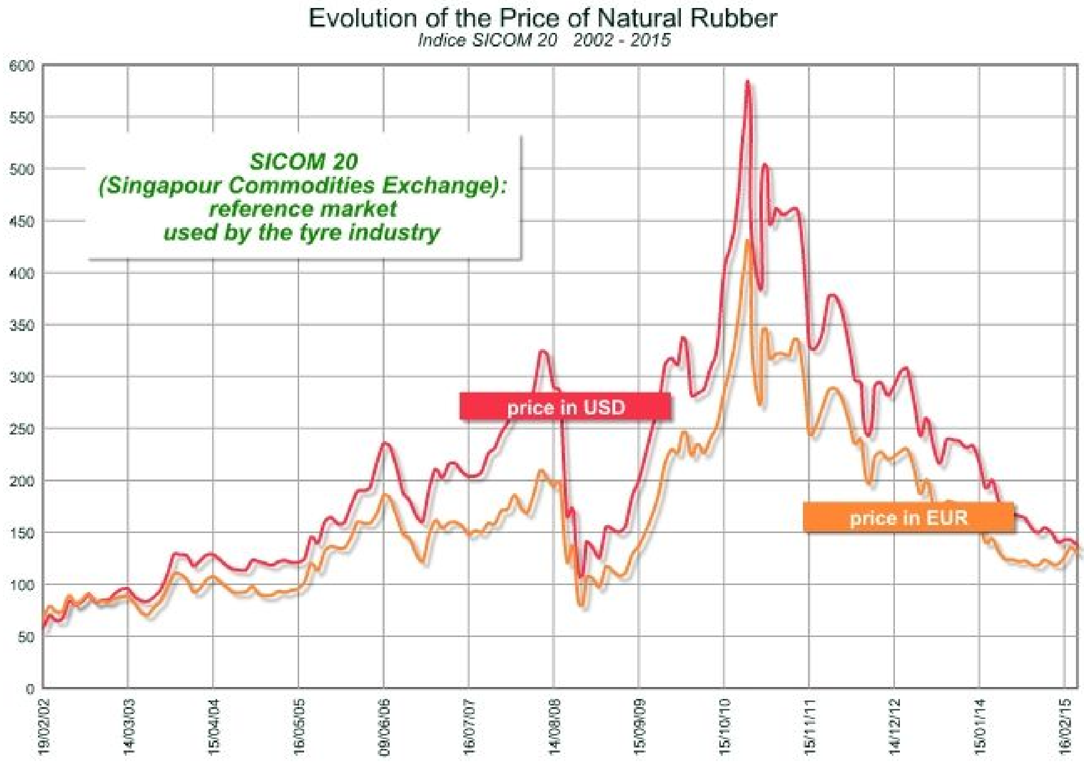

Figure 12:NR price evolution in US Dollars and Euros along the period 2002-2015.

Source: Société Internationale de Plantations d´Hévéas

But even when natural rubber is an essential element for modern life as we understand it, diminishing acreage dedicated to the crop (growers are switching to more profitable crops, such as oil palm), increasing global demand, political aspects and crop diseases are endangering natural rubber supply (Cornish *et al*., 1996).

2.1.4 Political aspects

As it has been stated in several occasions, rubber extraction has supposed the slavery and exploitation of humans for many years in the Americas (Ullán de la Rosa FJ, 2004 and IWGIA and CAAAP edts., 2012). Nowadays the political situation in some of the countries where rubber is extracted in not good at all. The armed conflict in many countries like Nigeria, Colombia, Liberia and Laos is the main factor affecting rubber plantations (personnel communications in Pulido-SierraI *et al.,* 2012). In the Firestone plantation there have been some attacks by illegal rubber tappers

(Worldnomads, 2012). All these conflicts and instability in the rubber producing countries are other reasons for unstable productions that must be solved.

2.1.5.1 Advantages and disadvantages of *Hevea brasiliensis* culturing

2.1.5.1.1 Advantages

Apart of being the most economic profitable rubber source, *Hevea brasiliensis* is also exploiting its wood as a by-product. It has not been used as a source of wood for timber due to its poor durability, but it has traditionally been used as a cheap source of fuel wood in those countries where plantations are abundant. It has also been used for brick burning, tobacco curing and also for drying rubber (Killmann *et al.*, 2000). Deep research has helped in the last decades to convert rubber wood into a very important by-product. It is a light colored wood that can be a cheap substitute (once treated) of more expensive and traditional woods such as teak, oak or ash (Balsiger *et al.*,2000). The uses that are nowadays been exploited are mainly flooring, furniture, wood panels and indoor building components. Havea's wood is available at relatively low cost since it is a by-product and it is considered as an "environmentally friendly" wood, since it is planted for latex extraction and then replanted once it is cut (Killmann *et al.*, 2000). Even when the use of timber has some disadvantages (see section I.V.II, "disadvantages"), the demand of this wood has increased tremendously and some large international companies such as IKEA are using it for their articles (Killman *et al.,* 2000), converting latex, in some cases, in a by-product of rubber wood (Afzal M, 1998).

2.1.5.2 Disadvantages

2.1.5.2.1 Latex Allergy

Sensitization and development of latex allergy is developed by exposure to products containing residual latex proteins and chemical additives in latex products. Latex proteins are potent allergens capable of inducing fatal anaphylaxis (Sell *et al.,* 2013). The first appearances of rubber allergies were reported in the late 1980´s and it´s now widespread (Cornish K, 1996). *Hevea brasiliensis* has got very allergenic proteins and the continued contact with them can cause this allergy. Proteins associated to NR are considered as the main cause of latex sensitivity, and the content may vary according to source, lot and manufacturing process. Since allergic reactions can be severe, finding a non-allergenic rubber source is needed. Cornish described in 1996 how she found some alternative rubber sources with little or none allergenic properties. She affirms that *P. argentatum* Gray has a protein profile different from the one in latex obtained from *Hevea* (Cornish K, 1996).

Results of experiments carried out by Cornish *et al.* in 2005 indicated that proteins present in production lots of ammoniated guayule latex are not detected by human IgE, and therefore are not cross-reactive with *Hevea brasiliensis* (Hev-b) latex allergens. This suggests that devices manufactured from guayule latex as an alternative rubber source should be safe for use by Hev-b Type I latex allergic individuals.

2.1.5.2.2 Diseases

Since Asiatic current plantations come from handful seeds that were collected in Brasil in the late XIX century, it has a very narrow genetic variability and it becomes endangered when threatened by a disease or plague (Venkatachalam *et al*., 2013).The most important and dangerous disease that *Hevea brasiliensis* suffers at the moment is the Southamerican Leaf Blight disease (SALB). It caused the decline in large scale of plantations of *Hevea* trees in America (Whaley WG, 1948) and it is still confined to the tropical Americas (Prabhakaran Nahir KP, 2010). Under natural conditions, where trees commonly grow sparsely, serious damage to Hevea is unusual, but under large-scale plantations it can become lethal. The trees growing in between act as non-susceptible species (Whaley WG, 1948). Breeding and selection programs are needed in order to minimize or avoid the disease.

Other significant diseases affecting the rubber tree are Abnormal Leaf Fall (ALF) and Secondary Leaf Fall (SLF), They were causing the main diseases of the rubber tree in India few years ago. Another one that can cause the drying of the main stem is called pink disease (Prabhakaran Nair KP, 2010).

Although insect pests are minimal in rubber tree (Prabhakaran Nair KP, 2010) massive seasonal invasion by the litter-dwelling beetle *Lupropstristis,* has become a very serious annoying pest in rubber plantations in the Western Ghats in southern India.

It eats the leaves of *Hevea brasiliensis* and prefers them rather than others like cashew, mango, jackfruit, etc (Sabu *et al*., 2011).

The “Tapping-Panel Dryness”, so-called “Brown Bast”, is a stress related physiological disorder generally present in some high yielding clones. It occurs when the harvesting of latex exceeds the physiological capacity of its regeneration. Even though it is not a disease, it provokes losses in latex production from 15 to 20% (Rubber Board, 2002), what constitutes a loss of profit of about 360 billion USD/ year (Okoma *et al.,* 2011). Reducing the tapping intensity or 3 to 12 months of tapping rest is considered curative measures for this disorder (Prabhakaran Nair KP, 2010).

2.1.5.2.3 Wood problem

Even when it has some interesting properties that make Hevea’s wood interesting, some disadvantages should also be taken into account. It is a non-durable timber; it is very susceptible to insect and fungi attack, the logs are small compared to other species, the wood could have seasoning physical defects such as cupping, twisting, bowing and checking, the remaining latex causes clogging of saw teeth and it has a low conversion rate and productivity rate (Balsiger *et al.,*2000).

2.1.5.2.4 Oil palm

One of the threats to the natural rubber market is the very competitive and very fast growing market of palm oil and its side products. Both are markets in expansion, but Malaysian *Hevea brasiliensis* harvested areas have started to diminish whilst for oil palm it has been growing for the same period (1990-2011) (FAOSTAT, 2013). Even when in the rest of the countries the harvested area hasn´t decreased if the huge and continuous growth of oil palm plantations doesn´t stop, either the natural forest or the *Hevea* plantations would have to leave place for the new crop.

The economy of the country is very important in terms of deciding which products are going to succeed in the market. In some countries like Malaysia, the economy is changing to an industrial basis, and it means that very laborious works like latex extraction, are increasing their costs (van Beilen *et al.,* 2007), what means also a global natural rubber price rise and the displacement of some of these crop areas to cheaper countries (Pulido-Sierra *et al.,* 2012).

2.1.6 Alternative rubber sources

Rubber is synthesized in over 7,500 plant species, confined to 300 genera of seven families, named, *Euphorbiaceae, Apocynaceae, Asclepiadaceae, Asteraceae, Moraceae, Papaveraceae* and *Sapotaceae* (Cornish *et al.,* 1993, in Mohan Jai *et al*., 2009). Only a few of them synthesize polyisoprenes in the trans configuration (Rose *et al*., 2005). A selection of the most interesting cis-configuration poly isoprene producers and their characteristics is shown in Table2.

Rubber source	Content (%)	Rubber Mw (kD)[a]	Protein (kD)[b]	Production T/Y (year)	Yield (kg ha^{-1} y^{-1})
Rubber tree ***H. brasiliensis***	30-50 in latex, 2% of the dw	1310	14.6 (REF)24 (SRPP)	9000000 (2005)	500-3000
Guayule ***P. argentatum*** **Gray**	3-12	1280	26 (GHS) 44 (RPP)	10000 (1910)	300-2000
Russian dandelion ***T. koksaghyz***	Trace-30	2180	-	3000 (1943)	150-500
Rubber rabbitbrush ***Chrysothamnus nauseosus***	<7	585	-	-	-
Goldenrod ***Solidago virgaurea minuta***	5-12 of root dw	160-240	-	110-155	110-155
Sunflower ***Helianthus sp.***	0.1-1	279	-	-	-
Fig tree ***Ficus carica***	4 in latex o	69/190	25,48?	-	-
Ficus bengalensis	0.3 in bark	1500	31,55?	-	-
Ficus elastica	18 in latex	1-10	376 (LPR)	-	-
Lettuce ***Lactuca serriola***	1.6-2.2 in latex	1380		-	-

Table 2: Alternative sources of Poly-cis-isoprene.

Legend: dw: dry weight; GHS: Guayule Homologue of SRPP; LPR: Large Protein from Rubber particles; REF: Rubber Elongation Factor; RPP: Rubber Particle Protein; SRPP: Small Rubber Particle Protein.
[a] Average molecular weight
[b] Main protein(s) associated with rubber particles or implicated in rubber biosynthesis

Source: van Beilen , J. et al., (2007a)

2.1.6.1 Guayule (*Parthenium argentatum Gray*)

It´s a native plant of the north plateau region of Mexico and the Big Bend area of Texas (Whaley GW, 1948). The temperature there ranges from (-18)ºC to 49,5 ºC (Venkatachalam *et al.,* 2013). It was discovered in 1852 and was firstly described in 1859. It is a shrub belonging to the *Compositae* family, usually restricted to limestone soils and very low rainfall regions (250-380mm per year). Under these conditions it can live for 30-40 years. Rubber is found in a considerable amount in the stem and in the root, and it appears in latex of individual cells instead of latex vessels or tubes. Its rubber content increases over a period of several years. The amount that the shrub can produce differs with several factors like location, age of the plant, strain and season. It can go from 7% to 22% or even more if the conditions are good and we use selected types for cultivation (Whaley GW, 1948). A picture of the plant can be seen in Figure 13.

Figure 13: Parthenium argentatum Gray (Guayule) plant.

Source: CIRAD (2015)

In the 1940´a very big research programme was established in the EEUU around guayule rubber. A rubber alternative source was being searched. Since the needs for natural rubber had gone up and down several times so did the research on guayule shrub. Along with the research program the duration knowledge of the species and the quality of the rubber productive material improved seriously reaching top a point in which it could be taken into account as an alternative source of rubber in case of emergency. Guayule rubber doesn´t satisfy the quality needed for all rubber uses due to its high resin content, but it has been used with other compounds for uses were its resin is desirable (Whaley GW, 1948). It is actually used and commercialized as an alternative source of natural rubber for biomedical applications due to its low allergenic power (Venkatachalam *et al.,* 2013). It is the only non tropical plant that has been cultivated in the early 20th century as a commercial rubber alternative source. It is cultivated by Yulex Corporation as a source of hypoallergenic latex (Yulex, 2015).

Even though guayule has very good quality rubber and it´s the only alternative rubber source being commercialized at the moment, it is a difficult species to work with from a breeder´s point of view; it is a perennial shrub that requires a relatively large amount of land for breeding programs, it is physiologically immature for the first two years and the reproduction is essentially asexual (by apomixis), making breeding to depend mainly, on the selection of high-yielding plants (van Beilen *et al.*, 2007).

2.1.6.1 Russian dandelion (*Taraxacum koksaghyz* Rodin)

As van Beilen *et al.* pointed in 2007, the ideal rubber-producing crop plant would be an annual crop, fast growing and producing large amounts of biomass. It would be plastic and adaptable to market changes and easier to introduce in crop rotation and farming systems. *Taraxacum koksaghyz* R. may fit these requirements better than guayule.

It was discovered in 1931 in the Tien Shan Mountains of Kazakstan in an expedition that was part of a Russian Government program, aiming to discover new or better plants that could supply all critical materials originated from plants (Whaley GW, 1948). See TKS plant in Figure 14.

By 1935 *Taraxacum koksaghyz* R. was the most promising alternative rubber source in Russia and in 1939 it was planted in a quite considerable extension and it was yielding 28.35kg/Ha, with some of the plantings producing several times that much. EEUU introduced it in the 40´s as part of an emergency program, looking for an alternative rubber source in War time. 300 Ha were planted along 42 states and they got sufficient rubber for chemical and physical analysis as well as for performance tests. They discovered the excellent rubber quality that in some cases was even better than the one coming from *H. brasiliensis* (Whaley GW, 1948).

During the World War II, *P.argentatum, Solidago altissima* and *Taraxacum koksaghyz* R. were used successfully for rubber production for army vehicles tires (Mooibroek *et al.*, 2000). After the World War II rubber from *Hevea brasiliensis* became available again, cheaper and easier to get and the Russian dandelion culture was abandoned in all countries. It only remained in Russia until 1950s. The 67,000 Ha of Russian dandelion crops covered 30% of total Russian NR consumption. It was finally abolished may be because of economic reasons (van Beilen *et al.*, 2007 b).

Some years later research of alternative rubber sources have started again and in 2007 van Beilen *et al.* reported a yielding of 150-500 kg/Ha per year. Based on the prediction that Polhamus made in 1962 (an increase of 15- 25 % of dry weight potential rubber production obtained by breeding programs) and the possibility of hybridizing TKS with *T. officinale*, they also predicted a theoretical rubber yield of 1,200-1,800kg per year and hectare (van Beilen *et al.*, 2007 b).

It is a member of the tribe *Cichoriae*, family *Compositae*. It grows erect or in a manner which it is very close to the ground but its leaves are always disposed in rosettes with a bunch of 25-50 gray-green colored leaves (Whaley GW, 1948). The optimum flowering and fruiting time is end of May or beginning of June (van Dijk *et al.,* 2010). During the first year of TKS life only a small number of plants produce flowers, but most of them produce flowers in the second year. Apparently flowering plants have a greater fresh weight than those non flowering plants, and a smaller % of rubber content. Plants not flowering the first year were reported to be more desirable from an economical point of view (Krotkov G, 1945).

Rubber from roots and leaves have different qualities. Leaf rubber has a high percentage of associated resins and it is a very poor product, while root rubber has a low percentage of associated resins, resulting in a high quality product. This is the reason why leaves should be removed before processing the roots (Whaley GW, 1948). Figure 15 shows how rubber is arranged in the laticifers of the plant.

The somatic number of chromosomes is 2n=16 and cross-pollination is the usual method for reproduction (Krotkov G, 1945). Because of a self-incompatibility system that prevents self-pollination TKS, breeding it is quite complicated (van Beilen *et al.*, 2007a). In the other hand it can be grown by tissue culture and it´s relatively easy to transform, what makes it susceptible of becoming a model rubber-producing plant (van Beilen *et al.*, 2007 b).

Figure 14: TKS plant in a pot in the greenhouse.

Taraxacum koksaghyz R. by-products include inulin (25-40% of root dry weight), which could be used in food applications and non food applications (van Beilen *et al.*, 2007a), for example being used in the production of the common polyester polyethylene terephthalate (PET) (van Beilen *et al.*, 2008). TKS inulin is quite similar to the one obtained from chicory, and it has been tested for bioethanol and biobutanol as biofuels. Fermentation of unhydrolyzed inulin media with *C. saccharobutylicum* P262 produced fair amount of solvents. The residual root bagasse (after extracting rubber and inulin) and leaves of TKS can also be used for cellulosic biofuel production (Bharathidasan, 2013).

Future work is necessary in order to make it economically profitable; it includes molecular studies, using the tools of genomics, metabolomics, proteomics and marker assisted breeding (van Beilen et al., 2007a).

2.2 Inulin

Inulin, a non digestible carbohydrate, is a fructan that is not only found in many plants, but has also been part of human's daily diet for several centuries (Frank and De Leenher, 2005). It is a mixture of poly- and oligosaccharides which almost have the same chemical structure GF_n (G: glucose, F: fructose and n: number of fructose units linked to one another). The links between the molecules are of a very special type: the β (2-1) form, which makes this molecule indigestible for all higher animals (Paul Coussement, 1999).

2.2.1 History

Inulin was first isolated in 1804 by the german scientist Rose. It was extracted with boiling water from *Inula helenium* and it was in 1818 when Thomson called it inulin (Franck *et al.,* 2005). Together with oligofructose it is present in more than 36,000 plants (Carpita *et al.,* 1989).

2.2.2 Uses

2.2.2.1 Human consumption

It is a non-digestible carbohydrate present in common human diet. Industrially it is obtained from chicory roots and it is used as a functional food ingredient. It offers interesting nutritional properties and important technological benefits (it is taste free, increases the stability of foams and emulsions, etc.). It represents a key ingredient for the food industry.

2.2.2.2 Pharmaceutical uses

It is also a much desired element in pharmaceutical industry (due to a number of unique chemical and physical properties), having many proved and potential applications. For example, soluble inulin is inert and non-toxic. Its glycosidic bonds make it indigestible by humans and higher animals. It passes through the human

43

digestive system relatively intact, until it reaches the large intestine where it is digested by bifid bacteria. From the stimulation of healthy intestinal micro flora some important by-products are obtained; butyric and propionic acids are relevant in suppressing colon cancer development. Dietary inulin may also reduce risk of cardiovascular disease and it has also been shown that it increases calcium and magnesium absorption and bone mineralization in young adolescents (Barclay *et al.,* 2010).

2.2.2.3 Biofuel

In the last decade some factors as the continuously increasing crude oil price, the depletion of crude oil resources and the alarming environment degradation (global warming is, in a very high degree, attributed to the use of fossil fuels) have increased the interest in finding alternatives to fossil fuels and, in particular, sustainable biofuels from biomass have brought an important attention. Inulin meet the needs for biofuels as biobutanol and bioethanol production (Tashiro and Sonomoto, 2010)

The use of inulin as a raw material for bioethanol production is still too expensive, and the process is relatively complex. In order to decrease the costs of production it is essential to minimize the sugar losses and achieve efficient conversion of inulin into fermentescible sugars. The development of new strains of microorganisms able to ferment inulin directly is one of the goals where efforts have been made (Neagu and Bahrim, 2012).

It has also been tested in order to produce biobutanol. It is a fuel with superior qualities than bioethanol. Its energy content is 30% more than the one in bioethanol and it is close to gasoline. Its application in existing gasoline supply channels is facilitated by its low vapor pressure. It is less hydrophilic, volatile hazardous to handle and flammable than ethanol. It can be used in unmodified internal combustion engines, blended with gasoline at any concentration (up to 100%) (Bharathidasan, 2013).

Even when biobutanol has proven superior fuel properties, it needs to compete on costs with ethanol in order to capitalize the large biofuel markets (Green, 2011).

Solventogenic *Clostridium* species metabolize a wide range of pure carbohydrates, such as starch, sucrose, glucose, fructose, galactose, cellobiose, xylose, arabinose, glycerol, lactose, and inulin (Ezeji, 2014). A high effort is being made in the study of new *Clostridium* strains and systems-level metabolic engineering approaches were taken to develop superior strains (Jang *et al.,* 2011)

Even though biobutanol is very interesting as a biofuel it still has some limitations. Some of them are the butanol toxicity for the microorganisms employed, low heating value compared to gasoline or diesel fuel, degeneration of microorganisms, lower octan number and potential corrosiveness (Bharathidasan, 2013).

2.3 Genetics

Nowadays TKS remains as a wild species and, in order to become a competitive rubber and inulin producer, a breeding program is required. As a plant breeding program we understand "the application of genetic principles in manipulating plants by hybridization and selection to improve cultivars suited to specific environments and production practices, and to provide food, feed, fiber (and also fuel and drugs) for the betterment of mankind" (Soh S, 2011).

Modern plant breeding integrates conventional and molecular methods in order to obtain varieties with improved characteristics. In the last years marker assisted selection (MAS) is gaining importance. It helps to overcome many of the problems faced during conventional breeding. For the study of the heritability of the desired characters, a genetic linkage map is required.

2.3.1 Linkage Map

A linkage map is a representation of the linear arrangements of the innumerable loci (which include morphological, isozyme and DNA markers) along the chromosomes throughout meiosis (Kumar L, 1999). Sequence polymorphisms are detected by all types of DNA markers. They also monitor the segregation of a DNA sequence among progeny of a genetic cross in order to construct a linkage map (Young N, 2000). The distance between two loci is expressed in centimorgans (cM), which represents the propability of recombination between them (1 cM = 1% probability of recombination) (Kumar L, 1999).

For a good linkage map development the selection of the mapping population is a critical decision. The goal of mapping the project is the main factor influencing this decision. It will influence which parents are chosen for crossing, the population size, the choice of genetic population and which generations are used for DNA and phenotypic analysis. The need of sufficient DNA sequence polymorphism between parents must be kept in mind (Young N, 2000).

2.3.2 Genetic Markers

A genetic marker is any locus used in order to tag the close presence of a gene of interest. A genetic marker must have a phenotypic or biochemical expression easily identifiable in order to pinpoint one or more genes of difficult direct observation or lately expression (Cubero JI, 2003). It can be used with two objectives, in order to tag the locus controlling the phenotypic difference and/or to tag a close locus controlling an interesting character (Nuez *et al.*, 2003).

The genetic markers can be morphological (visible and easily detectable, hardly ever show co-dominance and are frequently pleiotropic), biochemical (the most important are the isoenzymes. They are co-dominant) and molecular (those constituted by DNA fragments. Many but not all are co-dominant and not pleiotropic not epistasic) (Cubero JI, 2003).

2.3.2.1 Molecular Markers

There are many kinds of molecular markers : RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphisms), SCAR (Sequence Characterised Amplified Region), Microsatellites (STMS: Sequence Tagged Microsatellite Sites; SSR: Short Sequence Repeats), STS (Sequence Tagged Sites), SNP (Single Nucleotide Polymorphisms), DASH (Dinamic Allele Specific Hybridization), ASO (Allele-Specific Oligonucleotides), SSCP (Sigle Strand Conformation Polymorphisms) (Cubero JI, 2003).

***AFLP:** It is a technique for DNA fingerprinting. It consists in the detection of genomic restriction fragments by PCR amplification and can be used for DNAs of any origin or complexity. The technique is robust and reliable (Vos *et al.*, 1995).

***SSR:** The microsatellites are multilocus probes that create complex banding patterns and are usually non-species specific occurring ubiquitously. They essentially belong to the repetitive DNA family (Joshi *et al.*, 1999). SSRs were firstly described by Hamada et al. in 1982 as short DNA sequences formed by 1 to 6 nucleotides motifs repeated in tandem. They are very abundant and uniform in the genome of most of the eukaryotes (Nuez *et al.*, 2003).

***STS:** Short DNA sequence (100-500 bp usually) easily recognizable, unique in the chromosome or in the genome of study (They are obtained sequencing cDNA achieving this way sequences of codifying DNA (EST: Expressed Sequence Tags) with which design specific primers for each cDNA. They can be used to physically localize a gene in a chromosome and to arrange big DNA fragments obtained from DNA cloning of any species (Cubero JI, 2003)

***COS** (Conserved Ortholog Set): Those markers are evolutionary conserved, single-copy genes, identified from large databases of express sequence tags (ESTs). They are very useful for syntenic studies among species (Fulton *et al.*, 2002).

***EST-SSR:** They are SSR markers developed from expressed sequence tags (ESTs). The rapid and inexpensive development of SSRs from EST databases has been shown to be a feasible option for obtaining high quality nuclear markers (Gupta *et al.*, 2003). The flanking sequences of EST-SSRs are located in well-conserved regions across phylogenetically related species because ESTs become from coding DNA. It is the reason why they are chosen for comparative mapping and relevant functional and positional candidate genes to study their co-location with quantitative trait loci (QTLs) (Duran *et al.*, 2010).

2.3.3 Synteny studies

The term synteny (from the Greek; syn 5 together, taenia 5 ribbon) is used in classical genetics to indicate the presence of two or more loci on the same chromosome. The relevance of the term dates from a pre-genomics era, when locating genes to chromosomes was much more complicated without the assistance of whole-genome mapping technologies. Today, synteny is a concept used to address questions of homology (McCouch SR, 2001). It is commonly used by biologists as the conservation of gene order on chromosomes of different species. This concept is also known as shared or conserved synteny. Modern techniques of sequencing and mapping made possible the comparison of general structures of genomes of different species. When organisms of relatively recent divergence show similar blocks of genes in the same relative positions in the genome, we talk about synteny (Britannica online encyclopedia, 2015a).

2.4 Hypothesis, Main Objective and Secondary Objectives

2.4.1 Hypothesis

Concerning NR production, the global situation shows some critical aspects:

1. The control of NR production id held by Asian countries that have the monopoly of *H. brasiliensis* culture , being the European continent completely excluded from the NR market.

2. The NR World demand is increasing whilst *H. brasiliensis* crop is being changed, in many locations, by the more economically profitable oil palm crop.

3. Important threats are endangering *H. brasiliensis* plantations compromising NR supply.

Considering all these aspects our hypothesis is “the establishment of TKS as the main natural rubber and inulin producer being economically profitable and available for the European market”.

2.4.2 Main Objective

To increase the knowledge of TKS cropping requirements in order to obtain the maximum amount of biomass, inulin and rubber production and establish a genetic base for future marked assisted selection of TKS.

2.4.3 Secondary Objectives

1. To evaluate the influence of "population" and "planting dates" of TKS on the production of biomass, inulin and rubber.

2. To evaluate potential interactions between the two main factors.

3. To determine the needs of water supply in TKS culture in order to obtain maximum production of biomass, inulin and rubber.

4. To determine possible correlations between agronomic aspects, rubber and inulin production in TKS culture.

5. To develop the first genetic linkage map of TKS, based on AFLP, COS, SSR and EST-SSR markers.

6. To establish possible homologies with other plant gene sequences and determine which are useful for downstream applications in the future.

7. To blast the EST-SSR sequences against known sequences from lettuce in order to establish a possible alignment of our TKS map with a lettuce map.

8. To develop a short synteny study of TKS and lettuce.

3. CHAPTER I:

EVALUATION OF ROOT BIOMASS, RUBBER AND INULIN CONTENTS IN NINE *TARAXACUM KOKSAGHYZ* RODIN POPULATIONS.

3.1 Introduction

Russian dandelion (*Taraxakum koksaghyz* Rodin, TKS) is a native plant of Kazakhstan (Krotkov, 1945). Different countries put large efforts on the use of TKS as an alternative rubber source until approximately 1950, mainly due to the cut of natural rubber supply from the rubber tree (*Hevea brasiliensis*) in World War II. However, it never became an established crop due to the competition with the cheaper rubber price of *Hevea brasiliensis* (Kirschneret al., 2012). Some forty years ago, alternative natural rubber sources started to be actively sought, since several factors threatening *Hevea brasiliensis* production and utilization, such as for example allergies to *Hevea* latex, crop diseases, diminishing cultivation area and changing rubber prices. Between the alternative sources of interest, TKS was again on the top of the list (Mooibroek et al., 2000).The quality of TKS rubber is comparable to that one from the rubber tree, and its average molecular weight of 2180 kD is even higher compared to the only 1310 kD of *Hevea brasiliensis*(van Beilen and Poirier, 2007a).

The European Union started in 2008 the international R&D project EU-PEARLS (Production and Exploitation of Alternative Rubber and Latex Sources). The present study was part of this project and had the aim to analyzethe agronomical performance of different TKS populations under different planting dates with respect to root biomass production and rubber and inulin contents.

3.2 Materials and methods

3.2.1 Plant material and field trial

Seeds of nine wild TKS populations (Table 1), collected in 2008 by the Kirschner team in an expedition to Kazakhstan were planted and evaluated in the experimental station of NEIKER in Arkaute (Spain). The populations descended from the area in the vicinity of the villages Saryzhaz, Kegenand Komirshi close to the Mongolian boarder (Kirschner et al. 2012). The term population considers here a collection of individual plants growing together in a specific isolated region (Figure 1, 2).

Figure 1 : Plants belonging to different populations of TKS in pots in the greenhouse.

Figure 2: Plants belonging to different populations of TKS in the field trial.

Table 1: Origin of TKS populations used in the population trial.

Sample Code(Population)	Original sample code	Location and Date of collection
20	_006	1 km N of NW part of Sarydzhaz, 4 June 2008
21	202	c. 6 km ENE of Saryzhaz, 2 Jun 2008
22	203	c. 5-6 km ENE of Saryzhaz, 2 Jun 2008
27	207	c. 1-2 km SW of Kegen, 3 Jun 2008
30	217	Along the road between Saryzhaz and Komirshi, 3 Jun 2008
35	219	c. 6 km SW. of Komirshi, 3 Jun 2008
36	236	c. 4 km NNW. of Kegen, 6 Jun 2008
37	237	2-3 km E of Zhalauly village, c. 6 km NW. of Kegen, 6 Jun 2008
38	246	c. 2 km SE of Kegen, 3 Jun 2008

[a]TKS seed populations collected in Kazakhstan in 2008 (Kirschner et al. 2012).

The research was performed in the experimental field of the “Model Farm of Arkaute” (42° 51' 0.29" N -2° 37' 21.59" W; 517 m elevation), close to Vitoria-Gasteiz (Basque Country, Spain), during the year 2009. The soil texture of the field was classified as loam. The annual accumulated precipitation was 629 mm; mean temperature was 11.7 °C and average humidity 85.1%. The analyzed main factors were planting date and population. Two planting dates and nine populations were evaluated.

TKS seeds were sown in wet paper beds in a culture chamber and seedlings were raised in multi pots in the greenhouse from March to April. Trials were set up according to a factorial design with single plant repetitions and elementary plots of 1.2 x 2 m^2. Young seedlings of 10 to 15 cm height were planted on ridges at depth of 6 to 7 cm in four rows and five plants per row, resulting in a spacing of 24 by 34cm^2. After planting a 30 minutes irrigation was applied. All Russian dandelion populations were planted May 15th (F1) and June 15th (F2), respectively, following the suggestions of Whaley and Bowen (1947). For weed control a green anti weed MIPED mesh (2.10m width) was used. In order to avoid moles, an ultrasound mole chaser was used. The soil did not get any extra fertilization and water was supplied when needed. No pests or diseases were observed during cultivation.

3.2.2 Biomass production

Harvesting was done end of October, when no further growth was registered due to low temperatures. The roots were dragged out manually, washed and plants were kept in a cool chamber at 4ºC until processing up to four days later. Initially, fresh root weight was measured using four single plant repetitions. Roots were dried during two days at 65°C. After cooling in a desiccator, they were weighted again to determine dry root biomass and stored. Rubber threads could be already appreciated in dried and liophilized roots (Figure 3).

3.2.3 Rubber and Inulin contents

Roots were milled until a very fine powder was obtained with a Moulinex AR10031 electric grinder. The flour was stored at room temperature in 50 ml Falcon tubes.

The method of Post et al., (2012) was used for the determination of dry rubber content by gravimetric analysis with some modifications.An aliquot of 200 mg of root powder was weighed in a 10 mlThermo Scientific TeflonFEP tube, mixed with 4ml of toluene, incubated at 70°C for 18 h in a water bath. The toluene supernatant was transferred to a clean tube and vaporized in athermoblock. The remaining sample was re-dissolved in 300 µl of toluene for 30 min. Rubber was precipitated by incubating with 600 µl of methanol for 30 min. All steps were performed under a gas extraction hood. After centrifugation at room temperature for 2 min at 16.000 xg, the precipitated rubber appeared as a white layer. It was washed twice; first with water for 30 min with vigorous shaking and then with acetone for other 30 min with vigorous shaking in an Infors HTEcotron shaking incubator. The rubber samples were vacuum dried in a HETO Speed Vac, Model DNA mini, for 3 min at 60°C and cooled down to room temperature before weighing.Measurements were done in four plants of every combination of population and planting date. Three repetitions per plant were carried out and average values were computed in order to have more consistent data.

Figure 3: A) Rubber threads of *Taraxacum koksaghyz* Rodin in a lyophilized root; B) Rubber threads of TKS in a dried root.

For inulin extraction and quantification the D-glucose/D-fructose UV-method of R-BiopharmKit was used, including the procedure for inulin hydrolysis suggested by the manufacturer.

An aliquot of 500 mg of root flour were weighed in a 50 ml Falcon tube and 15 ml of miliQ boiling water was added. The pH was adjusted to 6.6-8.0 using a Crisson pH-meter Basic 20+ and then filled up to 25ml volume. After vigorous shaking the sample was heated in a shaking water bath (Selecta Ultrasonic 320 OR) at 80°C, 90 u/min for 15 minutes. Afterwards the sample was filtered using a Serlab vacuum filtering equipment with Whatman filter paper n° 589/2. The extracted liquid was cooled down to room temperature and filled up with water to 25 ml volume.

For hydrolysis an aliquot of 12.5 ml were transferred to a 50 ml Falcon tube, 1.25 ml of HCL 25% were added and left in the shaking water bath at 80°C, 90 u/min for 15 min. Then the sample was neutralized with 5 M KOH and brought to 25ml volume with miliQ water.

First glucose and fructose contents in the sample were measured spectrophotometrically and afterwards the amount of these sugars in the hydrolyzed sample was determined. By subtracting the corresponding measurements, the amount of inulin present in the sample was obtained after converting to inulin contents using a specific formula. For quantification a UV-2401 PC; Shimadzu UV-VIS Recording Spectrophotometer was used, measuring at 340 nm in a quartz cuvette with 1 cm light path.

Measurements were done in three plants of every combination of population and planting date.

3.2.4 Data analysis

Statistical analyses were conducted using JMP software version 5.0 (JMP, 2002). Analysis of variance, based on a factorial design with 4 replicates for rubber content and 3 for inulin content, was used to analyze the effects of population and planting date. A two-way full factorial analysis of variance was carried out.Tukey's honestly significant difference (Tukey´s HSD) was used for comparing treatment means, with an alpha error probability level of 5%.

The Box Cox transformation [$(RDW^{-0.2} - 1) / -0.0384096976766$]was used to transform Root dry weight (RDW) data in order to normalize the distribution. We refer to the transformed data as RDW X.

3.3 Results

3.3.1 Morphological Aspects

Individual plants showed a considerable amount of morphological variation between as well as within populations. They varied with respect to main and side root ramifications, rosette numbers, inflorescence numbers, leaf numbers and leaf morphology (results not shown).

3.3.2 Rubber content

All data were normally distributed and equal variances were assessed with Levene's test of equality of variances (Howard Levene, 1960). For determining rubber content, population 20 was not taken into account, since homocedasticity couldn´t be adjusted when included, due to a very high variance within its values.

The analysis of variances revealed only a significant main effect for population number, while the main effect for planting date was not significant, neither the interaction between population number and planting date (Table 2a).

Table 2: Results of Analysis of variances for the TKS population trial at two planting dates.

A) Model Parameter effects

Response	Source	DF	Sum of Squares	F Ratio	Prob. >F	R^2
Rubber (%)	**Population**	**7**	**24.324746**	**4.914**	**0.0003**	**0.3765**
	Planting Date	1	0.019368	0.027	0.8692	<0.0001
	Population*Planting Date	7	6.315005	1.276	0.2823	0.0978
Inulin (%)	**Population**	**8**	**762.17933**	**5.815**	**0.0001**	**0.3313**
	Planting Date	1	24.22331	1.479	0.2319	0.0105
	Population*Planting Date	**8**	**840.70221**	**6.414**	**0.0001**	**0.3654**
Root DW X (g)	Population	8	96.62421	1.695	0.1209	0.1368
	Planting Date	1	23.87673	3.351	0.0727	0.0338
	Population*Planting Date	**8**	**200.76273**	**3.522**	**0.0024**	**0.2843**

B) Goodness of Fit of the Model

Response	Prob	RSq	RMSE	CV (RMSE) (%)
Rubber (%)	0.0027	0.47	0.8409	51.0
Inulin (%)	<0.0001	0.74	4.0476	15.7
Root DW X (g)	0.0034	0.46	2.6694	42.6

[a]**Legend:** Prob=probability, DF=degrees of freedom, RSq=root square mean RMSE=root of the mean square error, CV= coefficient of variation.

According to the Goodness of Fit test, the whole model was able to explain 47.45% of the variance in the response variable with a CV (RMSE) of 51 %. (Table 2b).

The sample least square means are displayed in Table 3.Tukey´s HSD test showed that population number 30 had significantly higher rubber content than populations 38, 36, 21, 27 and 22 and population number 38 had significantly lower rubber content than population 35.

Table 3: Results of Separation of Means using Tukey HSD tests.

Rubber (%) Response				
Level (POP)				**LSM**
30	A			2.96
35	A	B		2.22
37	A	B	**C**	1.68
22		B	**C**	1.43
27		B	**C**	1.42
21		B	**C**	1.40
36		B	**C**	1.25
38			**C**	0.82
Alpha = 0.05; Q = 3.17				

Inulin (%) Response					
Level					**LSM**
20- F1	A				36.30
37- F1	A	B			36.05
38- F2	A	B	C		33.92
38- F1	A	B	C		33.91
35- F2	A	B	C		30.05
21- F2	A	B	C		29.95
27- F2	A	B	C		28.40
20- F2	A	B	C		26.91
36- F2	A	B	C		26.89
27- F1	A	B	C	D	26.14
30- F2		B	C	D	23.58
22- F1		B	C	D	23.34
22- F2		B	C	D	22.79
30- F1		B	C	D	22.13
21- F1		B	C	D	21.58
37- F2			C	D	20.64
35- F1			C	D	15.32
36- F1				D	14.89
Alpha = 0.05; Q = 3.75					

Root DW X (g) Response			
Level			**LSM**
20-F1	A		9.96
38-F2	A		9.87
22-F1	A	B	8.51
21-F1	A	B	8.50
36-F1	A	B	7.98
27-F1	A	B	7.23
35-F2	A	B	7.02
30-F1	A	B	6.68
27-F2	A	B	6.46
35-F1	A	B	6.36
30-F2	A	B	5.63
21-F2	A	B	4.94
36-F2	A	B	4.86
22-F2	A	B	4.71
38-F1	A	B	4.17
37-F2	A	B	3.93
20-F2	A	B	3.80
37-F1		B	2.20
Alpha = 0.05; Q = 3.66			

[a]Levels connected by same letter are not significantly different

3.3.3 Inulin content

Inulin content in dry roots of TKS showed a highly significant effect for population. In addition, it was significantly influenced by the interaction between population and planting date, while no effect of the planting date could be observed(Table 2a).

In the Goodness of Fit analyses the whole model was able to explain 74.4% of the variance in the response variable with a CV (RMSE) of 15.7% (Table 2b).

The separation of means in Table 3 showed that according to the Tukey´s HSD tests many population and planting date levels had not significant different inulin contents. For example, the contents of populations 20, 38, and 27 at both planting dates, as well as those from levels 37-F1, 35-F2, 21-F2 and 36-F2 were statistically not different. The highest inulin contents of level 20-F1 was significant different from those of both planting dates of populations 30 and 22, and from levels 21-F1, 37-F2, 35-F1 and 36-F1. The largest significantly difference was found between population 20 and 36, both planted mid-May with a difference of 21.4 % in inulin content.

Separate analyses of each population per planting date revealed that the simple effect for planting date was significant for population 20 ($p < 0.008$), population 21 ($p < 0.0001$), population 35 ($p < 0.0025$), population 36 ($p< 0.0002$) and population 37 ($p < 0.0035$), but not for the other four populations (results not shown).

3.3.4 RDW

RDW of TKS was only significantly influenced by the interaction effect between population and planting date (Table 2a). The whole model was able to explain 46% of the variance in the response variable with a CV (RMSE) of 42.60% (Table 2b).

The separation of means analyses revealed as before a large degree of overlapping significances involving many levels (Table 3). The only significant differences detected by Tukey HSD tests were found between populations 20 and 37, both planted in May and between population 38 planted in June and population 37 planted in May.

Subsequent, separate analyses for each planting date demonstrated only a population effect for plants planted mid-May (F1; $p < 0.0048$).The simple effect for planting date F2 (mid-June) was not significant, ($p > 0.06$, results not shown).

In separate analyses for each population, the planting date effect was only significant for population 20 ($p < 0.002$), population 22 ($p < 0.05$) and population 38 ($p < 0.004$; results not shown).

3.4 Discussion

Depending on the purpose of utilization of the crop for rubber and/or inulin production, either one or another population and planting date should be selected. A significant interaction between population and planting date was found in our tests for RDW and inulin content but not for rubber contents, where only a significant population effect was detected.

A large degree of variation between single plants of the same population was detected for all traits under study and hindered a more distinct separation of population means in table 3. This variation is not surprising since the diploid TKS is an outcrossing species (Warmke H.E., 1943), compared to the commonly more known Dandelion (*T. officinalis*), which is triploid and apomictic (van Dijk *et al*., 2009).

Due to this large variation also population 20 had to be excluded from the statistical analyses for rubber content. However, visual analysis and repeated measurements of single plants gave the impression that more rubber could be obtained from this population than from all others when planted in June.

We also made an attempt to estimate the potential RDW, rubber and inulin yield per ha, based on our trial data and assuming a plant density of 240,000 plants per ha (Table 4).

Table 4: Potential rubber, inulin and root dry weight yield in the TKS population trial.

	EXPECTED YIELD/HA*			
LEVEL	RUBBER CONTENT (%)	RUBBER (Kg)	INULIN (Kg)	ROOT DRY WEIGHT (KG)
20-F1	1.33	35.57	970.84	2674.48
20-F2	5.56	29.37	142.14	528.22
21-F1	1.12	19.39	373.70	1731.68
21-F2	1.69	11.61	205.82	687.23
22-F1	1.19	20.67	405.33	1736.62
22-F2	1.68	10.94	148.35	650.96
27-F1	1.13	13.79	319.11	1220.76
27-F2	1.72	17.18	283.67	998.82
30-F1	3.33	35.19	233.89	1056.89
30-F2	2.58	20.94	191.36	811.55
35-F1	2.60	25.32	149.17	973.70
35-F2	1.85	21.36	347.02	1154.82
36-F1	1.01	15.11	222.79	1496.26
36-F2	1.49	10.05	181.33	674.34
37-F1	1.45	5.41	134.53	373.18
37-F2	1.91	10.39	112.27	543.93
38-F1	1.23	7.07	194.80	574.45
38-F2	0.41	10.66	882.21	2600.85

**** Assuming a plant density of 240,000 plants/ha***

Considering that population 20 when planted in May has also the highest potential inulin yield, this population could be a good candidate for future development of the crop. Other promising target populations are population 30 planted in May for rubber production due to its high rubber concentration and population 38 planted in June for inulin production due to its high biomass production (Table 4).

In any case these findings suggest that TKS breeding should be based on several rounds of selections of single plants with favorable characteristics, taking available information about populations into account, subsequent crossings between them and selection of genotypes with improved and combined characteristics.

Although statistically not significant, in general the aerial part of the plant and also the root size seemed to be better developed when TKS was planted in May than in June.

The rubber contents in our populations ranged from 0.4 to 5.6%. However, Buranov exposed in an EU-PEARLS conference in 2010 up to 24% rubber contents. Wild material was used in our studies, while Buranow referred to improved lineages. These data reflect the potential of crop improvement through breeding.

With respect to rubber yield, Whaley (1948) stated an amount of 28.4 Kg/ha,

which is similar to the yields obtained in our studies with certain populations at specific planting dates. However this amount is far away from the 150 to 500 kg/ha reported by van Beilen and Poirier (2007a).

An increase in rubber yield could be also achieved by applying a different extraction method such as Accelerated Solvent Extraction (ASE) (Richter et al., 1996). This newer technique extracts substances under high pressure at elevated temperatures and is superior to our classical toluene extraction method.

Percentages of inulin contents ranged in our studies from 15 up to 36%. Gorham (1946) reported a higher value of 42%, but van Beilen and Poirier(2007b) reported a similar range of 25 to 40%.

A further increase of root biomass, rubber and inulin yield might be possible by increasing the plant density per m^2. Munt et al. (2011) saw seeds of the related species *Taraxacum brevicorniculatum* in rows 2-5 cm apart and obtained fully developed plants with 40-50 cm long main roots without branching, facilitating in this way also the harvesting. They recommended a spacing of less than 5-10 cm apart.

Further studies considering different agronomical aspects, but particularly breeding activities in the way suggested above are necessary, in order to convert this species from a "weed" into an economic interesting crop for rubber and/or inulin production.

3.5 Conclusions

1. The analyses of our field trial involving nine TKS populations cultivated at two planting dates revealedsignificant population effects for rubber and inulin contents, no planting date effects, but significant interaction effectsbetween population and planting dates for Inulin content and root dry weight.

2. Large variations for all traits were observed between populations as well as between individual plants of a population, which hindered a clearly significant separation between mean values.

3. Populations 20, 30 and 38 have promising candidate genotypes for rubber and /or inulin production.

4. However, particularly further breeding is necessary for converting TKS in an acceptable crop for these purposes.

4. CHAPTER: II

HOW DOES WATER SUPPLY AFFECT *TARAXACUM KOKSAGHYZ* ROD. RUBBER, INULIN AND BIOMASS PRODUCTION?

4.1 Introduction

Taraxacum koksaghyz Rodin (TKS) has been suggested in many occasions as a possible alternative crop for good quality, NR and inulin production (Krotkov, 1945; Whaley, 1947 and 1948; van Beilen, and Poirier 2007a and 2007b). It was already used for rubber production for army vehicle tires during the 2nd World War by the Russian Government (Mooibroek and Cornish, 2000), but its usage and future research was abandoned due to the reopening of the commercialization of *Hevea brasiliensis* natural rubber at the end of the war (van Beilen and Poirier, 2007 b). TKS inulin has been never extracted in an industrial scale, even when its properties and quality are well known. It still needs more research on its economic viability.

A patent was registered by Wade and Swiger (2011) stating that all parts of the plants are profitable. Inulin and rubber can be extracted at the same time, making this crop economically viable.

Natural rubber is a biopolymer consisting of isoprene units $(C_5H_8)_n$ linked together in a 1, 4 *cis*-configuration. The molecular weight of an isoprene monomer in natural rubber (C_5H_8) is 68 Da. (van Beilen et al., 2007). Natural rubber is considered to be one of the most versatile agricultural products. It is a strategic material, being utilized in the manufacturing of more than 40,000 consumer products including more than 400 medical devices, surgical gloves, and aircraft tires (van Beilen et al., 2007).

Rubber is present in leaves and roots of TKS. However, leaf rubber has a low quality due to the associated resins. In contrary, the root rubber represents a high quality product. This is the reason why only root rubber is being pursued for commercial aims (Whaley, 1948). Until now no irrigation experiments for the evaluation of TKS rubber production have been published.

Another promising, alternative natural rubber source which is already being commercialized is guayule (*Parthenium argentatum* Gray). This species is known to be a semiarid and drought-tolerant shrub, but must be irrigated for maximum sustained production. From 1000 to 1300 mm of water from rainfall and/or irrigation per year is needed to attain maximum production. Most of the literature indicates that decreasing irrigation results in increasing rubber content, but decreases also shrub biomass (Foster and Coffelt, 2005).

Inulin is a natural renewable polysaccharide with a significant number of diverse pharmaceutical and food applications (Barclay, 2010). It is a polydisperse fructan with a degree of polymerization (DP) between 2 and 60 or higher. The fructosyl units are connected by (2 -> 1) linkages with an end glucose residue. The inulin DP depends upon many factors such as plant source, climate and growing conditions, harvesting time and storage conditions (De Leenheer et al., 1994; Coussement, 1999). Even the type of the tissue from which it is extracted has an influence (Van den Ende et al., 2000). The DP determines the type of use of the inulin, either for food or for pharmaceutical purposes. Inulin with low DP can be added to food as a low calorie sweetener, whereas inulin with higher DP can be used as a fiber-type prebiotic with several health promoting effects (Flamm et al., 2001). In plants, inulin-type fructans mainly occur in dicot species belonging to *Asteraceae* (Van Laere and Van den Ende, 2002). Important species are chicory (*Chicorium intybus* L.), artichoke (*Cynara scolymus*), Jerusalem artichoke (*Helianthus tuberosus* L.), dandelion (*Taraxacum officinale*), dahlia (*Dahlia variabilis*), yacon (*Polymnia sonchifolia*) (Van Laere and Van den Ende, 2002; Wilson et al., 2001; Schütz et al, 2006) and Russian dandelion (TKS) (Gorham, 1946; Krotkov, 1948; Mikhlin and Akhumbaeva, 1955). Even though TKS is still in a pre-commercial stage, the use of its inulin has been studied for the production of pharmaceutical preparations and dietary supplements (Schütz et al., 2006), as well as for producing biofuels such as ethanol and butanol. (Buranov, 2010; OARDC, 2014; OMAFRA, 2014; AGMRC, 2014). Also the residual root bagasse and leaves have been proposed for cellulosic biofuel production, helping in this way to promote the commercialization of TKS as a viable natural rubber source (Gorham, 1946; Wade and Swiger, 2011; Bharathidasan, 2013).

In 1948 Krotkov already mentioned that during the first and second year of growth there is a steady increase in the inulin content of TKS roots. It goes from the beginning of the vegetative growth in spring until the middle of the flowering period. Hendry assigned in 1993 temperate climate zones with seasonal drought to fructan-accumulating plants. However, at this moment the relation between fructans and mechanisms for drought and freezing protection was not clear. Later on, in 2001, Wilson et al. directly related fructan metabolism with the exposure of plants to cold temperatures and moisture stress. They also evaluated seasonal changes of fructans in dandelion roots and correlated these changes with seasonal fluctuations in soil temperature and precipitation. They concluded that 50% of fructan variation can be explained by soil temperature and 15% of the variability was attributed to differences in rainfall.

Numerous are the studies on chicory, Jerusalem artichoke and other inulin producers. The objectives were to diminish production costs and rising plant efficiency through the reduction of water supply (De Maestro et al., 2004; Monti et al., 2005; Vandoorne et al., 2012). However, this kind of research is almost inexistent in TKS. In 1947, Whaley and Bowen wrote a compilation of studies on TKS where also some irrigation experiments were mentioned, but no relation with rubber and inulin production was evaluated.

In the present study three irrigation regimes were assayed in a field in Northern Spain during 2010. The effects of water supply on biomass production, inulin and rubber contents and on some other morphological characters were analyzed.

4.2 Materials and Methods

4.2.1 Plant Material

A controlled cross between TKS parent 237-2 (low rubber producer) and parent 194-1 (high rubber producer) was performed by KeyGene (Wageningen, The Netherlands) in 2009 in order to establish a mapping population. This progeny was multiplied in vitro by KeyGene and five plantlets of 68 genotypes were sent to NEIKER Tecnalia. Plantlets were raised in pots with turf in the greenhouse and transplanted after one month to the field in 2010. After flowering, inflorescences were collected from all genotypes, dried at room temperature and seeds were extracted. Seeds were kept in the refrigerator at 4ºC for some months. A random mixture of these collected seeds was used for the irrigation experiment. They were spread in wet paper beds with nutritive medium at 18ºC for one week, until germination occurred. In mid-April 2011 they seedlings were planted in a shaded greenhouse chamber and young TKS plants were raised for planting in the field trial. Seedlings were irrigated manually. No special temperature and light conditions were applied (light range of 270-340 (µmol/m^{2}s); T^a range 15-25ºC).

4.2.2 Field Trial

The irrigation trial was performed in the experimental field of the "Model Farm of Arkaute" (42° 51' 0.29" N -2° 37' 21.59" W; 517 m elevation) belonging to NEIKER Tecnalia, close to Vitoria-Gasteiz (Northern Spain), during the year 2011. The annual accumulated precipitation was 547 (L/m^2), mean T^a was 11.6 °C and average humidity 86.8 %. The texture of the field was classified as Loam (Figure 1).

The trial was set up as a randomized block design with four repetitions. The analysed main factor was irrigation regime with three levels (see below). Elementary plots had a width of 1.5 m and were 3 m in length. The soil didn´t get any extra fertilization. Ridge planting was practiced and a green anti weed MIPED mesh was used. The seedlings raised in the greenhouse were planted beginning of June at a depth of 7-8 cm. They were planted in four rows, nine plants per row, corresponding to a plant density of 40x33 cm^2. After planting, 30 minutes irrigation was applied. In order to avoid moles, an ultrasound mole chaser was used.

Harvesting was done end of October. Four roots from the center of each elementary plot were dragged out manually, washed and full plants were kept in a cool chamber at 4ºC until processing up to four days later.

Figure 1: Irrigation trial in Northern Spain fields.

4.2.3 Irrigation regimes

The dosage calculations for irrigations were based on the performance of an hydrological balance in which hydrological contributions (effective precipitation, supplied irrigation and irrigation system efficiency) and the hydrological usage (evapotranspiration) by the crop were taken into account. The method used for calculations of evapotranspiration was based on the estimation of the crop evapotranspiration (ET_c) depending on the reference evapotranspiration (ET_0), estimated by the FAO 56 Penman-Monteith equation and the adoption of a dual crop coefficient (FAO, 1998). The basal crop coefficient (K_{cb}) of *Beta vulgaris* was taken as reference, due to its close similarity to TKS. The model uses a stress coefficient (K_s) that diminishes the K_{cb} value when the hydrological deficit of available water ranges the value from which the plant starts to suffer hydrological stress. K_e (Evaporative coefficient) represents the evaporative rate from the first centimeters of soil (10-15 cm), in variable humidity conditions from this level. Based on these considerations and the local conditions a reference dosage was calculated for TKS. Three irrigation regimes were applied in the field trial: R0 = no irrigation; R1 = half of the reference dose; R2 = full reference dose. The supplied water dosage was adjusted weekly.

The irrigation system was formed by an irrigation hose with exudation (CT-12 Agro, from Poritex) connected to a pressure regulator (0.7 bar) and a timer. 15 L/m^2*h were dispensed by the system.

4.2.4 Biomass and Morphological data

The aerial part of the plants and the roots were measured separately. Fresh root weight (FRW), root dry weight (RDW), fresh aerial part weight (FLW) and dry aerial part weight (DLW) were computed. For calculating the dry weights, roots and aerial parts were dried during 2 days at 65°C. After cooling in a desiccator, they were weighed and stored in paper envelopes in a dry chamber.

With respect to morphological traits, the number of leaves (LfN), number of rosettes (Rosette N) and maximum leaf length (Max.LL) were recorded. All measures were done in four plants in the four repetitions of the three irrigation regimes.

4.2.5 Root analysis

Roots were milled with a Moulinex AR10031 electric grinder until a fine powder was obtained. The flour was stored at room temperature in 50 ml Falcon tubes.

4.2.5.1 Rubber extraction and quantification

The method of Post et al., (2012) was used for the determination of dry rubber content by gravimetric analysis with some modifications. An aliquot of 200 mg of root powder was weighed in a 10 ml Thermo Scientific Teflon oak FEP tube, mixed with 4ml of toluene and incubated at 70°C for 18 h in a water bath. The toluene supernatant was transferred to a clean tube and vaporized in a Thermoblock. The remaining sample was re-dissolved in 300 µl of toluene for 30 min. Rubber was precipitated by incubating with 600 µl of methanol for 30 min. All steps were performed under a gas extraction hood. After centrifugation at room temperature for 2 min at 16.000 g, the precipitated rubber appeared as a white layer. It was washed twice under vigorous shaking in an Infors HT Ecotron shaking incubator; first with water for 30 min and then with acetone for another 30 min. The rubber samples were vacuum dried in a HETO Speed Vac, Model DNA mini for 3 min at 60°C and cooled down to room temperature before weighing.

Measurements were done in four plants of each repetition of each irrigation regime. Three repetitions per plant were carried out and means for each plant were calculated in order to have more consistent data.

4.2.5.2 Inulin extraction and quantification

For inulin extraction and quantification the D-glucose/D-fructose UV-method of R-Biopharm Kit was used, including the procedure for inulin hydrolysis suggested by the manufacturer.

An aliquot of 500 mg of root flour was weighed in a 50 ml Falcon tube and 15 ml of miliQ boiling water were added. The pH was adjusted to 6.6-8.0 using a Crisson pH-meter Basic 20+ and then filled up to 25ml volume. After vigorous shaking, the sample was heated in a shaking water bath (Selecta Ultrasonic 320 OR) at 80°C, 90 u/min for 15 minutes. Afterwards the sample was filtered using a Serlab vacuum filtering equipment with Whatman filter paper n° 589/2. The extracted liquid was cooled down to room temperature and filled up with water to 25 ml volume.

For hydrolysis an aliquot of 12.5 ml was transferred to a 50 ml Falcon tube, 1.25 ml of HCL 25% were added and left in the shaking water bath at 80°C, 90 u/min for 15 minutes. Then the sample was neutralized with 5 M KOH and brought to 25ml volume with miliQ water.

First the glucose and fructose contents in the sample were measured spectrophotometrically and afterwards the amount of these sugars in the hydrolyzed sample was determined. By subtracting the corresponding measurements, the amount of inulin present in the sample was obtained after converting to inulin contents using a specific formula. For quantification a UV-2401 PC; Shimadzu UV-VIS Recording Spectrophotometer was used, measuring at 340 nm in a quartz cuvette with 1 cm light path.

A single measurement was done in four plants of each repetition of every irrigation regime.

4.2.6 Statistical analysis

Statistical analyses were performed using JMP software version 5.0 (JMP, 2002). A one-way ANOVA, between-subjects design with four replicates was used to analyze the effect of irrigation regime on rubber content, RDW, RFW, LDW, LFW, Leaf N and Max.LL ; ($p < 0.05$) (Table 1).

A root square transformation was applied to LfN, RFW and RDW in order to normalize distributions and a cube root transformation was done for rubber contents data for the same purpose. A Log_{10} transformation was applied to LFW and LDW and a Ln transformation to Max. LL data with the objective of establishing normal distributions. Percentage inulin contents and rosette number data could not be adjusted to a normal distribution. Therefore the nonparametric Wilcoxon/Kruskal-Wallis test was applied for the statistical analyses instead of ANOVA (Table 2). All data were subjected to Levene´s test for homocedasticity in order to identify outliers.

When significant differences were found, Tukey´s HSD multiple comparison test was used for the separation of means (Table 3).

Pearson Correlation analyses were performed between RDW, RFW, LDW, LFW, LfN, Max.LL and rubber percentage, while Spearman correlation analyses were applied for inulin and Rosette N data, since these were not normally distributed.

4.3 Results and Discussion

Data of rubber content leaf and root dry and fresh weight as well as leaf numbers and leaf lengths were analyzed using a one-way ANOVA, between-subjects design. Only a significant effect of rubber contents was detected which explained 13.63 % of the total variation due to irrigation regime (Table 1).

Table 1: Results of the One-way ANOVA analyses for studying the influence of irrigation regime on rubber contents, root FW, Leaf FW, FN, LeafL, LeafN, RootDW, LeafDW.

	Source	DF	SS	MS	F Ratio	R^2	p >
	Watering Group	2	0.5775	0.29	3.5532	0.1363	0.0369
Rubber contents	Within Groups	45	3.6572	0.08			
	Total	47	4.2347		**N=48**		
	Watering Group	2	0.7013	0.3507	0.7760	0.0333	0.4663
RootDW	Within Groups	45	20.333	0.4518			
	Total	47	21.035		**N=48**		**N=48**
	Watering Group	2	0.2980	0.1490	0.5962	0.0258	0.5552
LeafDW	Within Groups	45	11.2468	0.2499			
	Total	47	11.5448		**N=48**		**N=48**
	Watering Group	2	17,52	8,762	0,4116	0.0180	0.6651
Leaf number	Within Groups	45	958,01	21,29			
	Total	47	975,54		**N=48**		**N=48**
	Watering Group	2	0.5542	0.2762	1.3345	0.0560	0.2735
Leaf Length	Within Groups	45	9.3137	0.2070			
	Total	47	9.8661		**N=48**		**N=48**
	Watering Group	2	0.3966	0.1983	0.7573	0.0326	0.4748
LeafFW	Within Groups	45	11.7830	0.2619			
	Total	47	12.1800		**N=48**		**N=48**
	Watering Group	2	2.6329	1.3164	0.7295	0.0314	0.4878
Root FW	Within Groups	45	81.2064	1.8046			
	Total	47	83.8392		**N=48**		**N=48**

Tukey´s HSD multiple tests revealed that with R0 and R1 irrigation regimes a significantly higher amount of rubber percentage could be obtained than with R2 (Table 3). With respect to the non significant effects, the total variances explained by irrigation regimes for all other traits in Table 1 were very small not exceeding 5.6%.

Table 3: Comparison of means of total Rubber Contents using a Tukey-Kramer HSD test

Irrigation Regime			Mean value
R1	A		1,4928049
R0	A	B	1,4375338
R2		B	1,2374552

Levels not connected by same letter are significantly different

Also the non parametric Wilcoxon/ Kruskal-Wallis test for the non normally distributed characters inulin contents and rosette numbers did not show statistically significant differences between irrigation regimes (Table 2).

Table 2: Results of the non parametric Wilcoxon/ Kruskal-Wallis (Rank Sums) test for Inulin contents and rosette numbers under different irrigation regimes

	ChiSq [a]	DF	Prob > ChiSq
Inulin contents	0.6082	2	0.7378
Rosette Numbers	0.0668	2	0.9672

[a] One-Way test Chi Sqare approximation.

Table 4 displays the evaluated parameter means per treatment. Even though no statistical significant differences could be established except for rubber contents, a trend is visible that compared to no irrigation the rubber percentage, inulin contents, and root and leaf biomass production increases for the treatment with half reference dosage (R1) and decreases again for the full dosage.

Table 4: Parameter Means per treatment in the irrigation trial.

	R_0	R_1	R_2
Inulin (% in RDW)	30,58	32,54	31,25
Rubber (% in RDW)	3,51	3,56	2,14
RDW	3,21	4,40	3,82
Leaf DW	1,57	2,43	1,76
Leaf N	113,81	110,13	80,00
Leaf Length	6,81	8,09	10,50
Rosette N	2,38	2,13	2,38
Root FW	12,15	16,84	14,94
Leaf FW	8,77	16,46	11,98

[a] Mean values for % Inulin and rubber contents, RootDW, LeafDW,LeafN, Max Leaf Length, Rosette N, RootFW, Leaf FW.

[b] R0= no irrigation, R1= half of the reference dose; R2 = full reference dose.

However, according to the statistics under local weather conditions an additional water supply for optimum TKS development seems not to be necessary. So, apparently the normal precipitation was sufficient. Moreover, it has to be considered that TKS cultivation was done under an anti weed mesh which conserves to a certain degree soil moisture and reduces evaporation.

In previous studies a certain degree of drought tolerance was demonstrated for the alpine *Taraxacum* species *T. ceratophorum* (Brock and Galen, 2005). In fact, no real drought stress could be evaluated in our study and it would be convenient to repeat the trial at a different location with dryer climatic conditions.

With respect to the analyzed correlations (Table 5) it is worth to mention the strong positive correlation between leaf weights and root weights for both, fresh and dry weights. This finding could be exploited for indirect, visual selection of plants with large aerial biomass production to obtain also plants with big roots for TKS breeding purposes.

Table 5: Observed correlations between the evaluated parameters in the irrigation trial

	Inulin C.	Rubber C.	RDW	LDW	LfN	MaxLL	LfFW	RFW	RosetteN
Inulin C.		***-0.4327***							
Rubber C.	*-0.4327*			0.3925					
RDW				0.8583	0.6722	0.4237	0.8630	0.9948	***0.5662***
LDW		0.3925	0.8583		0.7480	0.4263	0.9465	0.8389	***0.6352***
LfN			0.6722	0.7480		0.3513	0.8025	0.6662	***0.7808***
MaxLL			0.4237	0.4263	0.3513		0.5363	0.4179	***0.3630***
LfFW			0.8630	0.9465	0.8025	0.5363		0.8495	***0.6681***
RFW			0.9948	0.8389	0.6662	0.4179	0.8495		***0.5765***
RosetteN			***0.5662***	***0.6352***	***0.7808***	***0.3630***	***0.6681***	***0.5765***	

[a] Correlations between Inulin and rubber percentages, Root dry weight (RDW), Leaf dry weight (LDW), Max. leaf length (MaxLL), Leaf fresh weight (LfFW), Root fresh weight (RFW) and Rosette number (Rosette N).

[b] Numbers in roman, bold refer to the non-parametric Spearman´s Rho correlation; while the other values refer to Pearson´s correlations. Empty spaces (except diagonal) indicate non significant correlations.

On the other hand the observed weak to moderate negative correlation between rubber and inulin percentages (-0.4327; Table 5) complicates the breeding for both traits at a time. It might require a differentiation of potential cultivars, depending on the intended utilization, either for inulin or for rubber production.

4.4 Conclusions

1. In our field trial only a significant influence of the irrigation regime on percentage of TKS rubber contents was observed, which explained 13.65% of the total variance under the assayed conditions.
2. No significant effects of irrigation regimes on inulin contents, biomass production and other morphological traits were detected.

3. Additional water supply for optimal growth seems not to be necessary for TKS optimum development under local weather conditions.
4. Since real drought stress could not be evaluated in our trial, further studies at dryer locations should be performed.
5. A strong positive correlation exists between root and leaf dry and fresh weights and a weak to moderate negative correlation between percentages of rubber and inulin contents.

6. GENERAL DISCUSSION

Source: blog.confortauto.com

6. GENERAL DISCUSSION:

When facing the culture of a wild species an exercise for improving the crop conditions and a breeding program is needed in order to get the optimum production index. Even when TKS crop was already studied in Russia and in the USA (Gorham, 1946; Krotkov, 1945; Krotkov, 1948; Whaley and Bowen, 1947; Whaley, 1948), a deep study of the special weather and soil conditions of Europe is needed if we aim to establish an alternative rubber source available to be grown within our continent. Taking those old studies as a starting point we commenced from the very beginning: the plant material. As Kirschner *et al*. wrote in 2012, “all available *ex situ* germplasm collections of TKS belonged to a single triploid apomictic clone that was misidentified as *T. koksaghyz*.” Harvesting of new and “true” TKS seeds was a need and finding the best agronomic practices for those plants and settling the genetic base for a breeding programme was a challenge for us. We started evaluating nine of the largest collected populations. They were always evaluated with the aim of obtaining the maximum rubber, inulin and biomass production. Together with the population variable we included in our trials the planting date and the harvesting date, but the last one couldn´t be taken into account due to a lack of plant material.

We found that depending on the purpose of utilization of the crop (rubber, inulin) either one or another population and planting date should be selected. We also found a large degree of variation between single plants of the same population. This variation is due to the fact that TKS is an out crossing species (Warmke, 1943). It suggests that TKS breeding should be based on several rounds of selection of single plants with desirable characteristics, taking into account our findings about populations. Crossings between them with improved and combined characteristics should end with the improvement and homogenization of the species in terms of production.

The large intra population variation forced us to exclude population 20 (_006 in Krischner *et al,* 2012) from the statistical analyses of rubber content, but it should be taken into account for future mass selection since visual analyses and repeated measurements of single plants pointed it out as a promising population. It is also promising for inulin production, since it produced the highest potential inulin yield when planted in May.

Population 30 (217, original name) was the best rubber producer and population 38

(246, original name) was the best inulin producer and they should also be selected for mass selection practices.

Even when the rubber contents reported in our populations where quite small (0.4-5%) we should be aware that these are preliminary studies with wild material and we hope that taking our studies into account , the mentioned percentages of rubber content by Buranov in 2010 (Buranov, 2010)- raising 24%- could be even overtaken. In respect to inulin content we got very close percentages to those reported by other authors (Gorham (1946) and van Beilen and Poirier (2007b)). Our 36% maximum amount is not far from the 40% or even 42% reported. But we remember again that these are preliminary studies.

The variable “harvesting date” still remains unexplored and should be essayed in future studies. We also missed plant density trial, but recent studies in the close relative *Taraxacum brevicorniculatum* (Munt *et al.*, 2011) pointed sawing seeds in rows 2-5 cm apart in order to obtain full developed plants with 40-50 cm main roots without branching. This practice should be tested in TKS and if suitable, it would make harvesting easier and may increase rubber, inulin and biomass yield.

Another very important aspect that should be studied when starting a new crop is water requirements. It depends very much on the water availability in each kind of soil and on the weather conditions. We wanted to test Northern Spanish soils as a possible growing location. The area is quite humid and TKS seemed to have enough water availability under local weather conditions for an optimum development. Looking to the parameters that we are interested in, only a significant effect of rubber contents was detected explaining 13.63 % of the total variation due to the irrigation regime. But the higher yields of rubber were obtained when no irrigation or just half of the watering dosage was applied. We could affirm that TKS could be grown in our field trials without water supply for an optimum rubber yield (it has to be considered that TKS cultivation was done under an anti weed mesh which conserves in certain degree soil moisture and reduces evaporation).

We wanted to check if the drought tolerance demonstrated for the alpine *Taraxacum* species *T. ceratophorum* (Brock and Galen, 2005) was also present in TKS but, no real drought stress could be evaluated in our study and it would be convenient to repeat the trial at a different location with dryer climatic conditions.

Irrigation effect was non significant for all other traits and the total variances were explained by the irrigation regimes which didn´t exeed 5.6%.

Furthermore we made a correlation study between all analyzed traits and got a very interesting positive correlation between leaf weight and root weight. This could be a very helpful finding that could be exploited for indirect, visual selection of plants with large aerial biomass production to obtain plants with big roots for TKS breeding purposes.

Other important aspect to study in good agronomic practices is the fertilization requirements. It still needs to be studied, but it was done by Munt *et al*. in 2011 for the close relative *T. brevicorniculatum*, what could be taken as a guide.

After agronomic aspects were more or less covered, a genetic study was needed urgently as a base for future breeding programs. No genetic linkage map was available for this species at the moment. We constructed an integrated, high density map of TKS containing over 500 markers on the eight linkage groups. Once the map was constructed we intended to do a comparative study with lettuce that is another species of the same family. Synteny studies are very interesting considering that they can help us to get new sources of heterologous probes for saturating regions including genes of interest (Kumar, 1999) and can help predicting the position of orthologous genes of agronomic interest in related species (Simon and Muehlbauer, 1997). Even when our synteny study was just an approach, we could potentially associate some homologous linkage groups between TKS and lettuce maps, but many additional markers should be evaluated in order to improve the alignment and see how they evolved. Testing COS markers developed by Fulton *et al.* in 2002 in our mapping population could be also interesting, since, if they are suitable for TKS, they could be used for comparative genomics with other higher plants of interest.

Once the genetic map is constructed the next step would be the establishment of a functional reference map of the TKS genome. It could be done in the future integrating QTLs for quantitative characters. Traits such as rubber and inulin content, biomass production, growth, etc. should be measured in the mapping population and QTL analyses should be carried out.

We should also include markers for candidate genes from interesting biosynthetic pathways as rubber and inulin formation. If some of the mapped QTL is co-located with a marker it might explain the potential influence of the underlying candidate gene.

The functionality of the map could also be increased if we use markers with underlying sequences; i.e. EST-SSRs. The sequences can be blasted against plant gene databases, sequenced markers of other maps and sequence scaffolds from other species. This search for homologies can detect interesting functions or molecules that could be used for explaining potential candidate genes, if they are co-located with QTLs in future studies.

When we blasted our sequences against the mentioned databases we got some interesting homologies. Three markers were related to stress responses, what we found remarkable, since sometimes rubber seems to be triggered by some kind of stress suffered by the plant. Particularly one of the sequences matched with a protein that was also found in *H. brasiliensis*. Since both of them produce rubber, it is highly possible that there is a potential candidate gene in the surroundings.

Another marker could be related to rubber biosynthetic pathway and rubber traits, since it seems to be involved in an isoprenoid biosynthetic process.

The ROP genes are genes primarily expressed in laticifers of the rubber tree (Quin *et al*., 2014). We obtained a blast with a ROP5 gene of *H. brasiliensis* and we suspect that it could have a similar function in TKS.

Regarding the potential candidate genes related to inulin we got four markers with sequences related to carbohydrate metabolic processes.

Apart of doing the QTL analysis and trying to co-locate them with our markers in order to look for candidate genes, a deeper study of the bibliography related to the rubber elongation factor (REF) associated to rubber particles in *H. brasiliensis* (Dennis et al., 1989), the prenyltransferase that elongates cis-polyisoprene rubber from the rubber tree (Light and Dennis, 1989) and rubber biosynthetic genes from TKS (Schmidt et al., 2010) is needed with the aim of developing molecular markers related to rubber formation in TKS. If they are found to be polymorphic they could be mapped in our mapping population and try to be co-located with QTLs. If so, their influence on rubber traits could be validated.

7. FUTURE WORK

7. FUTURE WORK

1.-Regarding agronomy, studies of harvesting date, fertilization and plant density should be developed. All them could considerably increase the amount of desirable traits.

2.-Drought studies should be carried out in order to check whether TKS could tolerate some water scarcity or not.

3. - A deep QTL study should be carried out;

4. - A search for new markers related to rubber pathways and molecules related to rubber formation should be accomplished.

5. - To check if the new markers are polymorphic and, if so, map them in our mapping population.

6. - Co-locate markers and QTLs in order to look for potential candidate genes.

8. BIBLIOGRAPHY

8. BIBLIOGRAFY

Afzal M (1998) An economic analysis of joint production between latex and wood among rubber smallholders in Melaka. Doctoral dissertation, Universiti Putra Malaysia.

AGMRC (2014)

http://www.agmrc.org/commodities__products/specialty_crops/russian-dandelion/

Alonso-Blanco C, Peeters AJM, Koornneef M, Lister C, Dean C, van den Bosch N, Pot J and Kuiper MTR (1998) Development of an AFLP based linkage map of Ler, Col and Cvi *Arabidopsis thaliana* ecotypes and construction of a Ler/Cvi recombinant inbred line population. Plant J 14(2):259-71.

Automotive World (2015) http://www.automotiveworld.com/news-releases/continental-trials-first-car-tires-taraxagum/

Backhaus RA, Walsh S (1983) The ontogeny of rubber formation in guayule, *Parthenium argentatum* Gray. Bot Gaz 144(3):391-400.

Balsiger J, Bahdon J and Whiteman A (2000) The utilization, processing and demand for rubberwood as a source of wood supply. Asia-Pacific forestry sector outlook study, Working paper series, Working paper nº: APFSOS/WP/50, FAO.

Barclay T, Ginic-Markovic M, Cooper P and Petrovsky N (2010) Inulin- a versatile polysaccharide with multiple pharmaceutical and food chemical uses. J. Excipients and Food Chem. 1 (3), 27-49.

Bharathidasan AK (2013) Production of biobutanol from inulin-rich biomass and industrial food processing wastes, Thesis, The Ohio State University, 309 pp.

Bradley S, Bushman A, Scholte A, Cornish K, Scott DJ, Brichta JL, Vederas JC, Ochoa O, Michelmore RW, Shintani DK, Knapp SJ (2006) Identification and Comparison of natural rubber from two Lactuca species.Phytochem 67: 2590-2596.

Britannica online encyclopedia (2015a) http://global.britannica.com/topic/synteny

Brock M and Galen C (2005) Drought tolerance in the alpine dandelion, Taraxacum ceratophorum (Asteraceae), its exotic congener T. officinale, and interespecific hybrids under natural and experimental conditions. Am. J. Bot. 98(2):1311-1321.

Buranov AU (2010) Russian Dandelion Seeds and Extraction processes, in October 14-15, 2010 meeting: The future of natural rubber. Montpellier, France.

Bushman BS, Scholte AA, Cornish K, Scott DJ, Brichta JL, Vederas JC, Ochoa O, Michelmore RW, Shintani DK and Knapp SJ (2006) Identification and comparison of natural rubber from two Lactuca species. Phytochem (67): 2590–2596.

Cadalen T, Mörchen M, Blassiau C, Clabaut A, Scheer I, Hilbert J-L, Hendriks T, Quillet M-C (2010) Development of SSR markers and construction of a consensus genetic map for chicory (*Cichorium intybus* L.). *Mol Breed* 25(4):699–722.

Carpita NC, Kanabus J and Hously TL (1989) Linkage structure of fructans and fructan oligomers from Triticum aestivum and Festuca arundinacea leaves. J Plant Physiol 134: 162-168. Carraher ChE (2003) Giant Molecules Essential Materials for Everyday Living and Problem Solving, Whiley J and Sons, Inc., Online ISBN: 9780471457190, 483 pp.

Chapman MA, Chang JC, Weisman D, Kesseli RV, Burke JM (2007) Universal markers for comparative mapping and phylogenetic analysis in the Asteraceae (Compositae). Theor Appl Genet 115:747-755.

Ciesielski A (2000) An introduction to rubber technology, Rapra Technology Limited, 183 pp, ISBN: 1-85957-150-6.

CIRAD (2015) www.cirad.fr

Clemson University (2010)

http://www.genome.clemson.edu/resources/online_tools/ssr).

Collard BCY, Jahufer MZZ, Brouwer JB and Pang ECK (2005) An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts. Euphytica 142:169-196. DOI: 10.1007/s190681-005-1681-5.

Compositae Genome Project Data Base (CGPDB) (2010)

http://compgenomics.ucdavis.edu/compositae_index.php

Conesa A, Götz S, García-Gómez JM, Terol J,Talón, Mand Robles M (2005) Blast2go:

a universal tool for annotation, visualization and analysis in functional genomics research.

Bioinformatics 21(18):3674-3676.

Cook OF (1941) Naming the cultivated rubber tree *Siphonia ridleyana*. J Wash Acad Sci 31: 46–65.

Cornish K (1996) Hypoallergenic natural rubber products from *Parthenium argentatum* (Gray) and other nonhHevea brasiliensis species.*United States Patent*. Patent number: 5.580.942; Dec. 3.

Cornish K, Siler DJ, Groesjean O and Goodman N (1993) Fundamental Similarities in Rubber Particle Architecture and Function in Three Evolutionarily Divergent Plant Species. J. nat. Rubb. Res , 8(4): 275-285.

Cornish K, Xie W, Shintani DK, Hamilton RG (2005) Absence of cross-reactivity between natural rubber latex from production lots of *Hevea brasiliensis* (Hev-b) and *Parthenium argentatum* (Guayule). in Proceedings of the Association for the Advancement of Industrial Crops, Murcia, Spain, 17-21 September pp 565-574.

Coussement P (1999) Inulin and oligofructose as dietary fiber: analytical, nutrition and legal aspects, in: Cho SS, Prosky L and Dreher M (Eds.), Complex Carbohydrates in Foods, New York, pp: 203-212.

Cubero JI (2003) Introducción a la mejora genética vegetal. Madrid, Spain, 567 pp.

De Leenheer L and Hoebregs H (1994) Progress in the elucidation of the composition of chicory inulin. Starch 46:193-196.

De Maestro G, Manolio G and Marzi V (2004) Jerusalem artichoke (*Helianthus tuberosus* L.) and chicory (*Cichorium intybus* L.): Potential crops for inulin production in the Mediterranean area. Acta Hortic. 629:365-374

DOI: 10.17660/ActaHortic.2004.629.47.

Dennis MS, Henzelg WJ, Bell J, Kohr W, and Light DR (1989) Amino Acid Sequence of Rubber Elongation Factor Protein Associated with Rubber Particles in Hevea Latex. J Biol Chem 264 (31): 18618-18626.

Doyle JJ and Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15.

Durand J, Bodenés C, Chanceler E, Frigerio JM, Vendramin G, Sebastiani F, Buonamici A, Gailing O, Koelewijn HO, Villani F, Mattioni C, Cherubini M, Goicoechea PG, Herrán A, Ikaran Z, Cabané C, Ueno S, Alberto F, Dumoulin PY, Guichoux E, de Daruvar A., Kremer A and Plomion C (2010) A fast and cost-effective approach to develop and map EST-SSR markers: oak as a case of study. Genomics 11: 570.

EU-PEARLS (2015)

http://www.wageningenur.nl/en/Research-Results/Projects-and-programmes/eu-pearls-projects.htm

Ezeji TC, Liu S and Qureshi N (2014) Mixed sugar fermentation by Clostridia and metabolic engineering for butanol production. In: Qureshi N, Hodge D and Vertes A (eds.) Biorefineries: Integrated Biochemical Processes for Liquid Biofuels, Elsevier BV, pp:191-204.

FAO (1998) http://www.fao.org/docrep/x0490e/x0490e06.htm

Fisher PJ, Gardner RC and Richardson TE (1996) Single locus microsatellites isolated using 5´anchored PCR. Nucleic Acids Res 24: 4369-4371.

Flamm G, Glinsmann W, Kritchevsky D, Prosky L and Roberfroid M (2001) Inulin and oligofructose as dietary fiber: a review of the evidence. Crit Rev **Food** Sci Nutr 41: 353-362.

Foster MA and Coffelt TA (2005) Guayule agronomics: establishment, irrigated production, and weed control. Ind Crops Prod 22: 27-40.

Franck A and De Leenheer L (2005) Inulin, Biopolymers online 6: 439-448.

DOI: 10.1002/3527600035.bpol6014

Fulton TM, van der Hoeven R, Eannetta NR, Tanksley SD (2002) Identification, analysis and utilization of conserved ortholog set markers for comparative genomics in higher plants. Plant Cell 14: 1457-1467.

Gebhardt C, Walkemeier B, Henselewski H, Barakat A, Delseny M and Stüber K. (2003) Comparative mapping between potato (Solanum tuberosum) and Arabidopsis thaliana reveals structurally conserved domains and ancient duplications in the potato genome. Plant J. 34(4):529-41.

Gorham PR (1946) Investigations on rubber-bearing plants: II. Carbohydrates in the roots of *Taraxacum koksaghyz* Rod. Can. J. Res., 24C: 47-53.

Green EM (2011) Fermentative production of butanol- the industrial perspective. Curr Opin Biotechnol 22: 1-7.

Gupta PK, Rustgi S, Sharma S, Shing R, Kumar N and Balyan HS (2003) Transferable EST-SSR markers for the study of polymorphism and genetic diversity in bread wheat. Mol Genet Genomics 270: 315-323.

Hamada HM, Petrino MG, Kakunaga T (1982) A novel repeated element with Z-DNA forming potential is widely found in evolutionarily diverse eukaryotic genomes. Proc Natl Acad Sci USA 79:6465-6469.

Hendry G (1993) Evolutionary origins and natural functions of fructas. A climatological, biogeographic and mechanistic appraisal. New Phytol 123: 3-14.

Hosler D, Burkett SL and Tarkanian MJ (1999) Prehistoric Polymers: Rubber Processing in Ancient Mesoamerica. Science 284(5422): 1988-1991.

DOI: 10.1126/science.284.5422.1988

IRSG (2015a) Rubber Statistical Bulletin 69: 10-12 April-June.

http://www.rubberstudy.com/statistics.aspx

IRSG (2015b)

http://www.rubberstudy.com/synthetic.aspx

Jang Y, Lee J, Malaviya A, Seung DY, Cho JH and Lee SY (2011) Butanol production from renewable biomass: Rediscovery of metabolic pathways and metabolic engineering. Biotechnol J 7: 186-198.

JMP®, Version 5. SAS Institute Inc. , Cary, NC, 2002.

Joshi SP, Ranjekar PK and Gupta VS (1999) Molecular markers in plant genome analysis. Curr Sci. 1999;77:230–240.

Killmann W and Hong LT (2000) Rubberwood- the success of an agricultural by-product. Unasylva 201, 51: 66-72.

Kirschner J, Stepanek J, Cerny T, De Heer P and van Dijk P (2012) Available ex situ germplasm of potential rubber crop *Taraxacum koksaghyz* belongs to a poor rubber producer, *T. brevicorniculatum* (Compositae-Crepidinae). Genet Resour Crop Evol, Published online 19 May 2012, DOI: 10.1007/s 10722-012-9848-0.

Krotkov G (1945) A review of literature on *Taraxacum kok-saghyz* Rod.. The Bot.l Review 11(8): 417-461.

Krotkov G (1948) Changes in the carbohydrate metabolism of *Taraxacum kok-saghyz* Rod. during the first and second years of growth. Plant Physiol. 25(1): 169–180.

Kumar LS (1999) DNA markers in plant improvement: An overview. Biotechnol Adv 17: 143-182.

Levene H (1960) Robust tests for equality of variances. In: Olkin I (Ed.), Contributions to Probability and Statistics. Palo Alto, California, Stanford University Press, pp. 278–292.

LI-COR (1997) 4200 automatic DNA sequencer MWG Biotech, Ebersberg, Germany.

Light DR and Dennis MS (1989) Purification of a prenyltransferase that elongates cis-polyisoprene rubber from the latex of Hevea brasiliensis. J Biol Chem 264 (31): 18589-18597.

McCouch SR (2001) Genomics and Synteny. Plant Physiol 125: 152–155.

Menendez CM, Ritter E, Schäfer-Pregl R, Walkemeier B, Kalde A, Salamini F, Gebhardt C (2002) Cold-sweetening in diploid potato. Mapping QTL and candidate genes. Genetics 162:1423–1434

Menz MA, Klein RR, Mullet JE, Obert JA, Unruh NC and Klein PE (2002) A high-density genetic map of Sorghum bicolor (L.) Moench based on 2926 AFLP, RFLP and SSR markers. Plant Mol Biol 48(5-6):483-99.

Mikhlin DM and Akhumbaeva BO (1955) Fructosans of kok-saghyz roots. Biokhimii͡a 21(2): 186-90.

Mohan M, Nair S, Bhagwat A, Krishna TG, Masahiro Y, Bhatia CR and Sasaki T (1997) Genome mapping, molecular markers and marker-assisted selection in crop plants. Mol Breed 3: 87-103.

Monti E, Amaducci MT, Pritoni G and Venturi G (2005) Growth, fructan yield, and quality of chicory (*Chicorium intybus* L.) as related to photosynthetic capacity, harvest time and water regime. J Exp Bot 56(415): 1389-1395.

Mooibroek H and Cornish K (2000) Alternative sources of natural rubber. Appl Microbiol Biotechnol 53: 355-365.

Munt O, Arias M, Hernandez M, Ritter E, Chulze Gronover Ch, Prüfer D (2011) Fertilizer and planting strategies to increase biomass and improve root morphology in the natural rubber producer *Taraxacum brevicorniculatum*. Ind Crops Prod 36: 289-293.

Nair KPP (2010) The agronomy and economy of important tree crops of the developing world. pp. xiv + 351 pp, ISBN 978-0-12-384677-8.

Neagu C and Bahrim G (2012) Fuel ethanol bioproduction from inulin rich feedstock. Innov Rom Food Biotechnol 11: 1-8.

Nhm (2015) http://www.nhm.ac.uk.

Nuez F, Carrillo JM and de Ron AM (2003) Introduction. In: Nuez F and Carrillo JM (Eds.) Los Marcadores Genéticos en la Mejora Vegetal. Editorial de la Universidad Politécnica de Valencia, Valencia, Spain. pp: 1-22.

NMCE (National Multy Commodity Exchange) (2013) Report 2012-2013.

http://www.nmce.com/

OARDC (2014) http://www.oardc.ohio-state.edu/images/E_Rubber.pdf

Okoma KM, Dian K, Obouayeba S, Angeline A, Elabo E, N´guetta ASP (2011) Seasonal variation of tapping panel dryness expression in rubber tree Hevea brasiliensis muell. arg. in Cote d'ivoire. Agric Biol J N Am 2(3): 559-569.

OMAFRA (2014)

http://www.omafra.gov.on.ca/CropOp/en/indus_misc/chemical/russd.html

Post J, Van Deenen N, Fricke J, Kowalski N, Wurbs D, Schaller H, Eisenreich W (2012) Laticifer-specific cis-prenyltransferase silencing affects the rubber, triterpene, and inulin content of *Taraxacum brevicorniculatum*. Plant Physiol 158(3): 1406–1417. doi:10.1104/pp.111.187880

Powell W, Machray GC and Provan J (1996) Polymorphism revealed by simple sequence repeats. Trends Plant Sci 1(7): 215-222.

Pulido-Sierra SI, Silva-Calvao ME, Ferreira-Neto JA and Rojo-Alboreca A (2012) Análisis del caucho natural en el Mundo. Spanish J Rural Dev 3(Special 3): 57-64.

Puskas JE, Gautriaud E, Deffieux A, Kennedy JP (2006) Natural rubber biosynthesis- A living carbocationic polymerization. Prog Polym Sci 31: 533-548.

Qin Y, Huang Y, Fang Y, Qi Jand Tang C (2014) Molecular characterization and expression analysis of the small GTPase ROP members expressed in laticifers of the rubber tree (*Hevea brasiliensis*). Plant Physiol Biochem 74:193-204.

Richter BE, Jones BA, Ezzell JL, Porter NL, Avdalovic N and Pohl C (1996) Accelerated Solvent Extraction: A Technique for Sample Preparation. Anal Chem 68 (6): 1033–1039. DOI: 10.1021/ac9508199.

Ritter E, Gebhardt C, Salamini F (1990) Estimation of recombination frequencies and construction of RFLP linkage maps in plants from crosses between heterozygous parents. Genetics 224:645-654.

Ritter E, Lucca F, Sánchez I, Ruiz de Galarreta JI *et al*. (2005) Genomic resources in potato and possibilities for exploitation. In: Haverkort AJ, Struik PC (eds) Potato in progress; science meets practice, Wageningen Academic Publishers, The Netherlands, pp 55-65.

Ritter E and Salamini F (1996) The calculation of recombination frequencies in crosses of allogamous plant species with application to linkage mapping. Genet Res 67: 55-65.

Rose K, Steinbüchel A (2005) Biodegradation of natural rubber and related compounds: recent insights into a

hardly understood catabolic capability of microorganisms. Appl Environ Microbiol 71:2803–2812.

Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds.) Methods in molecular biology, Totowa (NJ), Humana Press. pp 365–386.

Rubber Board (2002) http://rubberboard.org.in/ManageCultivation.asp?Id=147

Rubber News (2012)

http://www.rubbernews.com/article/20120712/NEWS/307129998/apollo-vredestein-uses-dandelions-guayule-in-tires

Rudd S (2003) Expressed sequence tags: alternative or complement to whole genome sequences? Trends Plant Sci 8(7): 321-329.

Schmidt T, Hillebrand A, Wurbs D, Wahler D, Lenders M, Gronover CS and Prüfer D (2010) Molecular Cloning and Characterization of Rubber Biosynthetic Genes from Taraxacum koksaghyz. Plant Mol Biol Rep 28:277–284. DOI 10.1007/s11105-009-0145-9

Schütz K, Muks E, Carle R and Schieber A (2006) Separation and quantification of inulin in selected artichoke (*Cynara scolymus* L.) cultivars and dandelion (*Taraxacum officinale* WEB. Ex WIGG.) roots by high performance anion exchange chromatography with pulsed amperometric detection. Biomed Chromatogr 20: 1295-1303.

Sefe KM, Regne F, Turetschek E, Glössl J, Steinkellner H (1999) Identification of microsatellites sequences in *Vitis riparia* and their applicability for genotyping of different *Vitis* species. Genome 42:1-7.

Sell AM and Laguila-Visentainer JE (2013) Natural rubber latex allergy. In: Pereira C (Ed.) Allergic Diseases- Highlights in the Clinic, Mechanismis and Treatments. Pp: 289-310.

Simko I (2009) Development of EST-SSR markers for the study of population structure in lettuce (*Lactuca sativa* L.). J Hered 100: 256-262.

Simon CJ and Muehlbauer FJ (1997) Construction of a chickpea linkage map and its comparison with maps of pea and lentil. J Hered 88: 115-119.

Société Internationale de Plantations d´Hévéas (2015) http://siph.com/

Soh AC (2011) Genomics and plant breeding. J oil palm res 23: 1019-1028.

Stoffel K, van Leeuwen K, Kozik A, Caldwell D[†], Ashrafi H, Cui X, Tan X, Hill T, Reyes-Chin-Wo S, Truco MJ, Michelmore RW and Van Deynze A (2012) Development and application of a 6.5 million feature AffymetrixGenechip® for massively parallel discovery of single position polymorphisms in lettuce (*Lactuca spp.*). BMC Genomics 13:185

Tanaka Y, Sakdapipanich JT (2005) Chemical Structure and Occurrence of Natural Polyisoprene. Biopolymers Online, DOI: 10.1002/3527600035.bpol2001

Tang S, Kishore VK, Knapp SJ (2003) PCR-multiplexes for a genome-wide framework of simple sequence repeat marker loci in cultivated sunflower. Theor Appl Genet 107:6-19.

Tashiro, Y and Sonomoto K (2010) Advances in butanol product ion from *Clostridia.* In: Méndez-Vilas A (Ed.) Current research, technology and education topics in applied microbiology and microbial biotechnology pp: 1383-1394.

Vijayaram TR (2009) A Technical Review of Natural Rubber. Int J Desa Manuf Technol 3(1): 25-37.

Truco MJ, Antonise R, Lavelle D, Ochoa O, Kozik A, Witsenboer H, Fort SB, Jeuken MJ, Kesseli RV, Lindhout P, Michelmore RW, Peleman J (2007) A high-density, integrated genetic linkage map of lettuce (Lactuca spp.). Theor Appl Genet 115(6):735-46.

Truco,M J, Ashrafi H, Kozik A, van Leeuwen H, Bowers J, Wo SRC, Michelmore RW (2013) An Ultra-High-Density, Transcript-Based, Genetic Map of Lettuce. G3: Genes Genomes Genetics 3(4): 617–631. http://doi.org/10.1534/g3.112.004929

Ullán de la Rosa FJ (2004) La era del caucho en el Amazonas (1870-1920): modelos de explotación y relaciones sociales de producción. An Mus Am 12: 183-204

Umar HY, Giroh DY, Agbonkpolor NB and Mesike CS (2011) An overview of world natural rubber production and consumption. J Hum Ecol 33(1): 53-59.

UNCTAD (2015)

http://www.unctad.info/en/Infocomm/Agricultural_Products/Caoutchouc/Characteristics/-Properties-of-NR/

UniProt (2015)

http://www.uniprot.org/uniprot/?query=pyridoxal+%225+phosphate%22+synthase+subunit+pdx+1+hevea&sort=score

van Beilen J and Poirier Y (2007a) Establishment of new crops for the production of natural rubber. **Trends** Biotechnol 25(11): 522–529.

DOI: http://dx.doi.org/10.1016/j.tibtech.2007.08.009.

van Beilen J and Poirier Y (2007b) Guayule and Russian Dandelion as Alternative Sources of Natural Rubber. Crit Rev Biotechnol 27(4): 217-231.

van Beilen J and Poirier Y (2008) Production of renewable polymers from crop plants. Plant J 54: 684-701.

van den Ende W, Michiels A, Van Wonterghem D, Vergauwen R and Van Laere A (2000) Cloning, developmental, and tissue-specific expression of Sucrose:Sucrose 1-Fructosyl Transferase from Taraxacum officinale. Fructan localization in roots. Plant Phys 123: 71-19.

van Dijk P, de Jong H, Vijverberg K, Biere A (2009) An apomixis-gene's view on dandelions. In: Schön I, Martens K, Van Dijk P (eds), Lost sex. The evolutionary biology of parthenogenesis. Springer, Dordrecht, Heidelberg, London, New York, pp. 474– 493.

DOI 10.1007/978-90-481-2770-2_22.

van Dijk PJ, Kirschner J, Štěpánek J, Baitulin IO, Černý T (2010) *Taraxacum koksaghyz* Rodin definitely is not an example of overcollecting in the past. A reply to S. Volis *et al.* (2009). J. Appl. Bot. Food Qual 83: 217–219.

van Laere A and Van den Ende W (2002) Inulin metabolism in dicots: chicory as a model system. Plant Cell Environ 25: 803-813.

Vandoorne B, Mathieu AS, Van den Ende W, Vergauwen R, Perilleux C, Jacaux M and Lutts S (2012) Water stress drastically reduces root growth and inulin yield in *Cichorium intybus* (var. *sativum*) independently of photosybthesis. J Exp Bot 63(12): 4359-4373.

Varshney RK, Graner A, Sorrells ME (2005) Genic microsatellite markers in plants: features and applications. Trends Biotechnol 23(1):48–55.

doi: 10.1016/j.tibtech.2004.11.005.

Venkatachalam P, Geetha N, Sangeetha P and Thulaseedharan A (2013) Natural rubber producing plants: An overview. Afr J Biotechnol 12(12)1297-1310.

Verheye W (2010) Growth and Production of Rubber. In: Verheye W (ed.), Land Use, Land Cover and Soil Sciences. Encyclopedia of Life Support Systems (EOLSS), UNESCO-EOLSS Publishers, Oxford, UK. http://www.eolss.net

Vos P, Hogers R, Bleeker M, Reijans M, Van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kupier M and Zabeau M (1995) AFLP: A new technique for DNA fingerprinting. Nucl Acids Res 23: 4407–4414.

Vuylsteke M, Peleman JD and van Eijk MJT (2007) AFLP technology for DNA fingerprinting. Nat Protoc 2: 1387–1398.

Wade J and Swiger D (2011) Patent Application Publication "Dandelion processes, compositions and products". Pub. Nº US 2011/0275142 A1.

Warmke HE (1943) Macrosporogenesis, fertilization, and early embryology of *Taraxacum kok-saghyz*. Bull Torrey Bot Club 70(2): 164-173.

Whaley WG (1948) Rubber-The Primary Source For American Production. Econ Bot 2(2):198-216. Online ISSN: 0013-0001. DOI: 10.1007/BF02859004.

Whaley WG and Bowen JS (1947) Russian Dandelion (kok-saghyz). An Emergency Source of Natural Rubber. Miscellaneous Publication Nº 618, United States Department of Agriculture, 212 pp.

Wilson RG, Kachman SD and Martin AR (2001) Seasonal changes in glucose, fructose, sucrose, and fructans in the roots of dandelion. Weed Sci 49: 150-155.

Wolf H and Wolf R (1936) Rubber. A story of glory and green. New York, Covici, Friede publishers.

Worldnomads (2012) http://safety.worldnomads.com/Liberia/78170/Politics-in-Liberia

Young ND (2000) Constructing a plant genetic linkage map with DNA markers. In: Ronald IKV and Phillips L (Eds.), DNA-based markers in plants, The Netherlands pp: 39-57.

Yulex (2015) http://www.yulex.com/home/

Zabeau M and Vos P (1993) Selective restriction fragment amplification: a general method for DNA fingerprinting. European Patent Office.Publication 0 534 858 A1 bulletin 93/13.

ERRATUM:

1.-Page 65 says:

"Table 4: Potential rubber, inulin and root dry weight yield in the TKS population trial.

	EXPECTED YIELD/HA*			
LEVEL	**RUBBER CONTENT (%)**	**RUBBER (Kg)**	**INULIN (Kg)**	**ROOT DRY WEIGHT (KG)**
20-F1	1.33	35.57	970.84	2674.48
20-F2	5.56	29.37	142.14	528.22
21-F1	1.12	19.39	373.70	1731.68
21-F2	1.69	11.61	205.82	687.23
22-F1	1.19	20.67	405.33	1736.62
22-F2	1.68	10.94	148.35	650.96
27-F1	1.13	13.79	319.11	1220.76
27-F2	1.72	17.18	283.67	998.82
30-F1	3.33	35.19	233.89	1056.89
30-F2	2.58	20.94	191.36	811.55
35-F1	2.60	25.32	149.17	973.70
35-F2	1.85	21.36	347.02	1154.82
36-F1	1.01	15.11	222.79	1496.26
36-F2	1.49	10.05	181.33	674.34
37-F1	1.45	5.41	134.53	373.18
37-F2	1.91	10.39	112.27	543.93
38-F1	1.23	7.07	194.80	574.45
38-F2	0.41	10.66	882.21	2600.85

**** Assuming a plant density of 240,000 plants/ha"***

and it should say:

"Table 4: Resume of N, mean and SD of Rubber (% in RDW), Inulin (% in RDW), RDW (g) and potential rubber, inulin and RDW weight yield in the TKS population trial.

	N	$\bar{x}$	SD	N	$\bar{x}$	SD	N	$\bar{x}$	SD	Expected Yield/Ha*		
Level	**RUBBER (% in RDW)**			**INULIN (% in RDW)**			**RDW (g)**			**RDW (Kg)**	**RUBBER (Kg)**	**INULIN (Kg)**
20F1	4	1,33	2,34	3	36,30	0,64	4	13,17	8,70	3160.8	42.04	1147.37
20F2	4	5,56	4,29	3	26,91	4,96	4	2,19	0,44	525.6	29.22	141.43
21F1	4	1,12	0,20	3	21,58	4,70	4	7,41	3,50	1778.4	19.92	383.78
21F2	4	1,69	0,70	3	29,95	1,57	4	3,46	1,71	830.4	14.03	248.71
22F1	4	1,19	0,78	3	23,34	8,53	4	9,92	9,41	2380.8	28.33	555.68
22F2	4	1,68	1,06	3	22,80	3,21	4	2,95	1,53	708.0	11.89	161.42
27F1	4	1,13	0,71	3	26,14	1,62	4	6,08	4,14	1459.2	16.49	381.43
27F2	4	1,72	0,88	4	28,40	4,85	4	4,04	0,67	969.6	16.68	275.37
30F1	4	3,33	1,75	3	22,13	0,66	4	5,85	4,82	1404.0	46.75	310.71
30F2	4	2,58	0,67	4	23,58	2,73	4	3,48	1,15	835.2	23.80	196.94
35F1	4	2,60	0,70	1	15,32	.	4	4,10	1,25	984.0	25.58	150.75
35F2	4	1,85	0,52	4	30,05	5,63	4	5,03	2,29	1207.2	23.33	362.76
36F1	4	1,01	0,69	4	14,89	5,07	4	6,51	3,13	1562.4	15.78	232.64
36F2	4	1,49	1,01	4	26,89	2,45	4	2,86	0,96	686.4	10.23	184.57
37F1	4	1,45	0,44	1	36,06	.	4	1,91	1,40	458.4	6.65	165.30
37F2	4	1,91	1,07	4	20,64	3,28	4	2,85	1,86	684.0	13.06	141.18
38F1	4	1,23	0,59	1	33,91	.	4	2,50	1,00	600.0	7.38	203.46
38F2	4	0,41	0,60	3	33,92	1,17	4	20,22	17,02	4852.8	19.90	1646.07

*240.000 plants/Ha were assumed for calculations.

Legend: F1: Planting date May; F2: Planting date June; RDW: Root Dry Weight. Expected Yield/Ha is based in means of rubber for 4 plants, inulin from 1 to 4 plants and RDW for 4 plants".

2.-Page 65 says:

"Considering that population 20 when planted in May has also the highest potential inulin yield, this population could be a good candidate for future development of the crop".

and it should say:

"Considering that population 20 when planted in May has also one of the highest potential inulin yields, this population could be a good candidate for future development of the crop".