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School of Agricultural Technology

Department of Crop Science

Senior Thesis

ENERGY PLANTS AS BIOFUELS BIOTECHNOLOGICAL METHODS TO INCREASE THEIR PRODUCTIVITY

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Περίληψη

Η ελάττωση του πετρελαίου και οι εκπομπές αερίων του θερμοκηπίου απο τον τομέα της αυτοκινητοβιομηχανίας, έκαναν την παγκόσμια κοινότητα να ανησυχεί σχετικά με την ζήτηση της ενέργειας και της αλλαγής του κλίματος. Τα βιοκαύσιμα, τα οποία προέρχονται από τα ενεργειακά φυτά αποτελούν εναλλακτική πηγή ενέργειας και μπορούν να αντικαταστήσουν την χρήση των ορυκτών καυσίμων. Επιπρόσθετα, τα ενεργειακά φυτά συμβάλλουν στην μείωση της κλιματικής αλλαγής, ισορροπώντας πλήρως τις εισροές κι εκροές του διοξειδίου του άνθρακα στο περιβάλλον. Παρ' όλα αυτά η εφαρμογή νιτρικών λιπασμάτων και εντομοκτόνων στις ενεργειακές καλλιέργειες, προκαλεί την εκπομπή του N2O στην ατμόσφαιρα με αρνητικές συνέπειες για το περιβάλλον. Η Ελλάδα ως μέλος της Ευρωπαϊκής Ένωσης, συμμετέχει στην παραγωγή βιοκαυσίμων. Στην Ελλάδα για την παραγωγή βιοκαυσίμων καλλιεργούνται κυρίως παραδοσιακές καλλιέργειες, όπως η αγριοαγκινάρα, το ζαχαρότευτλο, το σιτάρι, το κριθάρι και το γλυκό σόργο. Τα τελευταία χρόνια, η βιοτεχνολογία φυτών χρησιμοποιείται για την βελτίωση των χαρακτηριστικών των παραδοσιακών και μη παραδοσιακών καλλιεργειών που σχετίζονται με την βιοενέργεια. Οι λεύκες ειναι υποψήφια ενεργειακά φυτά με σκοπό την παραγωγή αιθανόλης απο κυτταρίνες. Η παρούσα διατριβή - πτυχιακή εργασία εξετάσει όλα τα παραπάνω, κι επίσης προβάλλει τα πρωτόκολλα βιοτεχνολογικών μεθόδων για την βελτίωση των χαρακτηριστικών της λεύκας ως ενεργειακή καλλιέργεια.

Abstract

The oil fuel depletion and the Greenhouse gas emissions from the transport sector, made the global community to concern about energy demand and climate mitigation. Biofuels deriving from energy plants are an alternative energy source, which can replace fossil fuels. Furthermore, energy plants participate to " neutral carbon" as a result the decrease of climate mitigation. However, the N fertilizers and pesticide applications in the field, emit nitrous oxide to the atmosphere and cause negative impacts to the environment. Greece, as part of the European Union participate in the production of biofuels. In Greece, traditional crops like artichoke, sugar beet, wheat, barley and sweet sorghum are mainly used for the production of biofuels. In recent years, plant biotechnology is used to enhance the bioenergy traits of the traditional and the non traditional energy plants. Populus spp. is a candidate energy plant which can be used for the production of cellulosic ethanol. This senior thesis deals with all of the above and also, protocols from biotechnological methods to enhance Populus spp. traits as energy crop, are presented.

INTRODUCTION

The oil fuels deplete in a rapid rate causing energy, social and economical problems. Furthermore, fossil fuels from industry and the transport sector increase the Greenhouse Gas (GHG) emissions and as a result they cause climate mitigation. Developing countries realize the need to produce new alternative sources of energy, which can replace fossil fuels. Biofuels deriving from plants are the only alternative energy source which is renewable and can thus meet our environmental concerns and socioeconomic challenges.

The agricultural sector constitutes the base of biofuel production. The "energy plants" or "energy crops", are the basic source for production of biofuels. The present study (senior thesis) deals with the most important energy plants which are used for the production of liquid (bioethanol and biodiesel) and solid biofuels. It also, deals with the biotechnological methods used for increasing their productivity. In the first part of this thesis a historical overview of biofuels is presented along with the current status of biofuels, the environmental impacts of energy crops and the climate mitigation. The land use and availability for energy crops is also presented. In the second part several methods for increasing productivity in some crops such as rapeseed, sugar beet, corn, swichtgrass and other are presented.

Furthermore, a description of experiments used for maximizing lignin contents and rooting capacity of poplars, as conducted at the laboratory of Molecular tree physiology of Purdue University is presented.

Part I

Energy plants used as biofuels

I.1 Historical overview of biofuels

The modern terms "energy plants" and "biofuels" are relatively new but have a history in the transport sector. In the early 1820's, Samuel Morey used ethanol to run his first combustion engine. In the early nineteenth century, Nicolas Otto made the prototype Otto engine which ran on ethanol derived from sugar and Rudolf Diesel used a peanut oil for his prototype engine. Henry's Ford prototype engine was able to run with ethanol which derived from a variety of energy plants *(Worldwacht Institute, 2007)*. In the early twentieth century, Germany produced ethanol from potato starch as a blend for gasoline. Also, in 1925, a train locomotives in Germany was powered by ethanol and thirteen years later, buses in Belgium were powered by biodiesel *(Reijnders L., Huijbregts M., 2009)*.

During the second world war, many countries like Brazil, Argentina, China and Japan, trying to reduce their fuel dependence, produced biofuels from vegetable oils and animal fats. After the second world war, the oil crisis turned Brazil and the USA to set separately, a program of ethanol production to minimize the oil fuel imports. Brazil program started in 1975 and produced ethanol from sugar cane. Two years later, started the USA's program and produced ethanol from corn and sorghum *(Reijnders L., Huijbregts M., 2009)*.

The decade of 80's, South Africa and European Countries produced biodiesel regularly but the volumes was not remarkable. In 1990, the USA and European countries started to produce biodiesel more seriously. In the early of twentieth first century, the demand of energy increased from industry and transport sector making the developing countries to concern for more energy *(Reijnders L., Huijbregts M., 2009)*.

I.2 Current status of biofuels

The countries which lead the world ethanol production are Brazil and USA. In 2006, the half world's production of ethanol was produced in the USA from corn crops which representing 2 to 3 per cent of the country's non diesel fuel. In Brazil, in 2006, was produced the two fifths of the global fuel ethanol supply from sugar cane (*Worldwatch Institute, 2007).*

European countries like Spain, Sweden, France and Germany are big producers using mainly cereals and sugar beets. China use corn, wheat and sugar cane as a feedstock to produce large amounts of ethanol for industry use. In india, sugar cane and cassava have been used for ethanol production (*Worldwatch Institute, 2007).*

Biodiesel has a similar growth in Europe. It comprises three quarters of Europe's total biofuels production, The EU biodiesel production reflects to 73 per cent of all biodiesel production globally. Germany accounted for 40 per cent of this production. (*Worldwatch Institute, 2007).* Below presented the producer countries of liquid biofuels globally (tables 1&2) and also, the European's Union (EU 25) production of liquid fuels (Figures 1 & 2)

Table 1. World ethanol production 2006 *(source: Worldwatch Institute, 2007).*

COUNTRY OR REGION	PRODUCTION (MILLION LITTERS)	SHARE OF TOTAL (PERCENTAGE)
United States	18300	47,9
Brazil	15700	41,1
European Union	1550	4,1
China	1300	3,4
Canada	550	1,4

COUNTRY OR REGION	PRODUCTION (MILLION LITTERS)	SHARE OF TOTAL (PERCENTAGE)
Colombia	250	0,7
India	200	0,5
Thailand	150	0,4
Australia	100	0,3
Central America	100	0,3
World total	38200	100

Table 2. World biodiesel production in 2006 *(source: Worldwatch Institute, 2007).*

COUNTRY OR REGION	PRODUCTION (MILLION LITTERS)	SHARE OF TOTAL PERCENTAGE
Germany	2499	40,6
United States	852	13,8
France	625	10,2
Italy	568	9,2
Czech Republic	153	2,5
Spain	142	2,3

COUNTRY OR REGION	PRODUCTION (MILLION LITTERS)	SHARE OF TOTAL PERCENTAGE
Malaysia	136	2,2
Poland	114	1,9
United Kingdom	114	1,9
Australia	91	1,5
Austria	85	1,4
Denmark	80	1,3
Philippines	68	1,1
Brazil	68	1,1
China	68	1,1
Others	490	8
Europe total	4504	73,2
Americas total	1113	18,1
World total	6153	100

Chart 1. Biodiesel production in European Union of twenty five (EU25) since 1992. *(Adapted from: Biofuels Barometer - June 2005, EUROBSERV' ER)*

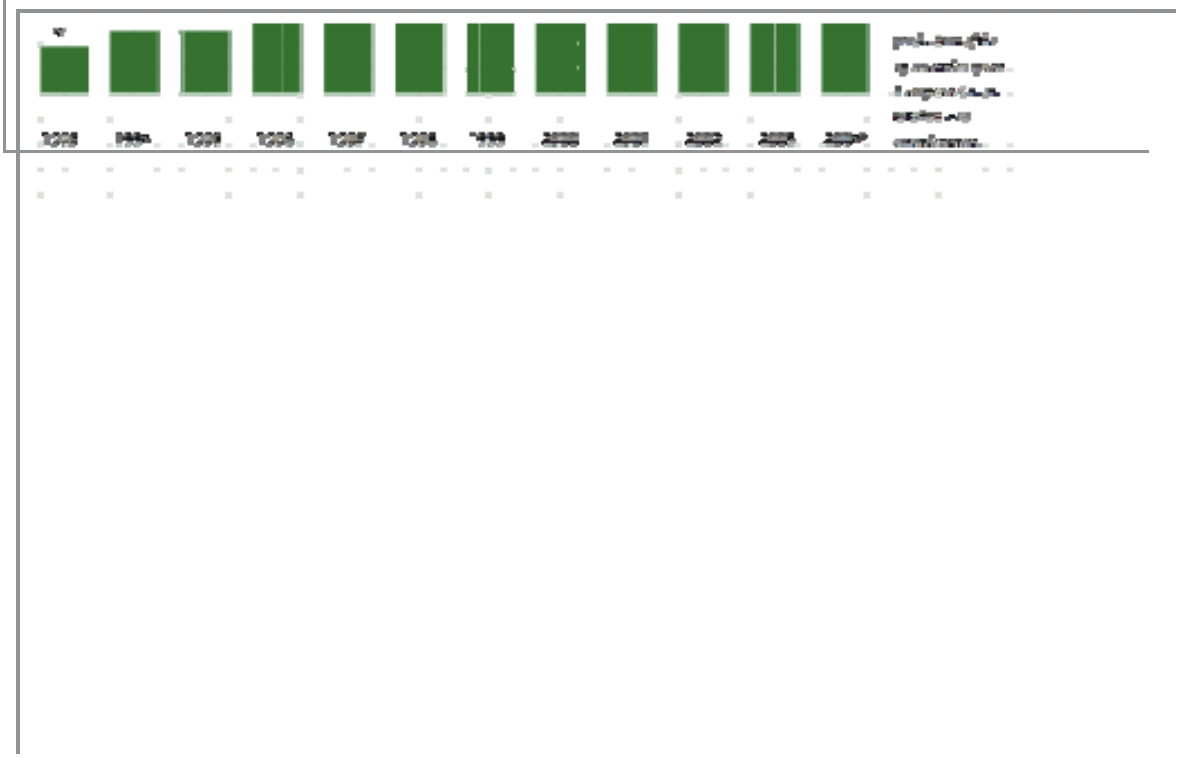

Chart 2. Ethanol production in European Union of twenty five (EU25) since 1993. *(Adapted from: Biofuels Barometer - June 2005, EUROBSERV' ER)*

I.3 Environmental issues relevant to biofuels utilization.

Nowadays, biofuels are proposed as an alternative energy source and many developing countries set scenarios for their future perspective of biofuels production. The wider utilization of biofuels have both positive and negative environmental impacts. The impacts of widespread cultivation of energy crops and feedstock developments cause issues with the GHG emissions, the changes in land use, the water use, the im-

pacts of increased nutrient and pesticide applications. Furthermore, the biofuels conversion and their final use, cause environmental impacts, such as the water usage and contamination and the air quality. *(The Royal Society, 2008).*

The method of LCA used to assess the environmental impacts and to prove which of them are sustainable. The LCA is a tool - method which evaluates the natural resource requirements and environmental impacts from the whole life - cycle of a product or service. *(The Royal Society, 2008).*

I.3.1 Greenhouse gas emissions and Climate mitigation

Biofuels derived from plants, reduce the climate mitigation because they participate to " neutral carbon" . Particularly, the plants take up the carbon dioxide (CO2) from the atmosphere and convert this into biomass. When the biomass burned, the CO2 returns to the atmosphere. However, biofuels are not related only with the CO2 emissions but also related with the nitrous oxide (N2O) emissions *(Reijnders L., Huijbregts M., 2009).*

The nitrogenous fertilizers and the mineralization of nitrogen into the soil releases N2O. The N2O impact to the environment is 296 times greater than CO2. In order to achieve greater annual production, the nitrogen fertilization to the arable crops increased at rates up to 350 kg/ha/yr. The IPCC estimates that 1% of added nitrogen returned to the atmosphere through the mineralization of soil organic matter *(The Royal Society, 2008).*

Furthermore the manufacture of nitrogen (N) fertilizers represents the largest emission of CO2 because of the high energy requirements. The winter wheat cultivation in US with smaller N applications of fertilizers than in Europe, represents approximately 60 percent of total CO2 emissions. This indicates the necessity of better management of N fertilizer to the field. Moreover, emissions are reduced with the use of legumes or organic manures as a N source. In the production of energy crops, the N fertilizer must be reduced because the environmental benefits from these crops is severely decreased. In Europe evaluated the establishment of belts of SRC willow among areas of conven-

tional agricultural crops with the aim of producing energy equal to that used in agricultural production within the farm *(Powlson S. D., Riche B. A., Shield I., 2005).*

I.3.2 Biofuels and Water use

Biomass crops impact the water quality positive. These crops have lower concentrations in nitrates, phosphates and pesticides compared to traditional arable crops. Biomass crops are perennials and they absorb water over a longer growing period in contrast with annual crops. Furthermore, the canopy of biomass crops can intercept rainfall during a large part of the year and decrease infiltration *(Powlson S. D., Riche B. A., Shield I., 2005).*

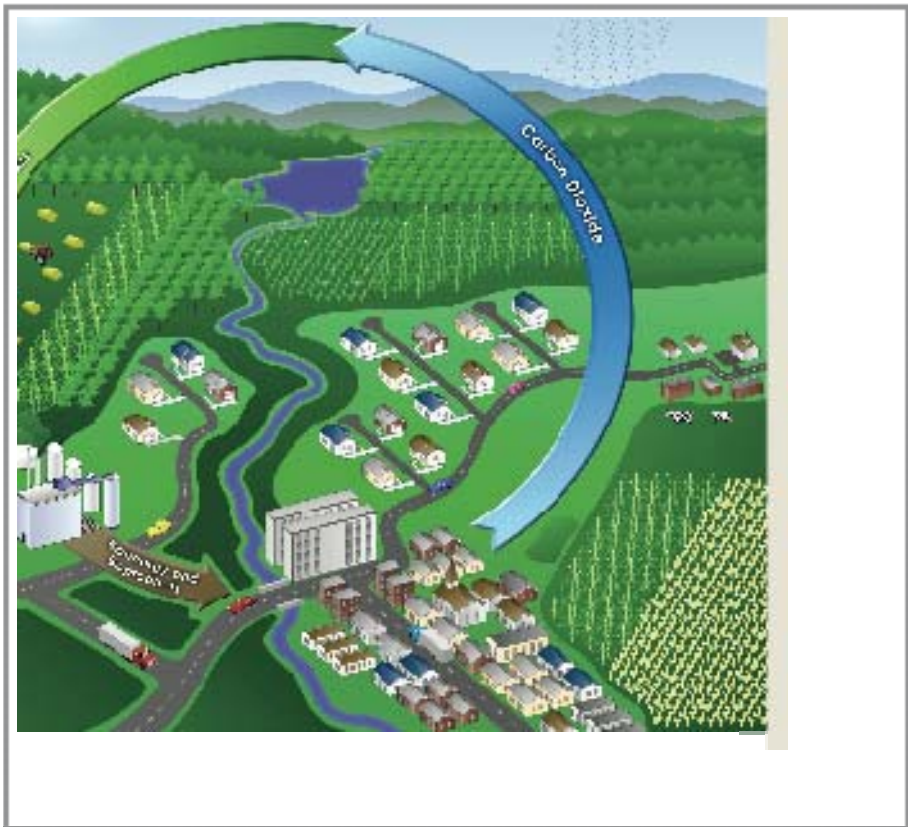

Figure 1. Reduce carbon dioxide emission of ethanol from biomass.

(source: www.orln.gov/info/orlnreview/v33_2_00/bioenergy.html)

I.4 Energy crops in Greece

The energy crops are plants that can be utilized as an alternative source for the production of liquid and solid fuels. The energy plants could be a traditional crops like sugar beet and corn for the production of bioethanol and sunflower for biodiesel. There also could be plants that are not cultivated commercially like miscanthus and artichoke of which the final product is used for bioenergy production. The main advantage of these plants is the stable production that can secure a great scale and long term raw material with homogenous qualitative characteristics for the production units of liquid biofuels and bioenergy. These cultivations provide high yields that enhance the economic outputs and minimize there needs in agrochemicals, transport costs and environmental consequences *(CRES, 2006).*

Below, some of the most important energy plants according to the bioenergy handbook of the Center of Renewable Energy Sources in Greece are presented.

I.4.1 Rapeseed (*Brassica napus and Brassica carinata*)

Rapeseed or canola (*Brassica spp.*) is an annual plant that belongs to the family of *Brassicaceae.* The plant of rapeseed propagates by seed and is mainly for oil production. The remaining residues from the oil extraction can be used as an animal feedstock since they are rich in proteins. Globally, it is the third most important oil plant after soybeans and palms. The seed is round and small. The oil concentration is between 30 to 50 per cent and the residues have proteins 10 to 45 per cent *(CRES, 2006).*

Figure 2. *Brassica napus*
(source: see p. 43)

The cultivation technics are the same with the winter wheats. A great consideration should be emphasized to weed control because the young plants are sensitive to weeds. It is necessary to apply pre and post emergent herbicides. Also, it is important to harvest the right period in

which the moisture of the seed is approximately 9 to 12 per cent *(CRES, 2006).*

Brassica napus is a precocious crop and it is widely spread to temperate climates. There are two types of cultivars, the winter and the spring crop. *Brassica carinata* originates from Ethiopia and is relative of *Brassica napus*. The plant is tall with wide leaf texture. Experiments found that the plant has a great adaptability and productivity to the Mediterranean soil climatic conditions. Furthermore, it can be cultivated as a winter crop in areas with a mild winter and in areas with heavy winters it can be cultivated as a spring crop *(CRES, 2006).*

Experiments which took place in Italy, Spain and Greece show us that the seed production is 150 to 300 kilos/ 1000 square meters (m2) and the dry biomass is 300 to 800 kilos/ 1000 m2. The yield depends from the variety and the soil climatic conditions during the growing period. The energy production from 1000 m2 of the rapeseed is 60 to 115 liters of biodiesel *(CRES, 2006).*

I.4.2 Sunflower *(Helianthus annuus)*

The Sunflower is an annual plant and belongs to the family of *Asteraceae (Compositae)*. In 2005, the global sunflower production was 31 million tons from 234 million m2. The 20 million ton produced from European Union of twenty five (EU25) *(CRES, 2006).*

Figure 3. *Helianthus annuus* (source: see p. 43)

In Greece, the sunflower crop has been reduced during the last years. The sunflower cultivation is located in the North Eastern part of Greece (Thrace) and it is a great source of vegetable oil for human consumption. The total cultivated area, in 2005, was 80 thousands square meters and corresponds to 16 thousand tons of seed. Alternatively, the sunflower can be used as feedstock source for biodiesel production. The EU25 is the largest biodiesel producer globally. More than 10% of biodiesel originates from sunflowers. Italy, which is the third largest biodiesel producer in the European Union, uses the sunflowers as its basic

source for biodiesel. The energy yield from 1000 square meters is 150 to 300 kilos of seed corresponding to 60 to 115 liters of biodiesel. *(CRES, 2006).*

I.4.3 Sweet Sorghum *(Sorghum bicolor L. Moench)*

Figure 4. *Sorghum bicolor*
(source: see p. 43)

Sweet sorghum is a C4 annual plant with a great photosynthetic ability, high yield in dry biomass, high percentage of soluble saccharose and cellulose. The irrigation and fertilization needs are relatively low. It also, presents great adaptation to variable climate conditions *(CRES, 2006).*

The yield of the crop depends on the soil climatic conditions and cultivation technics. The raw weight of the crop varies from 8 to 10 tons/ 1000 m2 in Germany, 9 tons/ 1000 m2 in Spain, 14 tons/ m2 in Greece. Sorghum is cultivated for many years in Greece for research proposes in different soil types and different cultivation technics *(CRES, 2006).*

Sweet Sorghum can be cultivated from Northern to Southern parts of Greece, in rich and poor soils. The key factor to achieve high yields is water. The nitrogen fertility doesn't effect the quantity of the yield *(CRES, 2006).*

The ratio of sucrose, varies from 9 to 13 per cent for the raw inedible part (stem) of the plant. The yield of saccharose that can be produced is 1,2 tons/ 1000 m2. Furthermore, it is important to underline that the best period to achieve the above yields is early September for the precocious varieties and 10 days later for the late varieties. The theoretical energy yield that can be produced from the row stems is 700 to 900 liters ethanol / 1000 m2 *(CRES, 2006).*

Another advantage of sweet Sorghum is the huge amounts of left residues (bagasse) which comes from the process of raw material. Those residues have a great

thermodynamic value and they can cover the energy needs for the production and conversion process of sweet Sorghum to ethanol. The European Union continues researches to assess Sweet Sorghum as an energy crop for the production of bioethanol as a transport fuel *(CRES, 2006).*

I.4.4 Wheat *(Triticum aestivum L.)* - Barley *(Hordeum sativum/ Vulgare L.)*

Wheat (also known as "common wheat" or "bread wheat") and barley are annual plants. They belong to the family of cereals "*Graminae"*. Globally, wheat is considered as the most important cereal. In 2005, the global wheat production was 628 million tons from which the 124 million tons was produced in EU25. On the other hand, the global production of barley was 137 million tons in 2005, from which 53 million tons were produced in EU25 *(CRES, 2006).*

Figure 5. *Triticum aestivum*
(source: see p. 43)

In Greece, wheat is the most widespread annual plant. The cultivated area, in 2005, was 8,5 million m2 and the corresponding yield was 1,8 million tons. According to the Cereal Institute of Thessaloniki, the yield of hard wheat varies from 150 to 800 kilos/1000 m2. Respectively, the yield of soft wheat varies from 200 to 900 kilos/ 1000 m2. The yield seed percentage of the total weight for the above ground part of the plant of both types of wheat (hard and soft) varies from 30 to 56 per cent. (CRES, 2006)

Figure 6. *Hordeum sativum*
(source: see p. 43)

Barley cultivation is scattered all over the country. In 2005, the total cultivated area was 980 thousands m2, and the corresponding yield was 220 thousand tons. The yield of barley varies from 150 to 700 kilos/ 1000 m2. The yield seed percentage of the total weight for the above ground part of the plant varies from 23 to 54 per cent (CRES, 2006).

In the last five years, wheat and barley are used as an alternative raw material for bioethanol production. Spain has an important activity in the sector of bioethanol. In 2004, the bioethanol production was 194.000 tons. *(CRES, 2006).*

Finally, the yield seed that produces from 1000 m2 wheat varies from 150 to 800 kilos and the corresponding yield fuel varies from 45 to 240 liters of bioethanol *(CRES, 2006).*

I.4.5 Sugar beet *(Beta vulgaris L.)*

Beta vulgaris is a biennial type of beet which is cultivated commercially for the high concentration of saccharose in its roots. The roots of the beets contain 20 per cent saccharose of its raw weight. It is the second important source of saccharose after the sugar cane. In 2005, the total global production of sugar beet was 242 million tons which were cultivated in 55 million m2 from which the 5 million m2 were cultivated in the United States of America and the 22 million m2 were cultivated in Europe *(CRES, 2006).*

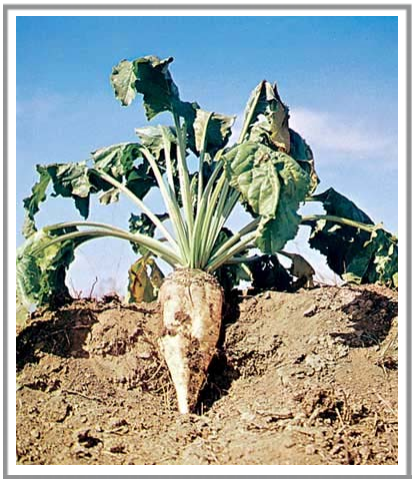

Figure 7. *Beta vulgaris*

(source: see p. 43)

In Greece, the cultivation of sugar beets is scattered all over the country. In 2002, Greece had a decrease in cultivation area of 420.000 thousands m2 and the production decreased from 3 million tons to 2,4 million tons. The sugar beet yield, in Greece, is 6000 kilos/ m2, which is the highest yield of other European countries. Most of the sugar beet production in Greece, goes for human consumption and for animal feed *(CRES, 2006).*

Sugar beet has an alternative use in recent years. It is a great feedstock for bioethanol production. One of the biggest producers of bioethanol from sugar beets is France. In France, 80 per cent of the total ethanol production, derives from sugar beets and the rest from cereals *(CRES, 2006).*

Finally, the volume of bioethanol that we can produce from a 1000 m2 varies from 550 to 700 liters bioethanol *(CRES, 2006).*

I.4.6. Corn *(Zea mays L.)*

The global corn production was 695 million tons and with corresponding cultivated area of 1.471 million m2 in 2005, from which the 300 million m2 were cultivated in the United States of America, 60 million m2 in Europe and 2.4 million m2 in Greece *(CRES, 2006).*

Figure 8. *Zea mays*

(source: see p. 43)

Corn is a widespread crop in Greece. During the years of 1993 to 2005, the corn yield presented an increase from 2,1 to 2,3 million tons. The Cereal Institute of Thessaloniki reports that the yield seed percentage of the total weight for the above ground part of the plant varies from 35 to 50 per cent *(CRES, 2006).*

It is widely known that many different products derive from corn. But in the last fifteen years, corn is also used as an alternative source for bioethanol production. The United States of America is one of the biggest bioethanol producers. In 1989, the US bioethanol production was 8 million ton and it went up to 28 million tons, in 2003 *(CRES, 2006).*

The bioethanol yield that can be produced from 1000 m2 varies from 240 to 360 liters *(CRES, 2006).*

I.4.7 Swichtgrass *(Panicum virgatum L.)*

Swichtgrass is a perennial C4 grass plant. It is widely spread plant in Northern and Central America. We can also find it in South America and Africa. The root system of the plant can surpass the three

Figure 9. *Panicum virgatum*

(source: see p. 43)

meters depth in soil. The root buds shoots many lateral stems with 10 mm diameter. Swichtgrass can be 2,5 meters tall. The field installation is applied with seed in May, when the soil temperature is 10 to 15 degrees Celsius. Also, the seeding should not be, more than 1 cm deep, in the soil. The optimum plant density should be approximately 200 to 300 plants per m2 *(CRES, 2006).*

The new stems from the root buds regrow in the last fifteen days of March, every year. The stems are sensitive to frost but the plant has the ability of regrowth even from a serious necrosis from low temperatures. The growth rate of Swichtgrass is 15 cm per day. The flowering occurs at the end of July until early August. It produces very small seeds. The weight of 1000 seeds is approximately 0,7 to 2,0 gr. After flowering, the plant starts lossing moisture. The best period to harvest the crop is by the end of November until January, with 25 per cent moisture *(CRES, 2006).*

The crop of Swichtgrass can produce great quantities of biomass. Also, the plant can produce biomass in difficult growing conditions without fertilizers and herbicides. Experiments show that the crop needs 400 mm of irrigation for a satisfactory production. Fertilization and irrigation are playing a significant role in the productivity of the plant which varies from 1 to 2 dry tons of biomass / m2. The energy yield is 18 to 36 Giga Joule (GJ) /m2/ year. Swichtgrass is a great feedstock for the production of liquid or solid biofuels *(CRES, 2006).*

I.4.8. Artichoke *(Cynara cardunculus L.)*

Artichoke comes from the Mediterranean region. The plant was well known by ancient Egyptians, Greeks and Romans. Nowadays, artichokes are considered as a wild plant which germinates near the river shores, continental and island areas of the Mediterranean. Also, it has been transfered in Australia, California, Mexico, Argentina, Chile and Uruguay. (Skoufogianni E.,

Figure 10. *Cynara cardunculus*
(source: see p. 43)

2006)

Cynara cardunculus has two subspecies, the *flavescens* and the *cardunculus*. *Flavescens* are located in Portugal and in the North West areas of the Mediterranean. On the other hand, *cardunculus* are located in the Central and South East areas of the Mediterranean. (Skoufogianni E., 2006)

Artichoke is a perennial plant (fifteen years). It belongs to the family of Asteraceae (Compositae). The crop seeding occurs in the fall period. The crop density should be 1000 to 1500 plants per 1000 m2. During the winter, the plant develops as a rosette and in spring it develops the flower head. In the summer, the above ground part of the plant dries up but the roots are active. In the next fall, the buds, from the above ground part of the plant, develop a new rosette, chiming the hour of the new biological cycle. The roots are developed very deep in the soil, as a result, the plant has the ability to absorb water and nutrients from a deep soil zone. The above advantage, keeps the artichoke productive in areas with a semi dry climate. (Skoufogianni E., 2006)

Experiments which took place in Greece show that the plant can be 2,6 meters high (Dalianis, 2006). The production of dry matter varies from 1,7 to 3,3 tons/ m2. In contrast, the Spanish yield varies from 0,4 to 1,5 tons/m2. Also, it had been estimated that 1000 m2 of artichoke can produce 28 to 40 litters biodiesel. (CRES, 2006)

The calorific value for every single part of the plant is 3.474 kcal/kg for the leafs and the bract leafs. The seed value is 5.912 kcal/kg because of its oil. The total energy yield from the whole plant varies from 18 to 27 GJ / m2 / year (CRES, 2006).

Figure 11. Artichoke trial field in the farm of University of Thessaly. (source: Skoufogianni E, 2006)

Part II

Biotechnological methods to increase the productivity of energy plants

II.1 Introduction

The trees of poplar (Populus. spp.) are playing a significant role in rural and industrial economy. In industry, the wood of poplars converted into a pulp and paper products. In recent years, scientists from developing countries turned their interests to utilize poplar trees as a biomass feedstock for the production, mainly, of liquid fuels and secondary of solid fuels *(Davis M. John, 2008).*

In forest science, poplars constitutes a "model" for tree physiology, tree genetics and now as a bioenergy woody crop. Particularly, the genus Populus chosen as bioenergy woody crop and as a "model tree" because of their great characteristic in genetic and phenotypic diversity which exists between the genus, Also, the sexual compatibility between many species, generates hybrid species with combinations of traits that not present in naturally occurring trees. Another characteristic, is that Populus provide ease for clonal propagation, facilitating the sharing and immortalization of useful genetic materials for the global research community, as well as commercial growers of the material for bioenergy applications. Finally, the growth rates of Populus are extraordinary in greenhouse trials and in field sites which occupied by well adapted genotypes, making the genetic analysis easer for measurement of growth related physiological traits *(Davis M. John, 2008).*

The beneficial characteristics of Populus that described above, effort researchers to use and screen genotypes which grown in a common environment and have desirable characteristics, to identify particular genotypes. Many of clones that tested in genetic screening trials, has been utilized to improvement of poplars for manufacture a variety of products such as high quality papers. Genetic research continues to screen the genetic diversity of genus Populus to identify clones with favorable traits that are relevant to bioenergy . *(Davis M. John, 2008).*

II. 2 Botanical description of Poplar (*Populus*)

The genus *Populus* is closely related to the genus *Salix* both genera are in the family Salicaceae, division Magnoliophyta, class Magnoliopsida, subclass Dilleniidae. The key botanical features of the genus from the perspective of genetic improvement of bioenergy crops are the broad species diversity and ranges of adaptability within the genus, the propagation methods that enable genetic variation to be fixed in clonal plantations, and the sexual compatibility of many species within the genus. These characteristics combine to generate abundant genetic and phenotypic diversity that can be harnessed in bioenergy plantations *(Davis M. John, 2008).*

II.3 Taxonomy of *Populus*

The genus *Populus* L. is comprised of >20 to <90 species, depending on the authority being considered. Species classification can be a real challenge in poplars, in part due to the wide geographic distribution of many poplar species – members of the same species growing in dramatically different environments can appear distinct due to the plasticity of tree phenotypes. This can lead to the inappropriate assignment of new species status. Additionally, the range of some poplar species overlap, creating geographic zones in which interspecific hybridization can occur. Without definitive visible or molecular markers to sort out the hybrids from the pure species, such assemblies of trees can appear to be distinct from the parental species (thus adding a species where it does not belong), or to represent intermediate forms within a single species (thus subtracting a species where it does actually belong). Furthermore, poplars have a long history of vegetative propagation, clone cultivation and sharing among human populations, which can generate confusion in nomenclature over time *(Davis M. John, 2008).*

The most generally accepted classification of poplars recognizes 29 species that are placed into five widely recognized sections: *Aigeiros*, *Leucoides*, *Populus* (also

called *Leuce*), *Tacamahaca* and *Turanga*. The salient features of these widely recognized sections are outlined below *(Davis M. John, 2008).*

• *Aigeiros*. This section contains eastern cottonwood (*P. deltoides*) and European black poplar (*P. nigra*), both important species used in interspecific hybridizations with one another and with compatible species in section *Tacamahaca*.

• *Leucoides*. This section contains large-leaved poplars such as swamp cottonwood (*P. heterophylla*) native to the central and eastern U.S.

• *Populus*. The aspens are in this section, including the important Eurasian species white poplar (*P. alba*) and European aspen (*P. tremula*), as well as the important North American species bigtooth aspen (*P. grandidentata*) and trembling aspen (*P. tremuloides*).

• *Tacamahaca*. The balsam poplars are in this section, including the reference species for the genome sequencing effort, black cottonwood (*P. trichocarpa*), Japanese poplar (*P. suaveolens*, or *P. maximowiczii*) and narrowleaf cottonwood (*P. angustifolia*). Many species in this section are compatible with species in *Aigeiros* with the resulting hybrids showing dramatic hybrid vigor.

• *Turanga*. The most important species in this section is *P. euphratica*, a species with high tolerance for extreme heat and poor soil conditions.

Many poplar hybrids have been given "hybrid designations" that are not necessarily intuitive based on the parental species, for example the naturally occurring *P. balsamifera* × *P. deltoides* hybrids are designated *P.* ×*jackii* Sarg. (common name: Jackii poplar). These designations are historically rooted and the more recent convention has been to designate hybrid families according to the parental species involved [(species used as female) × (species used as male)] with the actual parental genotypes being coded numerically. Progeny are then assigned addition designations within the family *(Davis M. John, 2008).*

II.4 Genetic Improvement

The publication of the draft sequence of *Populus* based on the genotype Nisqually-1 creates a new resource for bioenergy applications in that it contains all the genes to build a tree. Breaking the code requires more work than simply obtaining the DNA sequence itself – the process of decoding the genome sequence is called annotation. An-

notation has begun for genes involved in constructing poplar cell walls. In many cases the genes likely to be involved in conditioning particular bioenergy traits have already been identified in poplar or in annual crop plants based on previous studies. For example, the phenylpropanoid and monolignol biosynthetic pathways that control lignin composition and lignin quantity have been a subject of biochemical studies for many years. In these cases, the challenge in poplar is to identify the various alleles that control trait variation within the species or interspecific pedigree of interest, since each gene may have many alleles within each species. Haplotype diversity within the reference genotype Nisqually-1 was 2.6 point mutations or small indels per 1,000 bases, reflecting the level of heterozygosity within the reference genotype whose genome was sequenced. The lignin biosynthetic pathway is comprised of about twice as many genes in poplar compared to *Arabidopsis* . Similarly, there are almost twice as many *cellulose synthase* genes in poplar compared to the ten *CesA* genes in *Arabidopsis* *(Davis M. John, 2008).*

Genes that condition plant responses to the hormones auxin and cytokinin, known to have dramatic effects on tree growth, development and architecture, have also been annotated. In other cases, the genes involved in conditioning the traits of a particular poplar species are not known. A large-scale gene expression study performed on *P. euphratica*, which is highly drought and salt tolerant, identified 98% of its genes were shared by *P. trichocarpa* (the Nisqually-1 reference genome sequence). The authors suggested that it may be differential regulation of shared genes, as opposed to the evolution of brand-new suites of genes, that permits adaptation to extreme conditions in *P. euphratica*. The challenge ahead is to determine the relationships between allelic variation and trait variation, so that the alleles conditioning traits of interest can be identified *(Davis M. John, 2008).*

Once important alleles have been identified, they can be tracked within breeding and selection programs using marker-assisted selection. This promises to make breeding and selection more efficient, since parents and offspring with undesirable alleles (e.g. for growth) can be discarded prior to assessing traits in expensive field trials. In the bioenergy context, breeding lines of genotypes with extreme differences in biomass composition and crown architecture could be maintained separately to target various downstream conversion options. The overall goal is to identify individuals with the greatest

combination of desired alleles for clonal propagation and deployment *(Davis M. John, 2008).*

II.5 Transgenic manipulation of Gene Expression

Transgenic manipulation of gene expression in poplar relies on *Agrobacterium*-mediated delivery of DNA into the poplar genome. Most experiments reported in the literature use transgenic manipulation of poplar for reverse genetic analysis. In reverse genetic analysis, the experiment is intended to test the phenotype that is generated when a specific gene sequence is altered in its expression. The logic of reverse genetic analysis is that there are already many candidate genes implicated in controlling bioenergy traits, and these can be directly tested by mis-expressing them in transgenic poplar. The experiment boils down to a comparison of transgenic and non-transgenic lines. Candidate genes have been identified in several ways. Genetic analysis in other plant systems such as *Arabidopsis* creates a list of candidate genes in poplar, since one would expect that many genes involved in cell wall development might be conserved in structure and function in *Arabidopsis* and poplar. Biochemical characterization of genes and enzymes in several important pathways, including those leading to biosynthesis of lignin, cellulose and starch, have been carried out historically in non-tree systems but are likely to have important aspects that are conserved in poplar, since it seems unlikely that entirely independent genetic mechanisms would evolve anew in individual plant lineages. Perusal of the poplar genome sequence allows the poplar versions of these genes to be identified and transgenically manipulated. Microarray experiments also generate lists of candidate genes based on the presence of messenger RNA in specific cell types at specific times in which that gene's expression can be correlated with a known developmental process that is occurring simultaneously *(Davis M. John, 2008).*

Several aspects of transgenic manipulation are appealing, including the ability to reduce as well as increase transcript abundance by comparing multiple transgenic lines generated using the same genetic construct. Transgenic analysis in poplar requires ~1 year from vector construction to phenotypic analysis, and so the number of candidate genes that can be tested in a given period of time is limited mostly by labor (to carry out

the transformation steps in tissue culture, and to carry out phenotypic analysis of specific transgenic lines and non-transgenic controls) and space requirements (to grow the transgenic and non-transgenic lines in the greenhouse). Comprehensive reviews of the literature on transgenic manipulation of wood composition in poplars are available and only selected work will be highlighted here *(Davis M. John, 2008).*

Transgenic manipulation of poplar growth was accomplished by enhancing biosynthesis of the phytohormone gibberellin through overexpression of the gene encoding GA 20-oxidase, indicating a potential role for transgenic approaches to elevate poplar biomass accumulation and improve fiber properties by altering growth regulator biosynthesis. Increased growth mediated by enhanced nitrogen utilization may be the mechanism by which overexpression of a pine *glutamine synthase* gene increased growth in transgenic poplar trees. Although not necessarily predicted, enhanced growth was also observed under greenhouse conditions when lignin production was down-regulated by transgenic reduction of transcript encoding 4-coumarate: CoA ligase. Lignin was reduced dramatically relative to cellulose, with the most severe lines possessing about half of the lignin content of non-transgenic controls. This decrease is beyond that observed in interspecific hybrid pedigrees, and would be expected to increase the conversion efficiency of the biomass to ethanol. Transgenic manipulation is not limited to single genetic steps, but rather the feasibility of pathway engineering was demonstrated in poplar. These researchers introduced constructs that reduced *4CL* transcript and enhanced production of *coniferaldehyde 5-hydroxylase* transcript, then identified specific lines expressing single transgenes or pairwise combination of the transgenes. The latter lines produced about half of the lignin of non-transgenic controls, and most importantly demonstrated a significant increase (64%) in the ratio of syringyl (more easily extractable) to guaiacyl (less easily extractable) ratios of the lignin subunits. A recent, very comprehensive study in which the gene encoding cinnamoyl CoA reductase (CCR) was reduced using a transgenic approach, showed dramatic reductions in lignin (to about half of non-transgenic levels), significantly improved pulping efficiency, but detectable and significant decreases in growth in the field. These studies show that direct reverse genetic manipulation of lignin biosynthesis is feasible in poplar by introducing constructs that encode enzymes in the monolignol biosynthetic pathway *(Davis M. John, 2008).*

Enhancement of biomass traits can also occur unexpectedly, which may not be surprising given our relatively limited understanding of how cell walls are assembled in plants. For example, elevated cellulose content was incited by overexpression of a xyloglucanase gene. The mechanism by which cellulose increased is not clear, but could be due in part to the altered architecture of the cell wall environment that promoted altered deposition of cellulose *(Davis M. John, 2008).*

A completely different transgenic approach called *activation tagging* was used to create a population of poplar lines for forward genetic analysis. In forward genetic analysis, it is the phenotype that drives the entire experiment. Forward genetic analysis does not presume that any particular gene causes the phenotype, and so the power of this approach is that it is not biased by the scientist's choice of the transgene. Similar in concept to the forward genetic analysis of a QTL mapping experiment, activation tagging can reveal new, unexpected and/or previously unknown genes that play important roles in bioenergy traits. Activation tags are transcriptional enhancers that insert randomly in the genome, and their tendency to create dominant gain-of-function alleles is key in poplar, since tagged lines cannot be self-pollinated to reveal phenotypes for recessive alleles. A population of activation tagged poplar lines was created and screened for a dramatic dwarf growth phenotype in the greenhouse and in field plantings (Busov et al. 2003). The location of the tag in the genome adjacent to a gene encoding gibberellin 2-oxidase (*GA 2-oxidase*) was obtained using a method called plasmid rescue, but with the genome sequence now available can be inferred by obtaining sequence adjacent to the tag and comparing it to the whole-genome assembly. The dwarf phenotype was recapitulated by overexpressing *GA 2-oxidase* in transgenic poplar, thereby establishing the genetic basis of the dwarf phenotype. Further screening of populations designed for forward genetic analysis is warranted to enable discovery of novel genetic mechanisms conditioning bioenergy trait variation in poplar *(Davis M. John, 2008).*

Transgenic trees cannot be grown in field trials without specific governmental permits, so the direct value of transgenic poplar for bioenergy traits is not well understood relative to interspecific hybrid families produced by traditional breeding. However, the limited experience with transgenic manipulation for lignin reduction has been encouraging with respect to significantly reduced energy cost for pulping (Pilate et al. 2002; Leple et al. 2007) and no measurable ecological effects on interactions with insect

pests or associated soil microbial communities. Transgenic manipulation is usually performed in a single poplar genotype that regenerates efficiently in the tissue culture protocols used for transgenic plant production. Since that single poplar genotype may not be well adapted to the climatic and soil type conditions that are targeted for a bioenergy plantation, more comprehensive field testing of transgenes in diverse genetic backgrounds will be required to assess the usefulness of transgenic technology for bioenergy applications. Notwithstanding the limited inferences we can make regarding the potential application of transgenic technology, it is abundantly clear that a transgenic approach is a scientifically powerful way to test and ultimately prove a causative relationship between gene structure and gene function *(Davis M. John, 2008).*

From all the above information which are mainly derived from the book "Genetic Improvement of bioenergy crops" (Chapter 14 by Davis M. John), it is evident but populus plants show great genetic variation which can be used for genetic improvement programs.

II.6 Biotechnological method to enhance Poplars for biofuels production

Recent advantages in understanding of how lignin synthesized provide an opportunity to modify the content and the composition of lignin polymer. This research enable us to rationally assess the cost savings that could result from using genetically engineered poplar, instead of corn, as a feedstock for producing bioethanol. Efforts to improve the cell wall degradability should enable to achieve a dramatic reduction in the expense associated with producing ethanol from poplar biommas. *(Chapple Clint).*

I had the opportunity το work a small part of this research in the laboratory of "Tree molecular physiology" at Purdue University. The protocols which was used to

enhance poplars lignin content and rooting capacity. The protocols are the same for both lignin and rooting capacity enhancement. The plasmids, which were used was the pKVK-1 and pKVEK - 3.

II.6.1 Material and Methods

II.6.1.1 Protocol for "Yellow" Medium (Propagation medium for poplar 717)

(Received from Dr. Ma Oregon State University)

The compounds were added into the beaker and brought to favorable volume with ddH2O. Then, the pH was adjusted at 5.8 . The agar solution was dissolved in microwave for 8 minutes (1 litter of agar, depending the microwave machine, needs approximately 8 min.) and poured into Magenta boxes or tubes and autoclaved. Then, it was let to cool and used or stored at +4 degrees Celsius.

Table 3. Compounds which are need to make propagation medium for poplar 717 *(Received from Dr. Ma Oregon State University).*

COMPOUND	AMOUNT PER 1 LITER	AMOUNT PER 2 LITERS
Sucrose	20 g	40 g
MES	0.25 g	0.50 g
Myo-inositol	0.10 g	0.20 g

COMPOUND	AMOUNT PER 1 LITER	AMOUNT PER 2 LITERS
MS Basal Salts (Sigma M5524, 1/2 strength)	2.15 g	4.30 g
L-glutamine	0.20 g	0.40 g
FV Vitamins	10 ml	20 ml
Agar (Sigma)	8.0 g	16 g

II.6.1.2 Protocol for "Green" Medium (Propagation medium for poplar NM6)

(Received from Dr. Ma Oregon State University)

The preparation was the same with the "Yellow" propagation medium for poplar 717.

Table 4. Compounds which are need to make propagation medium for poplar NM6 *(Received from Dr. Ma Oregon State University).*

COMPOUND	AMOUNT PER 1 LITER	AMOUNT PER 2 LITERS
Sucrose	20 g	40 g
MES	0.25 g	0.50 g
Myo-inositol	0.10 g	0.20 g

COMPOUND	AMOUNT PER 1 LITER	AMOUNT PER 2 LITERS
MS Basal Salts (Sigma M5524, 1/2 strength)	2.15 g	4.30 g
L-glutamine	0.20 g	0.40 g
FV Vitamins	10 ml	20 ml
IBA (1mg/ml)	100 µl	200 µl
Agar (Sigma)	8.0 g	16 g

II.6.1.3 Protocol: Luria - Bertani (LB) media.

(Received from Dr. Meilan and Dr. Ma Oregon State University)

The LB media was used to grow liquid bacterial cultures and to grow bacterial colonies into plates.

Table 5. Compounds which are need to make LB media *(Received from Dr. Meilan and Dr. Ma Oregon State University).*

COMPOUND	AMOUNT PER 1/2 LITER	AMOUNT PER 1 LITER	AMOUNT PER 2 LITERS
NaCl	2.5 g	5.0 g	10 g

COMPOUND	AMOUNT PER 1/2 LITER	AMOUNT PER 1 LITER	AMOUNT PER 2 LITERS
Yeast Extract	4.0 g	8.0 g	16 g
Bacto - tryptone	8.0 g	16 g	32 g

II.6.1.4 Protocol of In vitro culture of Non-Sterile trees

(Dr. Ma, Oregon State University)

A few-grown poplar branches about 8-10 cm length were taken from the green-house. The leaves and petioles were cut off and the branches were putted in plastic bags with litter water to avoid moisture loss. Then in laboratory, the branches were washed with tap water and were putted into tubes with 30-40 ml of 70% Ethanol for 5 min (shaken at 200 rpm). Ethanol was poured out and was putted 20% bleach with 0,1% triton for 20-25 min (shaken at 200 rmp). Then, the branches were rinsed 4-5 times with sterille ddH2O. The ends of each branch section which were bleached were cut off. One section with 1-2 buds was placed in test tube with basic root medium (Yellow Medium) The branches were cultured in 25 C (room temperature) with 16 hr light and 8 hr in dark regime Roots and Shoots in 10-14 days.

II.6.1.5 Protocol: Transformation of E. coli via chemically component cells.

(Received from Dr. Meilan and Dr. Ma Oregon State University)

For each sample to be transformed 1 ml was placed in a micro centrifuge tube. A 100 µl of E. coli,was thawed on ice, for each sample (about 10 minutes). One selective media plate was placed for each sample on bench at room temperature or in 37 oC and inverted with the lid slightly off. The thawed cell suspension was dispersed into the micro centrifuge tube gently with a pipette. A 100 µl of cells was pipetted into each 1

ml tube and keept on ice. The DNA sample was added to cells (added ~ 5 μl DNA for ligations; added ~ 0,05 μl of stock plasmid DNA, depending the concentration). The mixture was incubated on ice for 30 to 45 minutes. The tubes were heat shocked in 42 o C water bath for exactly 60 seconds and immediately were removed to ice for 2 minutes. A 900 μl LB media was added to each tube, by taking up 100 μl from the first tube (1x) and added to the second tube (10x) then taking up an other 100 μl from the 10x tube and added to the third tube (100x). Then, were incubated with shaking at 37 o C for 30 to 60 minutes.

The next step had two alternative options. I followed the second option: 50 to 100 μl was transfered (in my case, I always transfered 50 μl) of cell suspension directly onto the center of warmed selective media plate for each sample separately. Alternatively, in option one: 1,5 ml suspension was pipetted to centrifuge tube and spun at 2000g for 5 minutes. Supernatant was decanted. The pellet was resuspended gently with pipetting. The suspension was spread around the plate until absorbed into the surface. Finally, the inverted plates were incubated at 37 oC overnight in the dark.

II.6.1.6. Preparation of plasmid DNA by Alkaline Lysis with SDS: Mini preparation

("Laboratory manual", Sambrook and Russell)

Materials:

Alkaline Lysis Solution I: 50mM glucose, 25 mM Tris-Cl (pH 8),10mM EDTA (pH 8). 100 ml were prepared in batches from standard stocks and autoclaved for 15 minutes at 15 PSI and was stored at 4oC.

Alkaline lysis solution II: 0.2 N NaOH (freshly diluted from a 10 N stock), 1% (w/v) SDS. The solution II was prepared fresh for each use and used at room temperature.

Alkaline lysis solution III: 5M Potasium Acetate (60 ml), Glasial Acetic Acid (11.5 ml), H2O (28.5 ml), the solution III was stored at 4oC and was used ice cold. Suitable antibiotic, Ethanol, Phenol: Chloroform (1:1, v/v)

STET:10 mM Tris-Cl (pH 8), 0.1 M NaCl, 1 mM EDTA (pH 8), 5% (v/v) Triton X-100. pH of finished solution was verified at 8.0 before use. It was not sterilized. TE (pH 8) containing 20 μg/ml R Nase A, LB media

Method:

The alkaline lysis procedure constitutes in three phases, the cell preparation, the cell lysis and the plasmid recovery.

Cell preparation: For each sample, 2 ml of rich medium LB was inoculated containing the appropriate antibiotic with a single colony of transformed bacteria. The culture was incubated overnight at 37oC with vigorous shaking. 1.5 ml of the culture was poured into a microfuge tube and was centrifuged at maximum speed for 30 seconds at +4 oC. The unused portion of the original culture was stored at 4 oC. When centrifugation was completed, the medium was removed by aspiration, leaving the bacterial pellets as dry as possible *(Sambrook and Russell, 2001).*

Cell lysis: The bacterial pellet was resuspended in 100 μl of ice cold Alkaline lysis solution I by vigorous vortexing. 200 μl of freshly prepared Alkaline lysis solution II was added to each bacterial sunpension. The tube was closed tightly, and the contents were mixed by inverting the tube rapidly five times. Vortexed was not used. The tube was stored on ice. 150 μl of ice cold Alkaline lysis solution III was added. The tube was closed and the Alkaline lysis III through the viscous bacterial lysate was dispersed by inverting the tube several times. The tube was stored on ice for 3-5 minutes. The bacterial lysate was centrifuged at maximum speed for 5 minutes at +4 oC in a microfuge tube and the supernatant was transfered to a fresh tube. (Optional) An equal volume of phenol : chloroform was added. The organic and aqueous phases were mixed by vortexing and then the emulsion was centrifuged at maximum speed for 2 minutes at 4 oC in a

microfuge. The aqueous upper layer was transfered to a fresh tube *(Sambrook and Russell, 2001).*

Recovery of Plasmid DNA: Nucleic acids from the supernatant were precipitated by adding 2 volumes of ethanol at room temperature. The solution was mixed by vortexing and then allowed to stand for 2 minutes at room temperature. The precipitated nucleic acids were collected by centrifugation at maximum speed for 5 minutes at 4 oC in a microfuge. The supernatant was removed by gentle aspiration as described previously. The tube was let to stand in an inverted position on a paper towel to allow all of the fluid to drain away. A kim wipe or disposable pipette tip was used to remove any drops of fluid adhering to the walls of the tube. 1 ml of 70% ethanol was added to the pellet and the closed tubes were inverted several times. The DNA was recovered by centrifuge at maximum speed for 2 minutes at 4 oC in a microfuge. all of the supernatant were removed by gentle aspiration as described previously. Beads of ethanol that formed on the sides of the tube were removed. The open tube was stored at room temperature until the ethanol had evaporated and no fluid was visible in the tube (5-10 minutes). The nucleic acids were dissolved in 50 µl of TE (pH 8) contained 20 µg/ml D Nase free R Nase A (pancreatic R Nase). The solution was vortexed gently for a few seconds and stored the DNA samples at -20 oC. *(Sambrook and Russell, 2001).*

II.6.1.7 Polymerase Chain Reaction (PCR)

(Based on the "Laboratory manual", Sambrook and Russell)

In order to run a PCR procedure, was made the appropriate master mix which was constituted from 18,5 µl ddH2O, 2,5 µl of10x PCR Buffer (Thermopol B9004S), 0,8 µl of 25mM MgCl2, 1 µl of dNTP, 0,5 µl for each primer (Knat6), 0,2 µl of Taq (5U/ µl). The master mix was accounted 24 µl and was mixed with 1 µl DNA template. The total volume was 25 µl.

Below shows how the PCR machine was programmed:

Step 1 = 94,0 oC for 1 minute, Step 2 = 58,0 oC for 1 minute, step 3 = 72,0 oC for 1 minute, step 4 = go to step 1 for 29 times, step 5 = 72,0 oC for 5 minutes, step 6 = 4,0 oC for ever, step 7 = End.

II.6.1.8 Electrophoresis

The edges of a clean dry plastic tray were sealed with tape to form a mold. The mold was set on a horizontal surface of the bench. The needed electrophoresis buffer (6x TAE) to fill the tank and to make the agarose gel was prepared. For the preparation of agarose gel (0,8 %) 150 ml of 1x TAE buffer and 1,2 g of agarose were poured in to a flask. Then, melted in the microwave for 1 minute. After melting, the agarose solution was left to cool down for a while. Then, the warm agarose solution was poured into the mold with the comb on top. The gel was allowed to set completely for 30 to 45 minutes at room temperature. When the gel was ready, electrophoresis buffer was added enough to cover the gel to a depth approximately 1 mm. Every sample of DNA (PCR products in my case) was mixed with Ladder. Then, 8 µl of mixed DNA sample was loaded into the wells which are formed from the comb. The lid of the tank was closed and attached the electrical leads. The machine was set at 120 volts for 60 minutes. After 60 minutes the gel was removed from the mold, was set it into a plastic pan and 20 µl Ethidium Bromide was added and shaken it for 2 hours. *(Based on the "Laboratory manual", Sambrook and Russell).*

II.6.1.9 Protocol - Inoculating with Agrobacterium and Co - cultivation

The Agrobacterium from frozen glycerol stock (-80 C) was streaked on solid LB media and grown 1 and 2 days at 28 degrees Celsius. An individual colony was selected and inoculated in 50 ml LB containing the appropriate antibiotics for the strain of Agrobacterium being use. An agrobacterium culture was grown overnight on an incubating shaker which was set at 250 rpm and 28 C. The OD 600nm was harvested approximately at 1.5 to 2.0. the bacterial cells was pelleted by spining for 30 min at

1992 g (3500 rpm). The cell suspension was diluted to OD 600nm of 0.4 to 0.6 with liquid CIM containing 25 μM acetosyringone. 30 to 40 explants was placed (leaf discs and wounded stem segments) in capped 50 ml disposable polypropylene, conical centrifuge (Falcon) tube containing 30 to 40 ml of diluted Agrobacterium culture. The tubes with the Agrobacterium suspension and the explants were shaken (orbitally) at 150 to 200rpm for 1 hour. The Agrobacterium culture was decanted into a flask and removed the excess Agrobacterium from explants by plotting them on sterile paper toweling using sterile forceps. The explants were aligned on fresh CIM (30 to 40 explants per plate). The plates were incubated in the dark for 2 days *(Dr. Meilan and Dr. Ma).*

II.6.1.10 Protocol - Callus induction and shoot regeneration

The inoculated explants were transfered into a Falcon tube and rinsed four to five times with 30-40 mL sterile ddH20 and once with washing solution. The explants were plotted to dry on sterile paper toweling and were placed onto CIM containing selection agent such as 25 mg/L kanamycin and 200 mg/L timentin. The explants were incubated in the dark for 2-3 weeks. Then, the explants were transfered to SIM containing 100 mg/L kanamycin and 200 mg/L timentin. The sealed plates were cultured in growth room under lights. The explants were sub-cultured onto fresh SIM every 2-3 weeks until shoots form. The explants were transfered with multiple small shoots to SEM containing 100 mg/L kanamycin and 200 mg/L timentin *(Dr. Meilan and Dr. Ma).*

II.6.2 Results

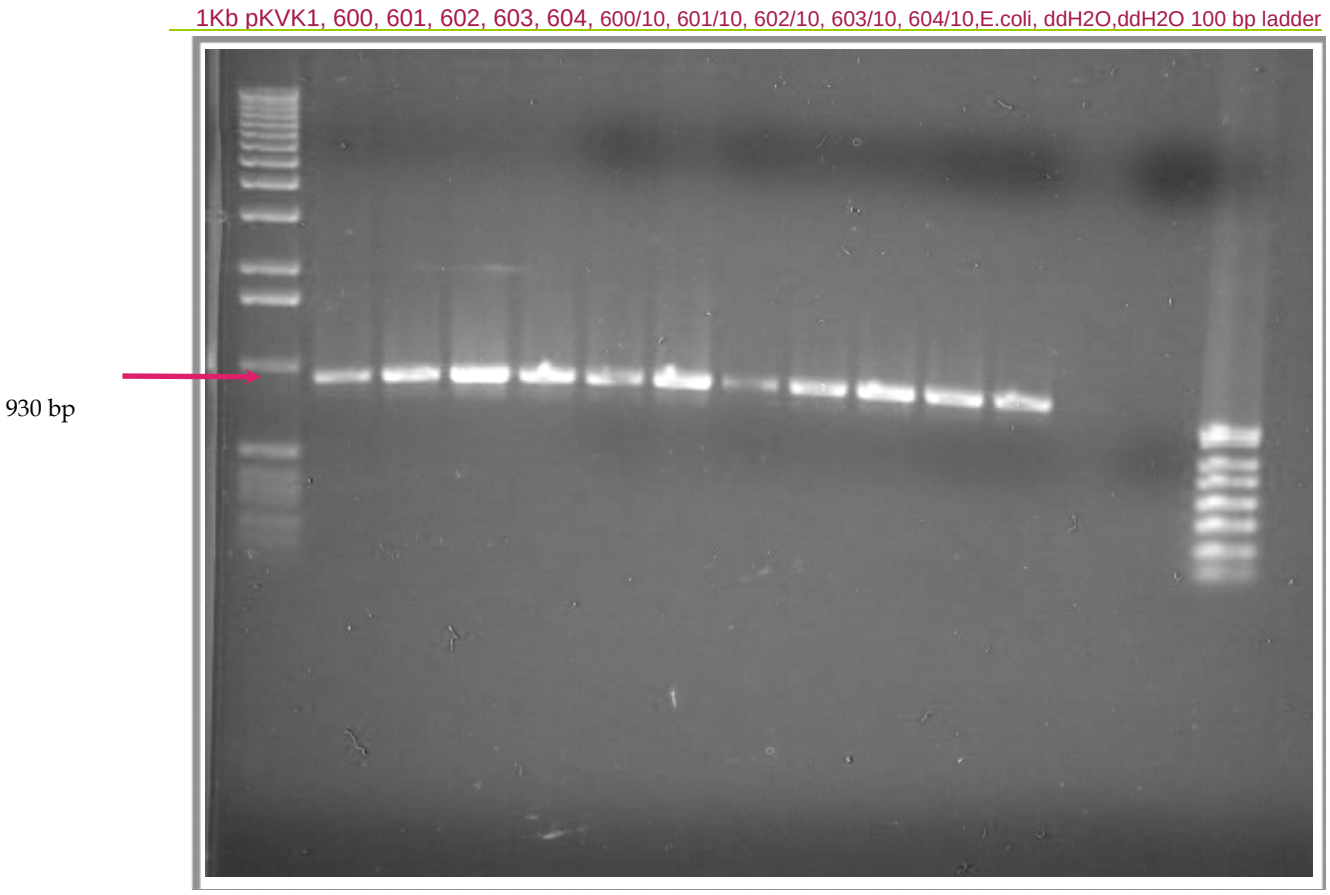

Figure 12. Transformation of E. coli with plasmid pKVK-1.

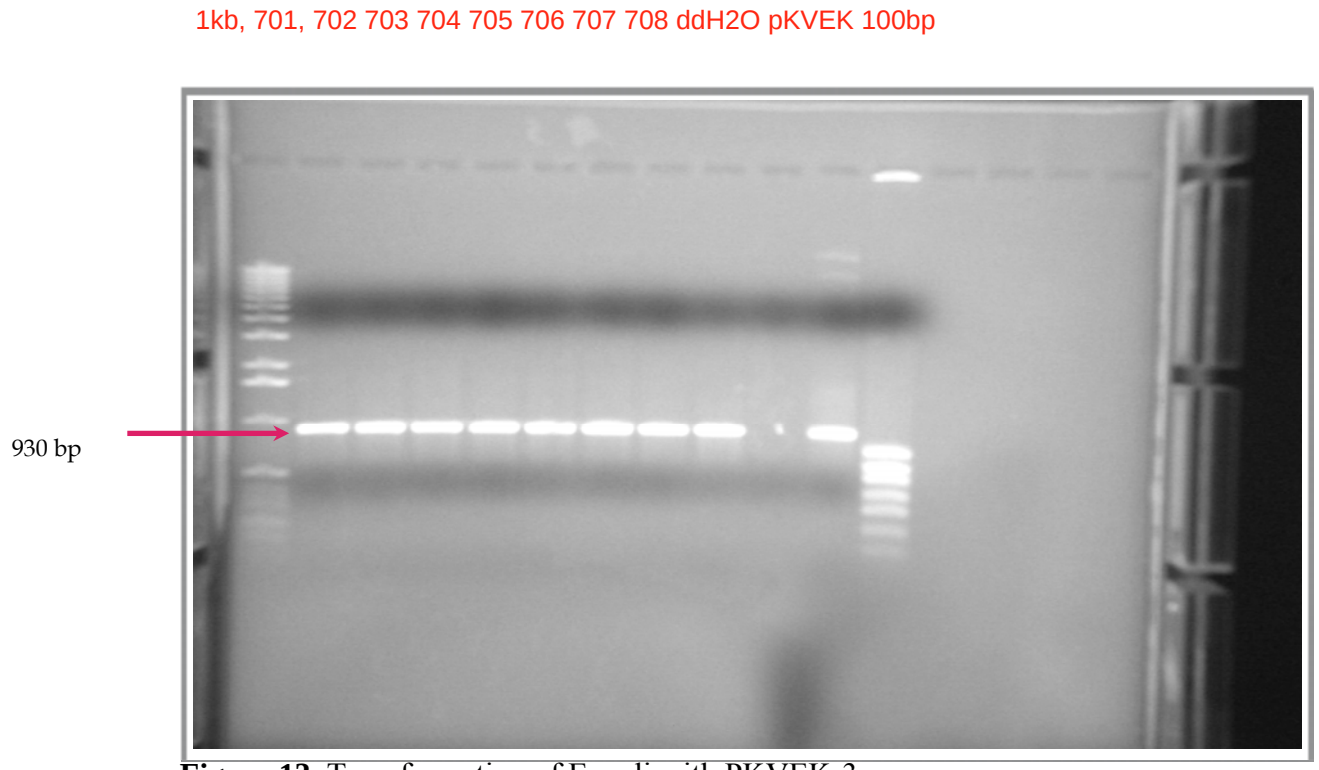

Figure 13. Transformation of E. coli with PKVEK-3.

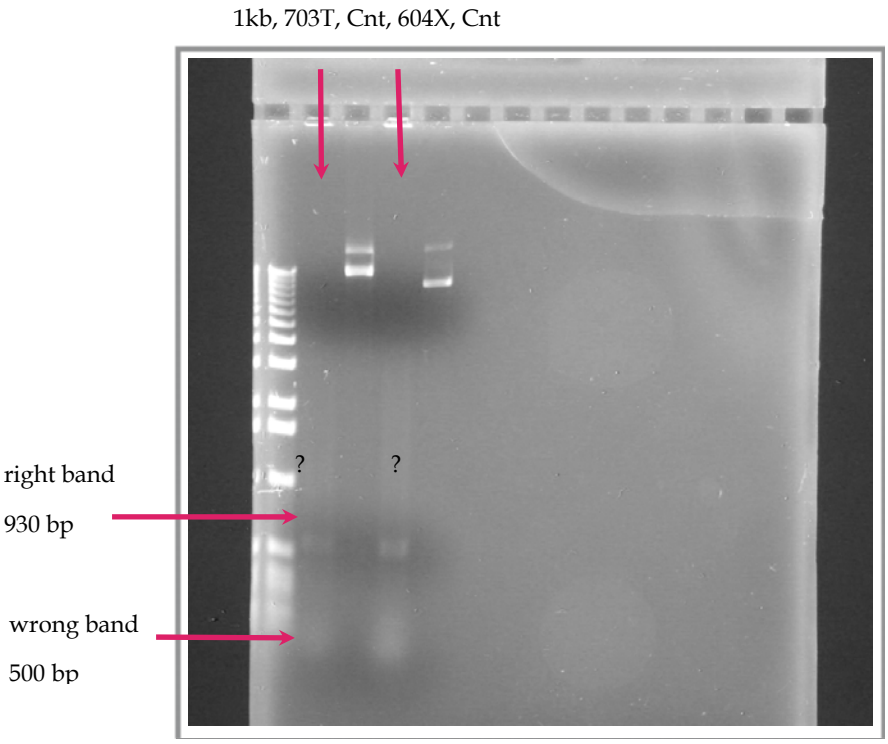

Figure 14. Transformation of Agrobacterium (AGL1) with plasmids pKVK-1 and pKVEK-3

II.6.3 Conclusion

The results of transformations with plasmids pKVEK - 3 & pKVK -1 into E. coli and into Agrobacterium, are presented. The transformation of E. coli with plasmids pKVEK - 3 and pKVK-1 was successful as we can see in figures 3 & 4. Unfortunately, the transformation of Agrobacterium with the same plasmids was not successful (figure 5). There are many reasons when the agrobacterium doesn't transform but I can not explain them since I do not have a strong background to biotechnology.

II.6.4 General Discussion

The concern of oil fuel depletion, the high energy demand of developing industrial countries and the climate mitigation from the GHG emissions, constitutes the most important factors which the world community turned to abundant the utilization of fossil fuels and replaced them with biofuels.

There are liquid (bioethanol and biodiesel) and solid (pellets) biofuels. Energy plants used as a basic feedstock for biofuels production. Brazil and USA are the leader producers of bioethanol and European Union is the leader producer in biodiesel.

Biofuels mostly can benefit the environment but on the other hand, there are concerns for negative environmental impacts. The energy plants participate to " neutral carbon" and reduce the climate mitigation. However, the utilization of N fertilizers and pesticides for greater yield cause nitrous oxide emission and effect the climate. Furthermore, the cultivation of energy crops to non agricultural lands, change the land use and creates biodiversity problems too.

Greece, as part of the European Union participates to the production of biofuels. The most important energy plants for the production of biofuels are rapeseed, sunflower, sugar beet, switchgrass, sweet sorghum, corn, wheat, barley and artichoke. From all the above energy plants, artichoke is a great challenge for the Greek biofuel production. Artichoke can provide to us liquid (biodiesel) and solid biofuels. Furthermore, artichoke

is a well adapt plant in the Mediterranean region and specially in Greece. The plant of Artichoke has the ability to provide high yields in dry areas and it doesn't need high fertilization.

The development of plant biotechnology, gave the ability to researchers to enhance the productivity of energy plants. The lignocellulosic ethanol production through poplar trees is a great challenge. Poplars are a great source of lignin. The genus of Populus evaluated as an important energy crop because Populus have a wide natural species with a bioenergy traits and we can find new genes, pathways and mechanisms which are relate to bioenergy. USA is one of the countries which invest in research of cellulosic ethanol and prospects to displace up to 30% of the nation's current gasoline use by 2030 *(USDA, 2006).*

Chart 1. *Biofuels Barometer - June 2005, EUROBSERV' ER*

Chart 2. *Biofuels Barometer - June 2005, EUROBSERV' ER*

Figure 1. www.orln.gov/info/orlnreview/v33_2_00/bioenergy.html

Figure 2. http://commons.wikimedia.org/wiki/File:Brassica_napus_2.jpg

Figure 3. http://www.planthogar.net/enciclopedia/fichas/556/girasol-helianthus-annuus.html

Figure 4. http://commons.wikimedia.org/wiki/File:Sorghum_bicolor_Bild0902.jpg

Figure 5. http://grow.ars-informatica.ca/gallery.php?img=19

Figure 6. http://www.flickr.com/photos/wicstun_tomo/458590109/

Figure 7. http://www.britannica.com/EBchecked/topic-art/571957/23324/Sugar-beet

Figure 8. http://commons.wikimedia.org/wiki/File:Zea_mays_Blanco2.279.png

Figure 9. http://blackwaternurseriesllc.com/Plant_List_2.html

Figure 10. http://commons.wikimedia.org/wiki/File:Cynara_cardunculus_InflorescenceFlowers_BotGardBln0906.jpg

Figure 11. (Skoufogianni E., 2006)

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