GENETIC DIFFERENTIATION OF EUROPEAN BLACK POPLAR (*POPULUS NIGRA* L.) CLONES AND POPULATIONS WITH RESPECT TO SOME ENZYMES INVOLVED IN BIOSYNTHESIS OF CELLULOSE AND LIGNIN

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submitted by **BİRCAN TAŞKIRAN** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Biology Department, Middle East Technical University** by,

Prof. Dr. Halil Kalıpçılar
Dean, Graduate School of **Natural and Applied Sciences** _____

Prof. Dr. Ayşegül Gözen
Head of Department, **Biological Sciences** _____

Prof. Dr. Zeki Kaya
Supervisor, **Biological Sciences, METU** _____

Prof. Dr. Nülüfer Tülin Güray
Co-Supervisor, **Biological Sciences, METU** _____

Examining Committee Members:

Prof. Dr. Orhan Adalı
Biological Sciences, METU _____

Prof. Dr. Zeki Kaya
Biological Sciences, METU _____

Prof. Dr. Füsun Eyidoğan
Program of Primary Education, Başkent University _____

Prof. Dr. Sertaç Önde
Biological Sciences, METU _____

Prof. Dr. Özlem Yıldırım
Biology, Ankara University _____

Date: 31.01.2020

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Surname: Bircan Taşkıran

Signature:

ABSTRACT

GENETIC DIFFERENTIATION OF EUROPEAN BLACK POPLAR (*POPULUS NIGRA* L.) CLONES AND POPULATIONS WITH RESPECT TO SOME ENZYMES INVOLVED IN BIOSYNTHESIS OF CELLULOSE AND LIGNIN

Taşkıran, Bircan
Doctor of Philosophy, Biology
Supervisor: Prof. Dr. Zeki Kaya
Co-Supervisor: Prof. Dr. Nülüfer Tülin Güray

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The European black poplar, (*Populus nigra* L.) is ecologically and economically important fast-growing trees. Its wide distribution range in northern hemisphere, easy vegetative propagation, rapid growth rate and hybridization ability, and also its biomass source potential make European black poplar a perfect candidate for lignocellulosic biomass production. In this study, specific activities of two important cellulose related (SuSy and UGPase) and three lignin pathway related (PAL, 4CL, and CAD) enzymes, were evaluated in natural *P. nigra* clones. In addition to cellulose and lignin related enzyme activities, cellulose, lignin, and glucose contents, as well as height and diameter of clones were examined to explore genetic variance components, heritability of traits, and relationships between these traits. Also, clones with lignocellulosic potential were identified to be used for future breeding studies and industrial applications. As a total, 285 clones (genotypes) represented by 4 ramets and replicated twice are used for the study. The results of our study indicated that the great portion of total variation was due to clonal variation ranging from 33.0% (in UGPase) to 64.1% (in glucose). Moderate to high clonal heritabilities were estimated for traits ranging from 0.50 (in UGPase) to 0.80 (in glucose). All lignin related traits showed

moderate heritabilities ranging from 0.53 (in CAD) to 0.61 (in PAL). Number of clones with desired cellulose and lignin properties were found. One of these clones, 62160 was among the highest 12 clones regarding UGPase, SuSy, cellulose, height, and diameter, but it was also among the lowest 12 clones with respect to PAL and CAD activities. Phenotypic correlations of cellulose content with SuSy (r_p=0.184) and UGPase (r_p=0.160) were low, but significant. Although PAL, 4CL, and CAD were positively correlated with each other, surprisingly there were no significant relationships detected between lignin content and these enzymes. The positive correlation among these phenylpropanoid pathway enzymes clearly demonstrated the flux of the substrate into the pathway, but no relationships between lignin content and them suggested that there might be more complicated regulations in the pathway which leads to change in lignin composition rather than lignin deposition.

Keywords: *Populus*, cellulose, lignin, enzymes, and genetics

ÖZ

SELÜLOZ VE LİGNİN BİYOSENTEZİNDE YER ALAN BAZI ENZİMLER BAKIMINDAN AVRUPA KARA KAVAK (*POPULUS NIGRA* L.) KLON VE POPULASYONLARININ GENETİK FARKLILAŞMASI

Taşkıran, Bircan
Doktora, Biyoloji
Tez Danışmanı: Prof. Dr. Zeki Kaya
Ortak Tez Danışmanı: Prof. Dr. Nülüfer Tülin Güray

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Avrupa kara kavağı (*Populus nigra* L.) ekolojik ve ekonomik olarak önemli ve hızlı büyüyen bir ağaçtır. Kuzey yarımküredeki yaygın dağılımı, vejetatif üreme ile çoğalabilmesi, hızlı büyüme oranı ve melezleşme kabiliyeti ve ayrıca biyokütle kaynağı olarak potansiyeli, Avrupa kara kavağını lignoselülozik biyokütle üretimi için mükemmel bir aday yapmaktadır. Bu çalışmada, selüloz ile ilişkili iki önemli enzim (SuSy ve UGPase) ve üç lignin yolağı ile ilişkili enzim (PAL, 4CL ve CAD) özgül aktiviteleri açısından *P. nigra* klonlarında değerlendirilmiştir. Selüloz ve lignin ile ilişkili enzim aktivitelerine ilaveten, klonların selüloz, lignin, glukoz içerikleri ve boy, çap ölçüleri, genetik varyasyon bileşenlerini ve bu karakterlerin kalıtımını ve aralarındaki ilişkiyi belirlemek için gerçekleştirilmiştir. Ayrıca, gelecekteki ıslah çalışmalarında ve endüstriyel uygulamalarda kullanılmak üzere, lignoselülozik potansiyeli olan klonlar belirlenmiştir. Çalışmada toplam, iki tekrardan alınan ve 4 ramet ile temsil edilen 285 klon (genotip) kullanılmıştır. Çalışmanın sonuçları, toplam varyasyonun büyük bir kısmının, %33,0 (UGPase'de) ve 64,1% (glukozda) aralığında değiştiğini ve klonal farklılıktan kaynaklandığını göstermiştir. Karakterler için 0,50 (UGPase'de) ve 0,80 (glukozda) aralığında değişen, orta dereceden yükseğe klonal kalıtım dereceleri tahmin edilmiştir. Tüm lignin ile ilişkili karakterler 0,53 (CAD) ve

0,61 (PAL) aralığında yer alan orta kalıtım dereceleri göstermiştir. İstenilen selüloz ve lignin özelliklerine sahip bazı klonlar belirlenmiştir. Bu klonlardan bir tanesi olan 62160 nolu klon, UGPase, SuSy, selüloz, boy ve çap açısından en yüksek olan 12 klon arasında yer alırken, PAL ve CAD aktiviteleri açısından ise en düşük değerlere sahip olan 12 klon arasında yer almıştır. Selüloz ile SuSY (r_f=0.184) ve UGPase (r_f=0.160) arasında ki fenotipik korelasyonlar düşük, fakat anlamlıdır. PAL, 4CL ve CAD enzimleri birbirleri ile pozitif korelasyon gösterirken, lignin içeriği ve bu enzimler arasında anlamlı bir ilişki tespit edilememiştir. Fenilpropanoid yolağında yer alan bu enzimler arasındaki pozitif korelasyon, yolaktaki substrat akışını açıkça gösterirken, lignin içeriği ve bu enzimler arasında ilişki tespit edilememiş olması, yolakta lignin depolanması yerine, lignin bileşiminde değişime neden olabilecek daha karmaşık bir düzenleme olabileceğini düşündürmektedir.

Anahtar Kelimeler: *Populus*, selüloz, lignin, enzim, and genetik

To my family,

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AMP: Adenosine monophosphate

ANOVA: Analysis of Variance

ATP: Adenosine triphosphate

Bp: Base pair

BSA: Bovine serum albumin

CAD: Cinnamyl alcohol dehydrogenase

C. Anatolia: Central Anatolia

CESA: Cellulose synthase

4CL: 4-Coumarate coenzyme A ligase

CSC: Cellulose synthase complex

CoA: Coenzyme A

Cov: Covariance

Dicot: Dicotyledonous plants

DTT: Dithiothreitol

E. Anatolia: Eastern Anatolia

EDTA: Ethylenedinitrilotetraacetic acid

EUFORGEN: European Forest Genetic Resources Program

FAO: Food and Agriculture Organization

FRA: Global Forest Resources Assessment

GLM: General Linear Model

G6PDG: Glucose 6-phosphate dehydrogenase

GWAS: Genome wide association study

HEPES: 4-(2-Hydroxyethyl)piperazine-1-ethanesulonic acid

HK: Hexokinase

INV: Invertase

IPC: International Poplar Commission

KOH: Potassium hydroxide

LDH: Lactate dehydrogenase

$MgCl_2$: Magnesium chloride

MCP: Mean cross product

MS: Mean square

NAD^+: β-Nicotinamide adenine dinucleotide oxidized

NADH: β-Nicotinamide adenine dinucleotide reduced

$NADP^+$: β-Nicotinamide adenine dinucleotide phosphate oxidized

NADPH: β-Nicotinamide adenine dinucleotide phosphate reduced

NGS: Next generation sequencing

Open. Pol: Open pollinated

QTL: Quantitative trait locus

PAL: Phenylalanine ammonia-lyase

PEG: Polyethylene glycol

PEP: Phosphoenolpyruvate

PGM: Phosphoglucomutase

PK: Pyruvate kinase

PMSF: Phenylmethylsulfonylfluoride

PP_i: Inorganic pyrophosphate

PVPP: Polyvinylpolypyrolidone

SA: Specific activity

S. Anatolia: Southeastern Anatolia

SPS: Sucrose phosphate synthase

SSR: Simple Sequence Repeats

SuSy: Sucrose synthase

TAPPI: Technical Association of Pulp and Paper Industry

TÜBİTAK: The Scientific and Technological Research Council of Turkey

UDP: Uridine diphosphate

UDP-Glu: Uridine diphoshoglucose

UGPase: Uridine diphosphoglucose pyrophosphorylase

UTP: Uridine triphosphate

LIST OF SYMBOLS

ε : Extinction coefficient

H^2: Broad sense heritability

kDa: Kilodalton

nm: Nanometer

r_p: Phenotypic correlation

r_g: Genetic correlation

U: Unit

Units / mg : μmol ml^{-1} mg^{-1} protein

CHAPTER 1

INTRODUCTION

1.1. European Black Poplar

European black poplar (*Populus nigra* L.) is one of the most important woody species with respect to its great potential for bioenergy production and other wood related products like timber, pulp, and paper (Porth and El-Kassaby, 2015). Besides being a pioneer tree species of riparian woodlands, European black poplar also appears to be good indicator of ecosystem diversity. It has a value for providing habitats to many living organisms, carbon and nutrient cycles, the rehabilitation of disrupted zones, the bioremediation of soils and the biofiltration of diffuse polluted water (Vanden Broeck, 2003; Gaudet *et al.,* 2008; Lewandowski and Litkowiec, 2017).

1.1.1. Biology, distribution, and importance

European black poplar is a member of the Salicaceae family (willow family) and its natural distribution area ranges from Europe to Siberia (Fig. 1.1.D) (Vanden Broeck, 2003; EUFORGEN*,* 2015). It is classified in Aigeiros section of the genus *Populus* (cottonwoods). It forms different population types from individual trees to metapopulations along rivers and streams especially with alluvial soils (Siler *et al.,* 2014; Velioğlu and Akgül, 2016). European black poplar trees can reach to 2.5 meters diameter and 30 meters height (Fig. 1.1.A). They can reach reproductive maturity within 10 to 15 years and live over 400 years (Stanton and Villar, 1996; Vanden Broeck, 2003). It has diploid chromosome number of 38 (x = 19, 2n = 2x = 38) and XY sex determination system (Gaudet *et al.,* 2008; Geraldes *et al.,* 2015).

Eventhough the majority of flowering plants (90%) are hermaphroditic (contains both male and floral parts in the same flower), European black poplar appears as a dioecious species (separate male and female individuals). Only 6 % of flowering plants are reported as dioecious (Renner, 2014). Wind pollination in black poplars, combined with their dioecy, has led to natural hybridization, allowing gene exchange with other poplar species especially with American *P. deltoides*. *Populus x euramericana*, the most common hybrid of black poplar, can be given as an example to the result of hybridization between *P. nigra* and *P. deltoides*. Yellow-green female catkins and purplish-red male catkins appear early Spring before the leaves (Fig. 1.1.B, C). In nature, black poplar trees can also reproduce through vegetative propagation, where genetically identical individuals, or ramets, arise clonally from roots or branches (Barsoum *et al.,* 2004; Çiftçi and Kaya, 2019).

European black poplar is a primary candidate species of many poplar breeding programs due to its hybridization potential, rapid growth ability, easy clonal propagation, quick adaptation to different environmental conditions, amount of biomass, harvest time flexibility, and low cultivation needs (Davis, 2008; Guerra *et al.,* 2013; Porth and El-Kassaby, 2015).

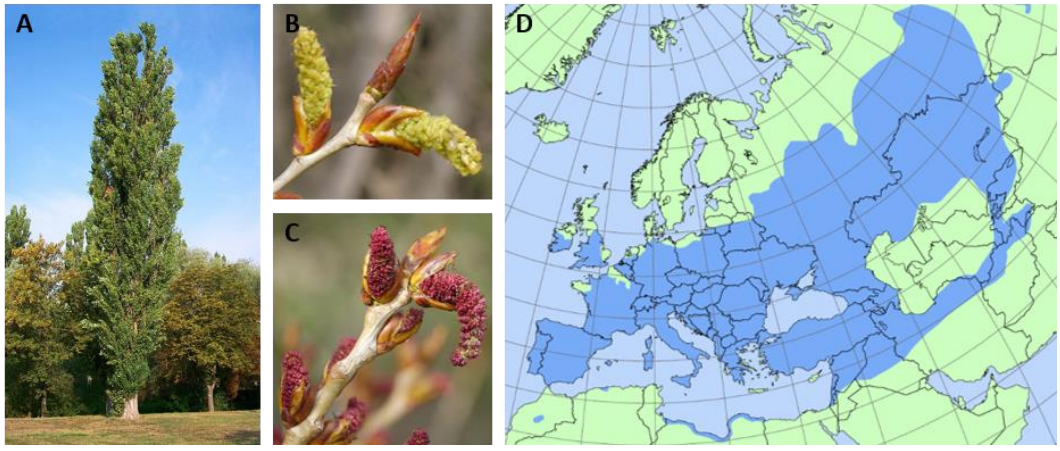


Figure 1.1. A. European black poplar tree (https://www.wikiwand.com/en/*Populus*_nigra), B. Female catkins, C. Male catkins, D. Distribution map of black poplar EUFORGEN 2015 (http://www.euforgen.org/species/*Populus*-nigra/) Retrieved on 10.11.2019

European black poplar is widely and naturally distributed in Turkey. The other native poplar species found in Turkey are *Populus alba* (white poplar)*, Populus euphratica*, and *Populus tremula* (aspen). *Populus deltoides* is not a native species, but an introduced species for plantations. It has been reported that over 145,000 hectar of pure poplar plantations exist in Turkey. Half of this is planted with commercially registered European black poplar clones, remaining with several hybrids of black poplar and *P. deltoides*. Row plantations with poplars have been practiced traditionally in Anatolia for centuries and contributed to rural and natural economy by providing 3.7 million m^3 wood annually (Toplu, 2005; Birler, 2010; Çiftçi *et al.,* 2017). According to the Country progress report of the National Poplar Commission, total forest area of Turkey is 22.3 million hectares in where about 18.3 million m^3 wood material are produced annually (Velioğlu and Akgül, 2016). Unfortunately, there is a shortage of raw wood material to meet the increasing demand. Therefore, wood material import has been started already. This increased wood demand could be met with fast growing and adaptive new clones of European black poplar.

1.1.2. Conservation and Breeding Efforts in Turkey

Although, *Populus nigra* L. is both economically and ecologically one of the most important tree species, it has been among the most threatened tree species in Turkey and Europe due to the long-term human impacts (Vanden Broeck, 2003; Çiftçi and Kaya, 2019). Therefore, under the framework of the European Forest Genetic Resources Program (EUFORGEN), conservation and breeding programs of black poplar were initiated almost 50 years ago. After the initiation of a countrywide conservation program by Poplar and Fast Growing Forest Trees Research Institute (Kocaeli, Turkey), five natural populations were identified in the Eastern Anatolia (in the Melet, Kelkit, Munzur, Karasu, and Pülümür river basins) as an *in situ* conservation study (Toplu and Kucukosmanoglu, 2003; Toplu, 2005). In 1990s, over 750 black poplar clones were selected from their natural populations. After several

field trials, four European black poplar clone banks have been established in Behicbey, Ankara, Elazığ and İzmit with 297 clones including 5 commercial clones as *ex situ* conservation program. Gazi, Anadolu, Kocabey, Geyve, and Behiçbey are the first commercially registered clones selected as a result of these efforts.

To determine the genetic diversity of black poplar clones transferred to clone banks, "Genetic characterization of Turkish Black Poplar genetic resources and development of molecular black poplar breeding program" project was conducted between 2010-2013 with collaboration of Middle East Techical University and Poplar Research Institute. The effects of drought and cold, cell wall compositions, and genetic diversity of these clones with simple sequence repeat (SSR) markers were among the several outcomes of this project (Taşkıran, 2014; Zeybek, 2014; Yıldırım and Kaya, 2017; Çiftçi *et al.,* 2017; Çiftçi and Kaya, 2019). However, to conduct breeding program for the purpose of efficient cellulosic ethanol production, it is crucial to understand the mechanisms of wood biosynthesis in European black poplar trees.

1.2. Wood

Wood represents the most important natural and renewable energy resource in our planet. It plays a crucial role in carbon cycle of terrestial ecosystem (Weih and Polle, 2016). The "wood" term is generally used to describe secondary xylem. Physical and chemical compositions of wood determine its mechanical strength and energy content. Wood is composed of cellulose (35–50%), hemicellulose (15–35%), lignin (15–35%), pectin (<10%), and integrated proteins (Plomion *et al.,* 2001; Takahashi and Schmidt, 2008).

Wood cells originate from the activity of vascular cambium. Formation of wood occurs as a result of complex developmental process called xylogenesis which includes four distinct stages: cell division, cell expansion, secondary wall synthesis, and programmed cell death (Demura and Fukuda, 2007; Déjardin *et al.,* 2010).

At the beginning of cell differentiation, primary cell wall forms. In primary cell wall, cellulose microfibrils are embedded in a matrix of pectins and hemicelluloses. When the cell reaches its ultimate size, formation of secondary cell wall starts at the inner side of the primary cell wall. Secondary cell wall is divided into three different layers, S_1, S_2, and S_3 because of differences at arrangement of cellulose microfibrils. Lignin deposition occurs at the end of the xylem differentiation in all layers (Sjostrom, 1993; Plomion *et al.,* 2001). This lignified secondary cell wall is also termed as lignocellulosic biomass and represents the main energy source of the wood (Wang and Dixon, 2011; Johnson *et al.,* 2018). Compositions and structures of cell walls vary among different species and even within the individuals of the same species, tissues, cells, and layers (Fig 1.2) (Sticklen, 2008; Loque e*t al.,* 2015).

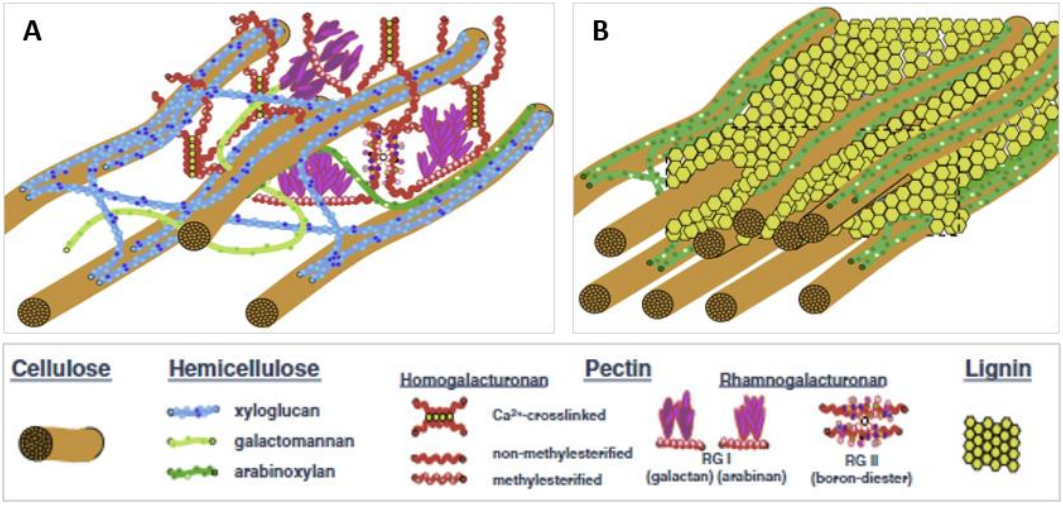


Figure 1.2. Cell wall composition of dicots. A. Primary cell walls, B. Lignified secondary cell walls (Adapted from Loque *et al.,* 2015)

Beside its biological and ecological roles, wood and its products have been used globally by humans mainly as fuel and industrial roundwood which later processed as paper, panel, shelters and so on. It has been reported that a total of 3.8 billion m^3 of wood was removed from forests around the world in 2016 of which around 50 % was

used as wood fuel and the other half was as industrial roundwood (Fig 1.3) (FAO, 2016). This amount (3.8 billion m^3) was 400 million m^3 higher than the amount announced by the 2010 Global Forest Resources Assessment (FRA) report for total wood removals (FAO, 2010). As a result of this increased demand and removal, the total forest area of our planet was reduced by around 23 million hectares between 2010 and 2016 years. The world's population was 6.4 billion in 2005, it is 7.6 billion today. It is estimated that it will increase to 10 billion in 2050. The demand for wood products will continue to increase as the number of humans grow (FAO, 2018).

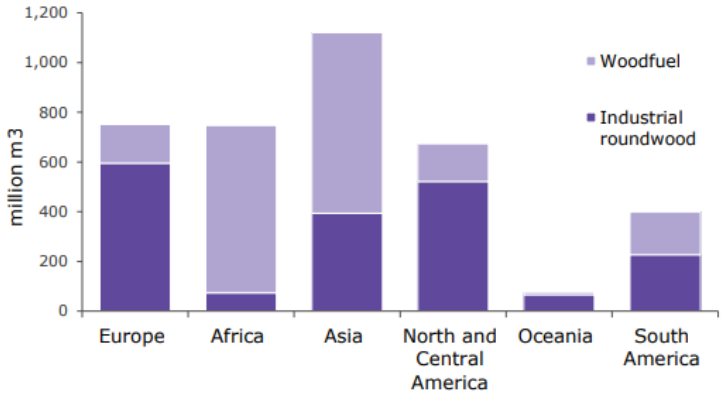


Figure 1.3. Removal of wood by region (After FAO 2016 main report)

The key questions we should ask is from which sources wood come and how it should be produced whether it will be enough for demand or not. There has been a slight shift in the sources of wood towards to plantations rather than natural forests. If we could increase the plantation supplies with fast growing trees like European black poplar, it will be possible to meet the increased wood demand and to decrease deforestration rate of global forests. Therefore, understanding the mechanism and genetics of wood biosynthesis and the factors affecting its composition have been gained great importance. That's why, in the following parts of this chapter, the main components

of wood, cellulose and lignin and the selective enzymes suggested to be related with their synthesis are briefly explained.

1.2.1. Cellulose

Cellulose is the most abundant biopolymer on earth and the main component of cell walls. Cellulose biosynthesis is not only limited to plants, but also many bacteria, protists, algae, and even animals synthesize cellulose for various biological functions. (Grimson *et al.,* 1996; Nobles *et al.,* 2001; Kimura *et al.,* 2001; Römling, 2002; Domozych *et al.,* 2012). About 40-50 % of wood dry matter is comprised of cellulose and the majority of this cellulose comes from secondary cell walls (Delmer and Haigler, 2002). With highly stable and extremely insoluble nature, and also by its strong interactions with other polymers, cellulose plays essential roles in the organization of plant cell walls which define the shape of cells by controlling cell growth and elongation.

Cellulose is an unbranched linear polymer of β-1,4-linked glucose residues in which every other glucose unit is rotated around 180º with respect to its neighbor (Fig 1.4) (Mcnamara *et al.,*2015; Eo *et al.,* 2016). To form organized microfibrils, cellulose chains are held together by hydrogen bonds and Van der Waals forces (Nishiyama *et al.,* 2002, 2003, 2009). In plants, these insoluble microfibrils can reach an amazing length up to 15,000 glucose units with 36 hydrogen bonded chains (Somerville, 2006). Further, cellulose microfibrils are assembled into macrofibers and fibers (Fig 1.5) (Rojas *et al*., 2015). Celluloses in primary and secondary cell walls display different characteristics. For example, while microfibril crystallinity and degree of polymerization are very low in primary cell walls, they are reported as very high in secondary cell walls (Müller *et al.,* 2006; Mellerowicz and Sundberg, 2008).

Cellulose synthesis takes place in the plasma membrane by membrane integrated cellulose synthase A (CESA) protein complexes (CSC). Plant CSC has been reported as a six-lobed rosette-like large structure which contains multiple CESA proteins

(Somerville, 2006; McFarlane *et al*. 2014; Kumar, 2015). Eventhough, it is suggested that 36 CESA proteins are organized in CSC (Saxena and Brown, 2005; Somerville, 2006), the exact number of CESA proteins in CSC is still uncertain (Xi *et al.,* 2017). Formation of primary and secondary cell wall involves different CESA isozymes and *CesA* genes (Meents *et al.,* 2018; Polko and Kieber, 2019).

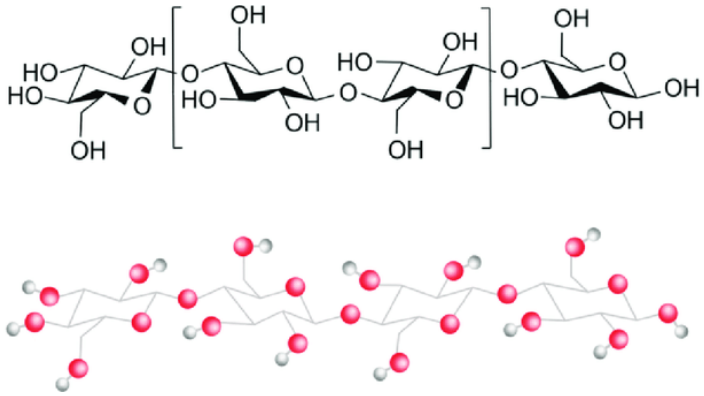


Figure 1.4. Basic structure of cellulose with β 1-4 linked cellubiose repeating units (Adapted from Eu *et al.,* 2016)

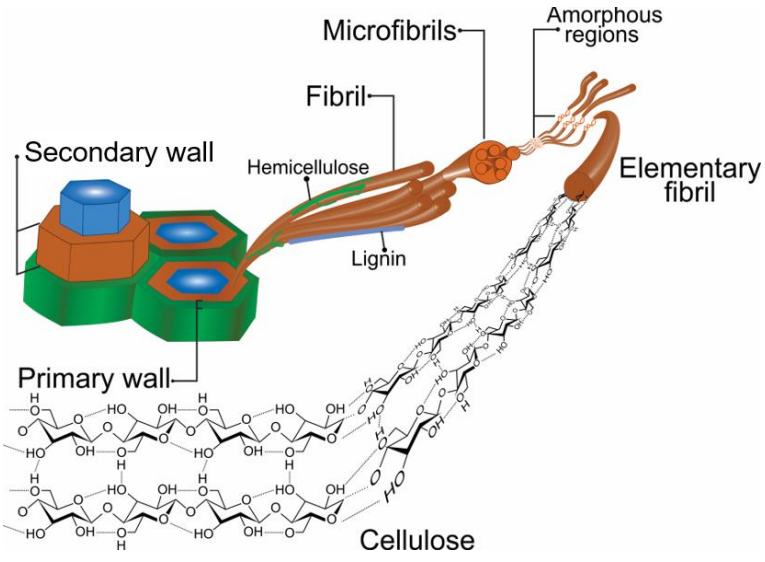


Figure 1.5. Hierarchical structure of cellulose derived from wood (After Rojas *et al.,* 2015)

CESA proteins use cytosolic uridinediphosphoglucose (UDP-Glucose) as donor sugar to synthesize β-1,4-glucan chain (Brown *et al.,* 2012; McNamara *et al.,* 2015; Verbancic *et al.,* 2018). UDP-Glucose can be synthesized by the activity of three different enzymes (Fig 1.6) which are sucrose synthase (SuSy), cytosolic invertase (INV), and UDP-glucose pyrophosphorylase (UGPase) (Delmer and Haigler, 2002; Joshi *et al.,* 2004; Barratt *et al.,* 2009; Fujii *et al.,* 2010; Tong *et al.,* 2018; Barnes and Anderson, 2018). Beside utilization by CesA proteins as a substrate, UDP-Glucose also serves as an immediate precursor for starch, callose, and pectin biosynthesis (Fujii *et al.,* 2010).

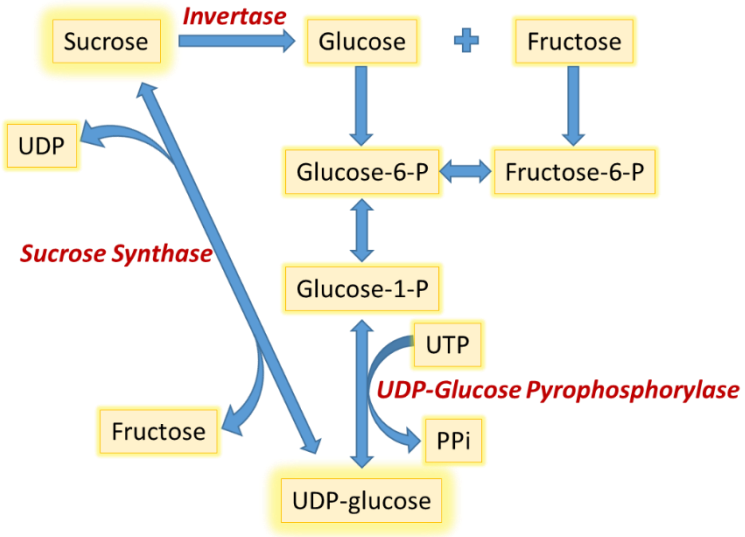


Figure 1.6. Overview of UDP-Glucose biosynthesis

1.2.1.1. Sucrose Synthase (SuSy)

In the kingdom of plants, it is well known that sucrose is the major product of photosynthesis (Dinh *et al.,* 2018). It is exported from source tissues to non-photosynthetic sink tissues as a carbon source for various metabolic pathways (Stein and Granot, 2019). To be metabolized, sucrose must be hydrolyzed by either cytosolic

invertase (INV; EC 3.2.1.26) or sucrose synthase (SuSy; EC 2.4.1.13) (Tong *et al.,* 2018). While invertase cleaves sucrose into glucose and fructose irreversibly (Barratt *et al.,* 2009), SuSy catalyzes the following reversible reaction (Fig 1.6) (Coleman *et al.,* 2009; Baroja-Fernandez *et al.,* 2012):

$$\text{Sucrose} + \text{UDP} \leftrightarrow \text{UDP-Glucose} + \text{Fructose}$$

Sucrose synthase is a member of the large glycosyltransferase enzyme family. SuSy proteins are usually accepted as homotetramers with a molecular weight of 90 kD, ~800 aa long (Schmolzer *et al.,* 2016). It has been suggested that there are two forms of SuSy found in the cells, soluble and membrane bound (Carlson and Chourey, 1996; Persia *et al.,* 2008). In the most widely accepted model for cellulose biosynthesis, UDP-glucose is provided to the cellulose synthase complex by membrane bound type SuSy (Carpita and McCann, 2000; Koch, 2004; Stein and Granot, 2019).

Based on the number of genes, considerable differences have been reported at the multigene family of SuSy between plant species. For example, six genes were identified in Arabidopsis and rice genomes (Baud *et al.,* 2004; Bieniawska *et al*. 2007; Hirose *et al.,* 2008); apple genomes with eleven distinct genes (Tong *et al.,* 2018); and only three genes were characterized in the maize and peas (Barratt *et al.,* 2001; Duncan *et al.,* 2006). In poplar genomes, 15 genes have been reported by An *et al*. (2014).

1.2.1.2. UDP-Glucose Pyrophoshorylase (UGPase)

UDP-Glucose pyrophosphorylase (UGPase) (EC 2.7.7.9) is a very important key enzyme for carbon metabolism and carries out the following freely reversible reaction (Fig 1.6) (Kleczkowski *et al.,* 2004):

$$\text{Glucose-1-P} + \text{UTP} \leftrightarrow \text{UDP-Glucose} + PP_i$$

It belongs to the nucleotidyltransferases enzyme class. It generally exists as a soluble cytosolic protein, but also as a membrane-bond one (Kleczkowski 1994a; Becker *et al.,* 1995). The direction of the reaction shows differences among source and sink

tissues. In source tissues, UGPase mainly produce UDP-glucose which eventually leads to sucrose formation by coupling the reaction with Sucrose Phosphate Synthase activity (SPS; EC 2.4.1.14), whereas in sink tissues SuSy is mostly responsible from UDP-glucose production and UGPase is mostly involved in its metabolism (Borokov *et al.,* 1996; Winter and Huber 2010; Coleman *et al.,* 2010).

The molecular weights of UGPase isozymes were calculated as about 51.7 kD in Arabidopsis (Meng *et al.,* 2008) and as 82 kD in wheat (Balan *et al.,* 2018). Over the last two decades, only two highly homologous UGPase genes have been identified in Arabidopsis (Ciereszko *et al*. 2001; Meng *et al.,* 2009b; Park *et al.,* 2010), rice (Abe *et al.,* 2002), and poplar (Meng *et al.,* 2007) genomes.

1.2.2. Lignin

Lignin is a natural phenolic polymer found mainly in secondary cell walls of plants (Fig 1.7) (Stewart *et al.,* 2009). After cellulose, it is the second most abundant biopolymers (about %30) in biosphere (Ralph *et al.,* 2004). Its complex structure and composition plays important roles in plants. Enhancing cell wall rigidity, providing hydrophobic properties and structural support, promoting mineral transport, being an important barrier against pests and pathogens, and involving in environmental stress responses can be given as several examples to these reported roles (Ithal *et al*., 2007; Bhuiyan *et al.,* 2009; Moura *et al.,* 2010; Schuetz *et al*., 2014; Liu *et al.,* 2018; Xie *et al.,* 2018).

Lignin is produced from phenylalanine by the general phenlypropanoid pathway in higher terrestial plants (Vanholme *et al.,* 2019). Monomers of lignin are synthesized in the cytosol and then transported to the cell wall to form lignin (Barros *et al.,* 2015). Lignin generally consists of three monomeric subunits; p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units which are polymerized from three monolignols known as p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, respectively (Fig 1.8) (Boerjan *et al.,* 2003; Weng *et al.,*2008; Yoon *et al.,* 2015).

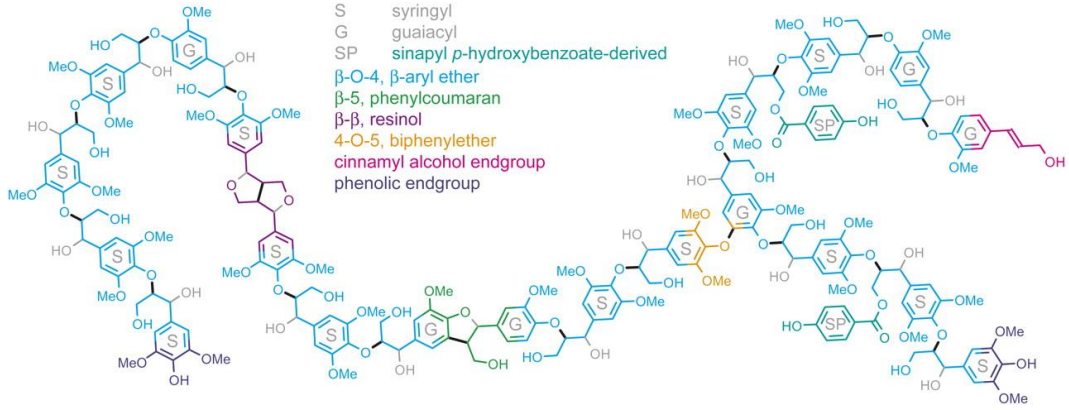


Figure 1.7. Representation of a lignin polymer from poplar, as predicted from NMR-based lignin analysis (After Stewart *et al.,* 2009)

In angiosperms, lignin mostly contains S- and G-units while in gymnosperms lignin is mostly composed of G- units and a small amount of H- units (Vanholme *et al.,* 2010). Based on lignin amount and composition, a great variation emerges between the individuals of the same species and among different species, tissues and cell types (Wang *et al.,* 2013). To illustrate, lignin in the cell wall of *Populus trichocarpa* trees was reported to be ranging from 16 to 28% and S/G ratio from one to three (Studer *et al.,* 2011).

Lignin and other cell wall polysachharides are collectively called lignocellulosic biomass and accepted as a renewable energy source for the production of biofuels. In regular processes, a chemical acid pretreatment at highly elevated temperatures is used to break down lignocellulosic materials, and all polysaccharides converted to monosaccharides by using different enzymes. Then the simple sugars are used to produce the desired chemical or fuel after fermentation by microorganisms or reforming by chemical catalysis (Loque *et al.,* 2015). Eventhough lignin is a very important component for plant fitness, it emerges as an undesirable polymer for these processes (Chen and Dixon, 2007; Li *et al.,* 2008). The need of chemical pretreatment which is costly and produces enzyme inhibitors can be eliminated by reducing the lignin content (Yang and Wyman, 2008; Min *et al.,* 2012; Wang *et al.,* 2018).

Therefore, understanding the lignin biosynthesis and gene expression has gained great importance to reduce lignin recalcitrance, to minimize lignin waste stream and also to increase saccharification efficiency in these commercial processes.

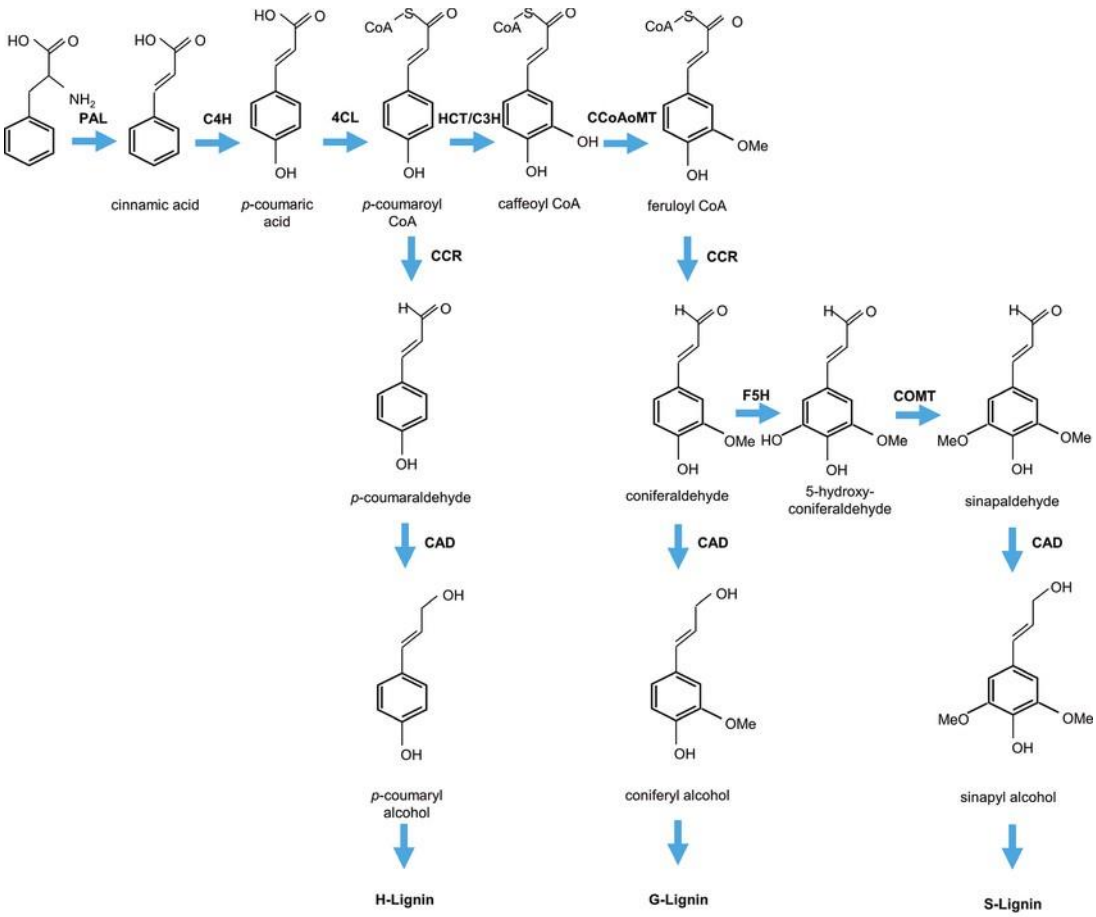


Figure 1.8. Lignin biosynthesis pathway in plants (After Yoon *et al.,* 2015). The enzymes involved in the pathway; PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; C3H, coumarate 3-hydroxylase; HCT, hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase; CCoAOMT, caffeoyl CoA 3-O-methyltransferase; CCR, cinnamoyl CoA reductase; F5H, ferulate 5-hydroxylase; COMT, caffeic acid 3-O-methyltransferase; CAD, cinnamylalcohol dehydrogenase

1.2.2.1. Phenlyalanine Ammonia-Lyase (PAL)

Phenylalanine ammonia-lyase (PAL; EC 4.3.1.24) catalyzes the initial step of phenylpropanoid biosynthesis in plants (Fig. 1.8). It produces trans-cinnamic acid by deaminating L-phenylalanine (Fig.1.9) (Vogt 2010). In plants, trans-cinnamic acid produced as a result of PAL activity leads to the formation of many important secondary metabolites which include flavonoids, alkaloids, condensed tannins, lignans, and phenolic glycosides, as well as lignin (Tsai *et al.,* 2006; Vermerris and Nicholson, 2006; Babst *et al.,* 2010; Vogt, 2010; Boecker *et al.,* 2011; Shi *et al.,* 2013). All of these products play crucial roles in defense of the plant against abiotic and biotic stress. Phenylpropanoid production rates and the flux to the phenylpropanoid pathway are determined by PAL enzyme activity (Wang *et al.,* 2014). PAL is inhibited with its own product trans-cinnamic acid (Fujita *et al.,* 2006).

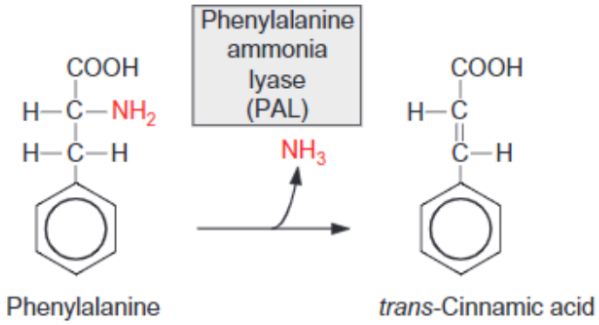


Figure 1.9. The deamination of L-phenylananine by PAL enzyme

Molecular weight of PAL enzyme is estimated in the range of 270-330 kDa (Camm and Towers, 1973; Appert *et al.,* 1994). PAL isozymes form a homo- or heterotetramer (Cochrane *et al.,* 2004; Reichart *et al.,* 2009). In poplar, PAL isozymes are encoded by a small PAL gene family with five genes (PtrPAL1-5) (Tuskan *et al*, 2006; Shi *et al*. 2010, 2013). It has been reported that PtrPAL2, 4, and 5 are mainly expressed in

xylem, while the other genes are widely expressed in every tissue (Tsai *et al.,*2006; De Jong *et al.,* 2015).

1.2.2.2. 4-Coumarate: Coenzyme-A Ligase (4CL)

Four-Coumarate: Coenzyme-A ligase (4CL; EC 6.2.1.12) is one of the key regulatory and stress response enzymes of the general phenylpropanoid pathway (Boerjan *et al.,*2003). It is well known that the first three steps of phenylpropanoid pathway are conserved in almost all dicots (Bonawitz and Chapple, 2010). The first reaction is catalyzed by the PAL enzyme, the next step is the hydroxylation of trans-cinnamic acid and it is catalyzed by cinnamate 4-hydroxylase (C4H). 4CL catalyzes the third step which includes the formation of activated CoA thioesters of hydroxycinnamic acids with two step reaction (adenylate formation followed by thioester formation) (Fig 1.8) (Vogt, 2010).

Metabolites produced by 4CL serve as precursors at the branch point to entry into different pathways of phenylpropanoid metabolism for the synthesis of lignins, flavonoids, and other phenylpropanoids (Fig 1.10) (Hahlbrock and Sheel, 1989; Lee at al., 1997; Lin *et al.,* 2015; Lavhale *et al.,* 2018). 4CL is able to ligate five cinnamic acids; 4-Coumaric acid, caffeic acid, ferulic acid, 5-hydroxyferulic acid, and sinapic acid, while 4CLs show a preference towards the first two in dicots (Rao *et al.,* 2015; Li *et al.,* 2015). Substrate affinity differences of 4CL isoforms may direct metabolic flux through different pathways (Allina *et al.,* 1998). Therefore, it can be said that the activity of 4CL enzyme determines and regulates the overall carbon flow to the phenylpropanoid pathway (Shigeto *et al.,* 2017).

In plants, the 4CL isoforms are encoded by a small number of genes. In *Arabidopsis*, four 4CL genes were found (Hamberger and Hahlbrock, 2004). In *Populus trichocarpa*, 17 4CL genes were identified (Shi *et al.,* 2010). Among these genes, Ptr-4CL3 and Ptr-4CL5 genes are reported to be encoding xylem-specific enzymes (4CL3

and 4CL5) (Chen *et al.,* 2013) and the interaction of these enzymes affects the metabolic flux direction and rate for monolignol biosynthesis (Chen *et al.,* 2014).

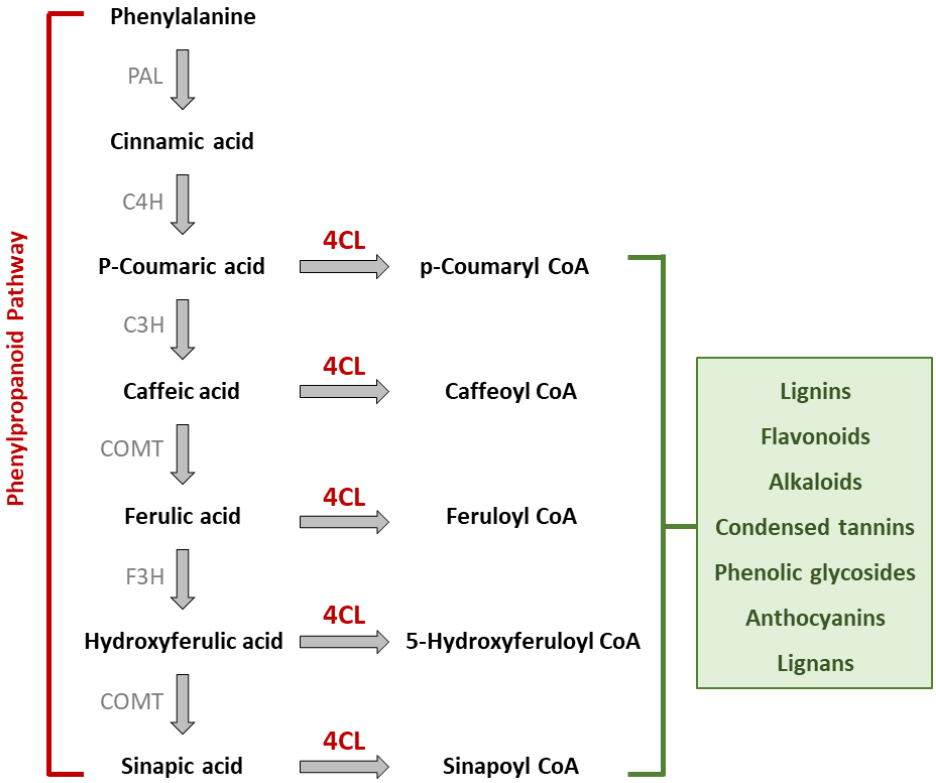
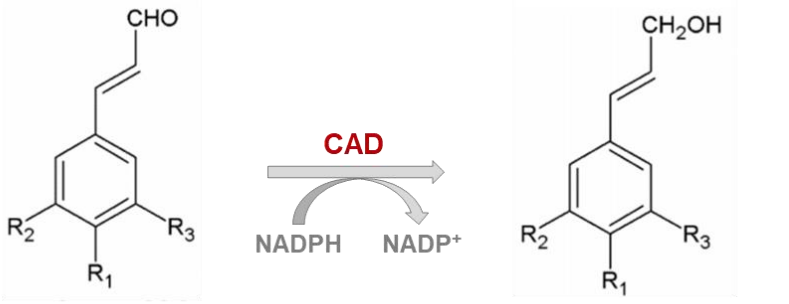


Figure 1.10. Role of 4-Coumarate:CoA ligase as a branch point enzyme. (Adapted from Lavhale *et al.,* 2018) PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; C3H, coumarate 3-hydroxylase; COMT, caffeic acid 3-O-methyltransferase; F3H, ferulate 3-hydroxylase

1.2.2.3. Cinnamyl Alcohol Dehydrogenase (CAD)

Cinnamyl Alcohol Dehydrogenase (CAD; EC 1.1.1.195) catalyzes the last step of monolignol biosynthesis, the reduction of hydroxycinnamaldehydes into cinnamyl alcohols using NADPH as a cofactor (Tronchet *et al*. 2010). In angiosperms, there are three main units found in lignin, p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units and they formed in the cell wall as a results of polymerization of their

corresponding monolignols (p-coumaryl, coniferyl, and sinapyl alcohols) which are synthesized by the activity of CAD enzyme (Fig 1.11) (Campbell and Sederoff, 1996; Saballos *et al.,* 2009; Kim and Huh, 2019).



R_1=H	R_2=H	R_3=H	Cinnamaldehyde / cinnamyl alcohol
R_1=OH	R_2=H	R_3=H	P-Coumaraldehyde / p-coumaryl alcohol
R_1=OH	R_2=H	R_3=OCH_3	Coniferaldehyde / coniferyl alcohol
R_1=OH	R_2=OCH_3	R_3=OCH_3	Sinapaldehyde / sinapyl alcohol

Figure 1.11. Reactions catalyzed by Cinnamyl alcohol dehydrogenase (CAD) enzyme

CAD proteins are a member of the oxidoreductase enzyme class. Its molecular weight was estimated about 40 kDa with two subunits (Goffner *et al.,* 1992; Kurt and Filiz, 2019). CAD enzymes are encoded by a small multigene family. In *Arabidopsis*, 9 CAD genes were identified (Sibout *et al.,*2003; Kim *et al.,* 2004) whereas 16 genes were identified in hybrid poplar (*P. deltoides x P. nigra*) (Barakat *et al.,* 2009). It is thought that different CAD genes play different roles and the number of genes directly involved in the lignification process is relatively low. To illustrate, only AtCAD4 and AtCAD5 genes have been reported to be involved in lignification in *Arabidopsis* (Sibout *et al.,* 2005). In addition to this, it was shown that, expression levels and functions of CAD genes display differences under different conditions in hybrid poplar. *Populus* CAD4 and CAD10 genes were found to be associated with lignin biosynthesis (Barakat *et al.,* 2009, 2010).

CHAPTER 2

LITERATURE REVIEW

2.1. Lignocellulosic plant biomass

Lignocellulosic plant biomass comprised mainly of cellulose, lignin and hemicellulose and it is an abundant, renewable and natural source of organic material (Somerville, 2006). Bioenergy crops like poplar, miscanthus, switchgrass, and willow are commonly used as an advantageous feedstock for cellulosic biofuel production. Cellulose and hemicellulose found in these crops are converted into ethanol and other advanced biofuels in this process. However, the recalcitrant nature of plant cell walls challenges the production of ethanol from such lignocellulosic biomass (Pauly and Keegstra, 2008; Carroll and Somerville, 2009). Since cellulose is embedded within a matrix of hemicellulose and lignin, fermentable sugar release becomes limited. Lignin acting as a mechanical barrier prevents the breaking down of cellulose and hemicellulose by hydrolytic enzymes (Vanholme *et al.,* 2008). Therefore, the release of sugar from lignocellulosic biomass is directly associated with lignin contents. Moreover, lignin inhibits fermentation processes, as it can absorbs hydrolytic enzymes (Keating *et al.,* 2006). Also, as cellulose and hemicellulose are highly water insoluble, it is very difficult to convert them into sugars efficiently.

In order to improve the process efficiency and the yields of fermentable sugar, it is necessary to make cell walls more accessible to enzymatic degradation. Chosing and developing plant varieties with more desirable cell wall compositions may contribute to useable biomass. Ideal feedstocks should contain high cellulose and low lignin content, together with low ash content and optimum S/G lignin ratio. Therefore, the amount and composition of cell wall polymers and cross-linking between them, wood density, cell wall crystallinity and saccharification efficiency have been considered as

targets for biomass improvements (Porth *et al.,* 2013; Furtado *et al.,* 2014). Understanding the chemistry, structure and biosynthesis of lignocellulosic biomass components is crucial to manage the selection and generation of desirable biomass varieties.

On the other hand, the diversity and complexity of these polymers are associated with many different genes and particular proteins. The poplar genomes contain 40000 genes (Tuskan *et al.,* 2006). These genomes are highly heterozygous and show natural genetic variation (Brunner *et al.,* 2004; Cronk, 2005). They also show high adaptive variations and among-individual genetic diversity (Keller *et al.,* 2011; Wullschleger *et al.,* 2013; Evans *et al.,* 2014). In a SNP genotyping array study of *P. trichocarpa*, 3543 candidate genes were reported to be associated with environmental adaptation and biofuel conversion from wood (Geraldes *et al.,* 2013).

Wood from poplar trees show good proportions of cell wall components and superior quality. Altogether, glucan content ranges from 40 to 62%, and lignin content from 15 to 29% of the total dry weight. The remaining 14 to 24% of dry weight accounts for xylan content (Cronk, 2005; Sannigrahi *et al.,* 2010; Studer *et al.,* 2011; Porth *et al.,* 2013a). Actually, the great variation in cell wall components and wood related traits makes genetic improvement of poplars possible which make them superior feedstock supply (Mosier *et al.,* 2005; Wegrzyn *et al.,* 2010; Studer *et al.,* 2011; Mansfield *et al.,* 2012).

Since woody plants are very rich in lignocellulose than other herbaceous crops like switchgrass and alfalfa, they are perfect candidates for the production of second-generation biofuels. With fast growth ability, high resistance to diseases, and low management cost, poplar is one of the leading candidates among woody fast-growing trees (Sims *et al.,* 2001; Wang *et al.,* 2016). Poplar is also the model tree for genetic studies since the genome assembly is available for poplar (Tuskan *et al.,* 2006). Its moderate genome size aids to molecular breeding strategies (Karp and Shield, 2008). Recently, many different approaches aiming to improve lignocellulosic potential of

energy crops have been carried on by different research groups. Linkage maps, genome editing, genetic transformation, gene silencing, hybridization studies, SSR markers, and genome wide association studies (GWAS) with next generation sequencing (NGS) based markers are the most commonly used approaches in poplar breeding programmes and biomass related studies (Porth and El-Kassaby, 2015; Ciftci *et al.,* 2017; Liu *et al*., 2018; Tuskan *et al.,* 2018; Wildhagen *et al.,* 2018; Clifton-Brown *et al.,* 2019; Ma *et al.,* 2019). Several progresses in these approaches related with cell wall polysaccharide and lignin modification are described below.

2.1.1. Cell wall polysaccharides modification

In order to increase specific cell wall sugars by genetic manipulation, glycosyltransferases and glycan synthases were considered as promising targets (Loque *et al.,* 2015). Surprisingly, cellulose synthase overexpression did not lead to boost in cellulose contents in poplar. However in the same study, silencing of endogenous cellulose synthases resulted in cell wall cellulose decrease and defective phenotype (Joshi *et al.,* 2011). Similarly, *Arabidopsis* CesAs mutants displayed reduced cellulose content and crystallinity and also dwarfism (Harris *et al.,* 2012; Fujita *et al.,* 2013). Recently, it was suggested that secondary cell wall formation may involve two type of cellulose synthase complexes in poplar. While expression of one type CSCs was increasing during the cell wall thickening, the other one is decreasing (Xi *et al.,* 2017).

Alternatively, it has been showed that overexpressing SuSy enzymes in poplar induce increase in cellulose contents of fiber cells in the range of 2 to 6%. However, this increase in transgenic plants did not affect the growth traits which remained similar to control plants (Coleman *et al.,* 2009). The achievement in this increase occurred without any apparent disturbing effect on biomass yield. On the other hand, it was reported that reduced expression of SuSy in poplar only reduced cell wall density but cellulose, hemicellulose, and lignin contents were not affected (Gerber *et al.,* 2014).

Another study related with UDP-glucose pyrophosphorylase showed that double mutant for UGPase observed with reduced cellulose synthase expression in *Arabidopsis* (Park *et al.,* 2010). Moreover, an increase in cellulose content in jute was observed as a result of overexpression of UGPase enzyme (Zhang *et al*., 2013).

Alternative strategy is to reduce xylan biosynthesis in order to increase the ratio of hexose sugars to pentose sugars in the cell wall. However, plants in this approach by using xyloglucan synthase mutants ended up with dwarfed phenotypes and collapsed xylem vessels (Jensen *et al.,* 2014). In *Arabidopsis*, silencing of gylcosyltransferases which are involved in xylan biosynthesis also gave similar results (Hao *et al.,* 2014). In poplar, the reduction in secondary cell wall thickness was observed as a result of hemicellulose disruption by RNA interference. This reduction aided enzymatic saccharification and glucose yield (Lee *et al.,* 2009).

In the study of Porth *et al*. (2013b), 48 SNPs associated with cell wall carbohydrate traits were identified by GWAS method in poplar. Their findings showed that 5.7% of total phenotypic variance was explained by the effect of these SNPs. Also, SNPs involved in cell wall crystallinity were characterized in the same study (Porth *et al*., 2013b). Moreover, in an association genetics study, some SNPs related with lignin and cellulose contents were reported in *Populus trichocorpa* (Wegrzyn *et al.,* 2010). Alternatively, it has been suggested that there is a certain relationship between identified SNPs and lignocellulosic traits in *Populus nigra* (Guerre *et al.,* 2013).

2.1.2. Lignin modification

Since lignin has negative effects on enzymatic hydrolysis of cell wall polysaccharides, there are so many research to alter lignin network and its content and structure in the secondary cell wall. The suppression of key phenylpronaid pathway enzymes is the most commonly used method to reduce lignin content. Phenylalanine ammonia lyase (PAL) is the first enzyme of phenylpropanoid pathway. It has been reported that PAL expression in *P. trichocarpa* is upregulated by wounding (Tsai *et al.,* 2006). As a result

of four mutant PAL genes, lignin content was decreased around 25% in *Arabidopsis* whereas the susceptibility to pathogens was also increased (Huang *et al.,* 2010).

Coleman *et al*. (2008) found that inhibition of coumarate 3-hydroxylase (C3H) expression reduced lignin content in poplar and S/G ratio was also changed. In transgenic *P. trichocarpa*, suppression of 4-Coumarate: CoA ligase (4CL) expression lead to lower lignin content and increased saccharification efficiency (Min *et al*., 2012). Similarly down-regulation of 4CL in hybrid poplars resulted in almost 50% of lower lignin content with respect to non-transgenic plants (Voelker *et al.,* 2010). By using, clustered requlary interspaced palindromic repeats (CRISPR) genome editing technologies, three 4CL genes were edited in hybrid poplar and 30 trangenic lines were generated. Lignin content and S/G monolignol ratios were reduced in 4CL1 gene mutants (Zhou *et al*., 2015).

Altering caffeic acid 3-O-methyltransferase (COMT) and cinnamyl alcohol dehydrogenase (CAD) which are the last enzymes in the monolignol biosynthesis has almost no impact on plant growth (Jung *et al.,* 2012). Here, CAD manipulation was used to modify lignin content and whereas COMT was used to change its composition. However, recently it was reported that CAD1 deficiency also resulted in modified lignin content and structure in poplar (Van Acker *et al.,* 2017). Moreover, up to 50% reduction in lignin content and resulting increase in cellulose content were observed as a consequence of down regulation of cinnamyl-CoA reductase (CCR) in poplar (Leplé *et al.,* 2007). Also C3H RNA suppressed poplars displayed reduced lignin content, but also stunted growth (Coleman *et al.,* 2008). Mostly, plants with down regulated lignin showed collapsed xylem vessels and dwarfed growth. Recently, the first enzymatic based study was reported with reduce lignin content without abnormal growth by creating a competitive pathway for decreasing shikimate availability to phenylpropanoid pathway (Eudes *et al.,* 2015).

There are also several studies using association mapping or quantitative trait locus (QTL) mapping methods to identify candidate genes related to lignin content and its

composition (Ranjan *et al.,* 2009; Wegrzyn *et al.,* 2010; Porth *et al.,* 2013b; Guerra *et al.,* 2013; Fahrenkrog *et al.,* 2016; Wildhagen *et al.,* 2018). For example, five putative QTLs for S/G ratio and lignin content were identified from *P. trichocarpa* populations (Muchero *et al.,* 2015)

As a mechanical barrier against pathogens, lignin accumulation plays an important role in plant disease resistance (Santiago *et al.,* 2013; Jannoey *et al.,* 2015). After infection with pathogens, cell wall accumulates a large amount of lignin which is rich in H unit (Zhang *et al.,* 2007; Miedes *et al.,* 2014). Rice PAL and C4H, *Arabidopsis* CAD5, rice 4CL and maize CCoAOMT (caffeoyl CoA 3-O-methyltransferase) genes were highly up regulated during various infections (Duan *et al.,* 2014; Trochet *et al.,* 2010; Liu *et al.,* 2016; Yang *et al.,* 2017). Similarly, various abiotic stresses such as drought, salt or heavy metal also cause lignin accumulation in the cell wall and expression levels of lignin related genes increase (Mao *et al.,* 2004; Hu *et al.,* 2009; Yıldırım and Kaya, 2017; Wildhagen *et al.,* 2018).

Overall, based on all previous studies, it can be concluded that the modification of lignin content and structure is possible through molecular manipulations. Also, engineering of lignin to reduce its accumulation can improve biofuel production efficiency. Lignin is a highly crucial polymer for plant fitness and survival (Liu *et al*. 2018), therefore it should be remembered that it is important for the maintanence of normal plant growth and development.

2.2. Justification of the study

It has been reported by many studies that poplar trees show natural genetic variation and cellulose and lignin contents change from one species to another. In order to improve lignocellulosic biomass, it is highly crucial to understand the mechanisms underlying the cellulose and lignin deposition. In this study, Sucrose synthase (SuSy) and UDP-glucose pyrophosphorylase (UGPase) enzymes were selected to examine their roles in cellulose deposition. Even though Cellulose synthase (CesA) complex is

known as the main cellulose producing machinery, it is not possible to isolate this complex to study its activity with the current methods. Phenylalanine ammonia lyase (PAL) as the first enzyme of the phenylpropanoid pathway, Four-coumarate: CoA ligase (4CL) as the first regulatory branch point enzyme, and lastly, Cinnamyl alcohol dehydrogenase (CAD) as the last enzyme of the pathway play significant roles in phenylpropanoid pathway. Therefore, these three key enzymes were chosen to understand their contribution to lignin deposition.

According to literature, the current study is the most comprehensive biochemical and physiological study which pools five key cell wall enzymes together in poplar in their natural states by using wide range of samples rather than transgenic forms. Investigating genetic diversity, the contribution of various factors to total variation, heritabilities of these traits, and also estimating relationships between these enzymes, cell wall components, and growth traits are highly crucial to understand the background of cell wall biosynthesis. The results and knowledge produced in this study might contribute to the future molecular marker assisted breeding studies.

After evaluating the genetic background and relationships among traits, a very important question was needed to be answered. How can we evaluate and utilize our European black poplar collection in terms of biomass production? Till now, only seven commercial clones were registered based on their phenotypic performances. However, as we can see from all studies and informations explained previous sections, it is highly crucial to chose and develop energy crops with lignocellulosic potential. As a fast-growing tree, *Populus nigra* has a great potential for biomass and bioenergy production. European black poplar clonal collections used in this study were selected from the natural range of the species in Turkey and results of sixty-years-efforts. The results of this study when combined with previous studies originated from our laboratory (Zeybek, 2014; Taşkıran, 2014; Yıldırım and Kaya, 2017; Ciftçi *et al.* 2017) which utilized the same clonal collection, it is possible to identify new potential *P. nigra* clones to be registered as commercial clones which will be suitable to different geographic regions of Turkey.

2.3. Objectives of the study

In this study, two important key enzymes namely, sucrose synthase (SuSy) and UDP-Glucose pyrophosphorylase (UGPase) of cellulose biosynthesis and three important phenylpropanoid pathway enzymes, phenylalanine ammonia lyase (PAL), 4-coumarate: CoA ligase (4CL), and cinnamyl alcohol dehydrogenase (CAD) were selected in order to examine following objectives in *Populus nigra*:

- To investigate genetic variations in these enzymes, cellulose, lignin, glucose contents and growth in *Populus nigra* clones which were gathered as a long-term *ex-situ* conservation program.

- To estimate the heritabilities of traits

- To examine the relationships among lignocellulosic traits and growth

- To determine of European black poplar clones with high lignocellulosic potential to be used future pulp and biofuel plantations.

CHAPTER 3

MATERIALS & METHODS

3.1. Materials

3.1.1. Plant Materials and Sampling

Ankara Behiçbey *Populus nigra* clonal experiment containing two-hundred and ninety-seven clones (genotypes) was established in 2009 as an *ex-situ* conservation program with the collaboration of the METU, Central Anatolia Forest Research Institute in Ankara and Poplar and Fast-Growing Research Institute in İzmit, Ministry of Forestry and Water Affairs of Turkey. Details of the Behiçbey European black poplar clonal experiment can be found in the project submitted to The Scientific and Technological Council of Turkey, "Genetic characterization of Turkish Black Poplar genetic resources and development of molecular black poplar breeding program" (project number TOVAG-110O570) and also in the study of Ciftci *et al.,* (2017).

Originally, each genotype was represented with five ramets within each of the three replicates in this experiment. After grouping these genotypes according to their original sampling locations within the biogeographic regions of Turkey, they were defined as populations of clone collection (Table 3.1) and instead of "genotype" term, "clone" term was used to define these clonally propagated genotypes in the current study.

Due to missing clonal information, including commercially registered five European black poplar clones and foreign clones, there were 285 genotypes available for the study. To meet the objectives, 285 black poplar genotypes as a total of four clonal ramets from two replications were sampled from Behiçbey Nursery at the end of June 2015 to maximize wood specific enzyme activities.

Table 3.1. *Information on clonal experiment of European black poplar established in Behiçbey Nursery*

Clone collection populations	Number of clones	Latitude (range)	Longitude (range)	Mean of altitudes (m)
C. Anatolia	81	37˚ 52’ N - 39˚ 57’ N	32˚ 35’ E - 32˚ 54’ E	1205
E. Anatolia	57	38˚ 25’ N - 39˚ 57’ N	38˚ 20’ E - 41˚ 15’ E	1829
Aegean	20	37˚ 42’ N - 38˚ 45’ N	29˚ 02’ E - 30˚ 33’ E	715
Black Sea	34	40˚ 15’ N - 40˚ 40’ N	36˚ 30’ E - 35˚ 50’ E	1163
Mediterranean	11	37˚ 05’ N - 37˚ 37’ N	36˚ 10’ E - 36˚ 53’ E	1027
S. Anatolia	10	37˚ 06’ N - 37˚ 46’ N	27˚ 23’ E - 38˚ 17’ E	748
Marmara	20	37˚ 47’ N - 40˚ 05’ N	30˚ 30’ E - 30˚ 05’ E	280
Foreign	18	Unknown	Unknown	Unknown
Open Pollinated	20	Unknown	Unknown	Unknown
Unknown	24	Unknown	Unknown	Unknown

For measurements of enzyme activities, rectangular wood samples (around 7 mm x 5 cm x 10 cm) were taken from the trunk wood of five year old trees above 130 cm from the ground after removing the bark. Samples were immediately frozen in liquid nitrogen and stored at -80 ˚C until use. Frozen tissues were ground in a mortar chilled with liquid nitrogen and crude extracts were obtained. Enzyme assays (Section 3.2.2) were immediately performed following the protein extraction (Section 3.2.1) to prevent possible protein degradation and activity loss due to faulty storage conditions.

In the study, altogether 10 variables were measured. The units of enzyme specific activities were calculated as “μmol min^{-1} mg^{-1} protein”. Throughout the manuscript, for the ease of understanding, they are abbreviated as “Units / mg” (Table 3.2).

Table 3.2. *Measured Populus nigra traits and their units*

Variables	Units
UGPASE activity SuSy activity PAL activity 4CL activity CAD activity	Units / mg protein or μmol min^{-1} mg^{-1} protein
Cellulose content[a] Acid soluble lignin content[a] Glucose content[a]	μg / ml
Height and diameter (Yıldırım, 2013)	cm

[a] Collected at the previous study by Taşkıran (2014). For the method details, see the Appendix C.

3.1.2. Chemicals

All chemicals and inhibitors used in protein extractions and enzyme assays were obtained from Sigma-Aldrich. Bradford protein reagent dye and bovine serum albumin (BSA) standards were purchased from Bio-Rad. PD-10 Sephadex G-25 columns were purchased from GE Healthcare. All enzyme activity measurements were performed spectrophotometrically by using Multiskan GO (Thermo Scientific) microplate reader with 96 well flat bottom plates (Corning).

3.2. Methods

3.2.1. Protein Extraction and Quantification

In order to study the enzyme activities in relation to cellulose deposition, the wood sections were ground with a mortar and pestle in liquid nitrogen. For SuSy activity, 1.6 mL of 100 ml Hepes-KOH buffer (pH 7.5) containing 5 mM $MgCl_2$, 2 mM EDTA, 2 mM dithiothreitol (DTT), 1 μM Pepstatin, 1 μM Leupeptin A, 20 μM PMSF, 0.1% (v/v) Triton X-100, 3% (w/v) Polyethylene Glycol were added to 0.1 g fresh weight

of wood powder. After chilling on ice for 20 min with two times vortexing, tissue debris was removed by centrifugation at 12,000 g, for 20 min at 4 °C. Immediately after centrifugation, 200 µL of crude extract was used for enzyme assays in a total volume of 250 µL reaction mixture, whereas the remaining supernatants were frozen at -80 °C as small aliquots (Schrader and Sauter, 2002).

To perform UGPase assay, 0.15 g powder was extracted on ice by adding 1.5 mL extraction solution containing 100 mM Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT, 1% (w/v) polyvinylpolypyrolidone (PVPP), 0.05 % (v/v) Triton X-100, 5 mM Aminocaproic acid, 1 mM PMSF, 1 µM Leupeptin, and 1 µM Pepstatin. The mixture was centrifuged at 14,000 g for 10 min at 4 °C. The resulting 10 µL supernatant was used for UGPase activity assay immediately in a total volume of 125 µL reaction mixture (Ciereszko *et al*., 2001).

Protein extractions for PAL and CAD assays were performed as described by Cabanè *et al.,* 2004). 0.15 g sample powder was mixed to 1.8 mL of 100 mM Hepes-KOH buffer (pH 7.5) containing 2 mM DTT, 5 mM $MgCl_2$, 5 mM EDTA, 1 µM Pepstatin, 1 µM Leupeptin A, 20 µM PMSF, 0.5% (w/v) PVPP-10, 0.5% (w/v) Polyethylene Glycol (PEG) and 10% (v/v) glycerol. Afterwards, the mixture was centrifuged at 18,500 g for 20 min at 4 °C. 1.5 mL of the supernatant was desalted using PD-10 Sephadex G-25 column (GE Healthcare) with 100 mM Hepes-KOH (pH 7.5) equilibration buffer containing 2 mM DTT, 5 mM $MgCl_2$, and 10% (v/v) glycerol. The resulting desalted extract was used for PAL and CAD enzyme assays.

4CL protein mixture was extracted by using extraction buffer (1.6 mL/ 0.2 g wood powder) containing 200 mM Tris-HCl (pH7.8), 30% (v/v) glycerol, 8 mM $MgCl_2$, 5 mM DTT, 3% (w/v) PVPP-10, 1 µM Pepstatin, 1 µM Leupeptin A, and 1 mM PMSF. The extracts were centrifuged at 4 °C and 18,000 g for 15 min. 1.5 mL of the supernatant was desalted using PD-10 Sephadex G-25 column (GE Healthcare) with 200 mM Tris-HCl (pH7.8) equilibration buffer containing 5 mM DTT, 8 mM $MgCl_2$,

and 10% (v/v) glycerol. The resulting supernatant was used for 4CL assays (Li *et al*., 2015).

Total protein content of the extracts for all enzyme assays was determined using Bio-Rad Bradford protein reagent dye with bovine serume albümin (BSA) as standard (Bradford, 1976).

3.2.2. Enzyme Activity Assays

3.2.2.1. Sucrose Synthase (SuSy) Assay

The activity of SuSy was monitored in sucrose synthesis direction by using a two-step end point assay that couples fructose and UDP-glucose dependent UDP production to NADH oxidation at 340 nm according to Schrader and Sauter (2002) with minor modifications (Fig. 3.1). All assays were carried out in triplicates and corrected for reagent and substrate blanks. The crude extracts were first incubated at 37 °C for 45 min in 100 mM Hepes KOH buffer (pH 7.5), containing 4 mM UDP-glucose, 20 mM fructose and 5 mM $MgCl_2$. The reaction was stopped by heating at 95 °C for 3 min. After cooling at -20 °C and vortexing, the UDP content of samples was quantified by pyruvate kinase/lactate dehydrogenase (PK/LDH) coupled reaction by using 50 µL of supernatant, 0.8 mM phosphoenolpyruvate (PEP), and 0.3 mM NADH as substrates in 100 mM Hepes KOH buffer (pH 7.5) containing 5 mM $MgCl_2$. After the addition of 1.5 µL PK/LDH (450 U PK/ 450 U LDH, Sigma-Aldrich) to a total volume of 200 µL, the decrease in absorbance was determined at 340 nm by using the microplate reader. Molar extinction coefficient of 6.22×10^3 $M^{-1}cm^{-1}$ for NADH at 340 nm was used for calculations. The amount of the enzyme required to oxidize 1 µmol NADH per minute was defined as a unit of SuSy.

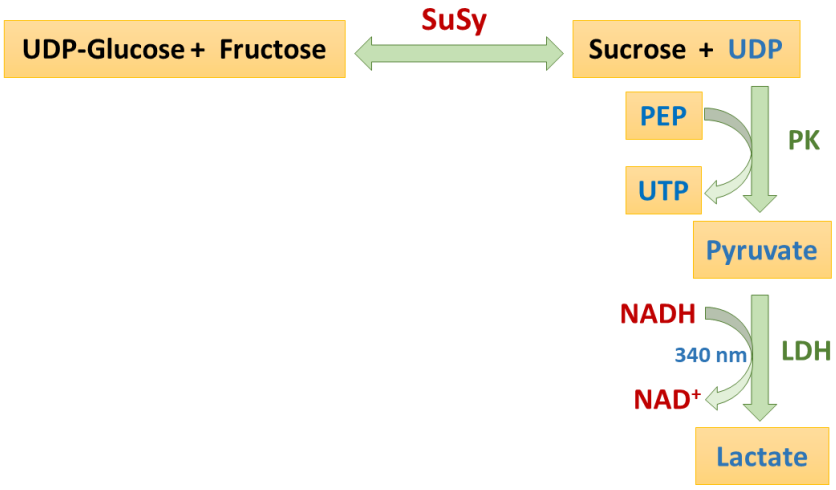


Figure 3.1. Steps of the Sucrose synthase (SuSy) enzyme assay: UDP, Uridinediphosphate; PEP, phosphoenolpyruvate as substrate of pyruvate kinase (PK); UTP, uridine triphosphate; LDH, lactate dehydrogenase

3.2.2.2. UDP-Glucose Pyrophosphorylase (UGPase) Assay

UGPase activity assay was performed as described by Ciereszko *et al*. (2001a) with some modifications. The enzyme activity was followed in the pyrophosphorolytic direction of UGPase using inorganic pyrophosphate (PPi) and UDP-Glucose as substrates in the presence of phosphoglucomutase (PGM), glucose 6-P dehydrogenase (G6PDH) and $NADP^+$ to couple the Glucose 1-P production for the formation of NADPH (Fig 3.2). The standard reaction mixture contained 100 mM Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$, 0.8 mM UDP-Glucose, 0.3 mM $NADP^+$, 0.5 Unit PGM, 0.5 Unit G6PDH, and 10 µL extract in 125 µL total volume. The reactions were initiated with the addition of 1 mM PPi. All assays were run in triplicates and corrected for substrate (PPi) blanks. The decrease in $NADP^+$ was measured spectrophotometrically at 340 nm for 30 minutes at 25 °C. Molar extinction coefficient 6.22×10^3 $M^{-1}cm^{-1}$ for NADPH was used for calculations. The amount of the enzyme required to reduce 1 µmol $NADP^+$ per minute was defined as a unit of UGPase.

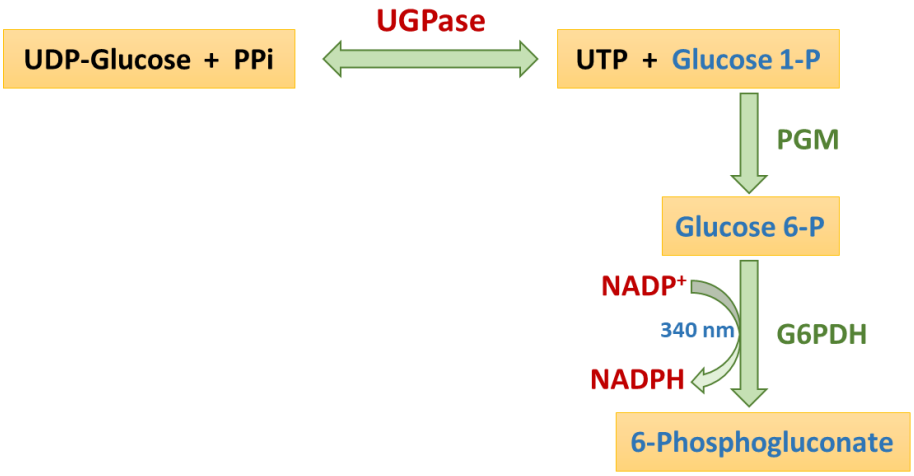


Figure 3.2. Steps of the UDP-Glucose pyrophosphorylase (UGPase) enzyme assay: UTP, Uridine triphosphate; PGM, Phosphoglucomutase; G6PDH, Glucose 6-Phosphate dehydrogenase

3.2.2.3. Phenylalanine Ammonia Lyase (PAL) Assay

PAL activity was detected spectrophotometrically by using L-phenylalanine as substrate and measuring the amount of trans-cinnamic acid formed at 290 nm ($\varepsilon_{transcinnamate}$ = 1x10^4 M^{-1}cm^{-1}) as described by Richet *et al.,* (2011) (Fig 3.3). In a total volume of 250 µL reaction mixture, 40 µL of the extract was mixed with 100 mM Boric acid buffer (pH 8.5) and the reaction was initiated with 15 mM L-Phenylalanine. The reaction was followed by using microplate reader at 30 ˚C. As a control, substrate blank was used. The activity was expressed as µmol of trans-cinnamate mg^{-1} protein min^{-1} (See Appendix C for specific activity calculation).

Figure 3.3. Phenylalanine ammonia lyase (PAL) enzyme assay

3.2.2.4. 4-Coumarate: Coenzyme-A Ligase (4CL) Assay

4CL enzymatic activity was performed according to Li *et al*. (2015) with minor modifications (Fig 3.4). The reaction mixture contained 40 µL crude extract, 200 mM Tris-HCl (pH7.8), 5 mM ATP, 5 mM $MgCl_2$, and 0.2 mM 4-coumaric acid in a total volume of 200 µL. The reaction was initiated by the addition of 0.15 mM Coenzyme A (CoA). As controls, both substrate and enzyme blanks were used. The increase in absorbance of the Coumarate-CoA ester was monitored at 37 ℃ spectrophotometrically at 333 nm for an hour with 5 min intervals ($\varepsilon_{coumaryl\text{-}CoA}$ = $21x10^3$ $M^{-1}cm^{-1}$). 4CL activity was expressed as 1 µmol of 4-Coumarate converted to product per minute per milligram of protein.

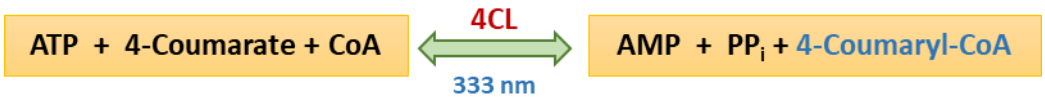


Figure 3.4. 4-Coumarate: CoA ligase (4CL) enzyme assay

3.2.2.5. Cinnamyl Alcohol Dehydrogenase (CAD) Assay

The CAD activity was measured by following coniferyl alcohol oxidation to coniferaldehyde at 400 nm ($\varepsilon_{coniferaldehyde}$= $21x10^3$ $M^{-1}cm^{-1}$) for an hour at 30 ˚C as described by Di Baccio *et al*. (2008) with minor modifications which includes slight differences in concentrations of substrates (Fig 3.5). The reaction was carried out in a total volume of 200 µL containing 100 mM Tris–HCl (pH 8.8), 0.5 mM $NADP^+$, 100 µM coniferyl alcohol, and 40 µL enzyme extract. Substrate ($NADP^+$) blank was used as control. All determinations were done in triplicates. Enzyme activity was expressed as µmol of coniferaldehyde formed min mg^{-1} protein.

Figure 3.5. Cinnamyl alcohol dehydrogenase (CAD) enzyme assay

3.3. Wood Traits

Cellulose, lignin and glucose contents of the studied poplar clones were previously given in detail by Taşkıran (2014). Briefly, cellulose contents were estimated spectrophotometrically at 630 nm by using cold anthrone reagent as described by Uppdegraf (1969). Lignin content determination was carried out according to the standard TAPPI Test Method T250 (Dence, 1992). Glucose contents of woods were calculated by using the combination of TAPPI Test Method T250 and the method of Hedge and Hofreiter (1962) (For the details of cellulose, lignin, and glucose estimation, please check Appendix C). Growth and diameter measurements used in the study were obtained from one year and two year old *P. nigra* clones (Yıldırım, 2013).

3.4. Data Analysis

Prior to the analysis of each trait, distribution, outliers, and homogeneity of the data were evaluated. A Shapiro-Wilks (Shapiro and Wilk, 1965) test for normality was performed on each quantitative trait. All traits were distributed approximately normal except for cellulose content. The log transformation was used to normalize cellulose content data. To check the assumption of equal variance for parametric tests, Levene's tests were performed (Levene, 1960). In the methods of univariate analysis, the average value of ramets for each clone in the replicate were used. On the other hand, average values of all measurements which represent the same clone were used for the methods of multivariate analysis.

In the study of Ciftci *et al*. (2017) where the same black poplar clonal populations were used, all samples were genotyped by using 12 nuclear microsatellite DNA markers to study the genetic composition. The results of the study revealed that the clone N0375 was sampled 84 times with different clonal labelling. In our study, to prevent such shortcomings, we developed two approaches while analyzing the data. In the first one, all measurements were taken into account without considering any repeated clones. With the second approach, all repeated clones except one representative were removed from the data and evaluated as all unique clones. Unless otherwise stated, the results of unique clones will be given in the text.

3.4.1. Investigating genetic variations in cell wall related enzymes, cell wall components and growth traits

A mixed model approach by the SAS® 9.2 Software (SAS Institute, Cary, NC, USA). (Littell *et al.,* 1996) was applied to study the genetic variation underlying the traits. This Mixed Model ANOVA allowed us to estimate desired main effects of clones and regions by evaluating the undesired replicate effect using the following linear model:

$$y_{ijk} = \mu + b_k + r_i + c_{j(i)} + e_{ijk} \text{ (Eq. 1)}$$

where y_{ijk} is the response measured to the jth clone of the ith region in the kth replicates, μ is the overall mean; b_k is the random effect of replicate k; r_i is the fixed effect of ith region; and $c_{j(i)}$ is the random effect of jth clone within region i; e_{ijk} is the random error effect within the experiment.

Variance components were estimated by using the restricted maximum likelihood (REML) method of the VARCOMP procedure in the SAS software. Duncan and Tukey's multiple range tests in PROC GLM of SAS were then used for comparisons of main effects. All statistical tests were conducted at 0.05 and 0.01 significant levels.

Clonal heritability (H^2) of traits was estimated according to the method developed by Falconer and Mackay (1996) as follows (Eq. 2):

$$H^2 = \frac{\sigma^2 c}{(\sigma^2 c + \left(\frac{\sigma^2 e}{r}\right))} \qquad \text{(Eq.2)}$$

where $\sigma^2 c$ is the clonal component of total variance, $\sigma^2 e$ is the error variance, and r is the number of replications.

3.4.2. Investigation of the relationship between the cell wall related enzymes, cell wall components and growth traits

To understand the relationships among the traits, phenotypic and genetic correlations of paired traits were estimated. Scatterplot matrix of studied traits was plotted by using JMP®, Version 14 (SAS Institute Inc., Cary, NC, 1989-2019) statistical package programme. Phenotypic correlation ($r_{p(x,y)}$) between pairwise traits *x* and *y* was estimated as described by Kaya and Temerit (1994) (Eq. 3):

$$r_{p(x,y)} = \frac{MCP_{c(x,y)}}{MS_{c(x)} MS_{c(y)}} \qquad \text{(Eq.3)}$$

where $MCP_{c(x,y)}$ is the mean cross product between clones within regions for traits *x* and *y*; $MS_{c(x)}$ and $MS_{c(y)}$ are the mean squares between clones within regions for trait *x* and *y*, respectively.

Genetic correlation ($r_{g(x,y)}$) between traits *x* and *y* was estimated based on Falconer and Mackay (1996) with the following bivariate equation (Eq. 4):

$$r_{g(x,y)} = \frac{Cov_{c(x,y)}}{\sqrt{\sigma^2{}_{c(x)}\, \sigma^2{}_{c(y)}}} \qquad \text{(Eq.4)}$$

where $Cov_{c(x,y)}$ is the clonal covariance between traits *x* and *y*, estimated as (Eq. 5)

$$Cov_{c(x,y)} = \frac{\sigma^2{}_{c(x+y)} - \sigma^2{}_{c(x)} - \sigma^2{}_{c(y)}}{2} \qquad \text{(Eq.5)}$$

and σ^2_c is the clonal variance component for traits *x* and *y*, respectively.

3.4.3. Determination of the potential lignocellulosic clones

Fifty clones representing the highest and the lowest mean values of the particular trait were determined by sorting average values of clones with EXCEL program (Microsoft, WA). Then, these clones were separately analyzed to examine possible changes on the relationship of the traits by using JMP®, Version 14 (SAS Institute Inc., Cary, NC, 1989-2019) statistical package. As a next step, the highest and the lowest 10 clones were selected. However, since a few clones were detected among the first 12 clones with respect to mean values of several traits, reporting the first 12 clones instead of 10 clones was preferred. Afterwards, the chosen clones of the trait were compared with the other related traits in terms of mean values and ranks.

CHAPTER 4

RESULTS

Prior to the analysis, normality, outliers, and homogeneity of variance were evaluated for each trait. Except cellulose content, all traits were normally distributed based on Shapiro-Wilks test results. In cellulose contents data, severe positive skewness was detected. To normalize this skewness, log transformation was performed. Other traits were not modified (Fig. 4.1). To check the assumption of equal variance for parametric tests, Levene's tests were performed (Levene, 1960). The results indicated that samples were obtained from populations of equal variances which leaded us to perform Mixed Model ANOVA by SAS software to check the contribution of different sources to total variation.

In this study, specific activities of five key enzymes acting on cellulose and lignin biosynthesis were measured and examined together with five other growth and cell wall traits. The wide-scale sampling strategy made it possible to perform appropriate statistical analysis to clarify the relationship between these enzyme activities and growth and cell wall characteristics.

As mentioned earlier, two approaches were followed for the evaluation of clones. Desriptive statistics of two approachs for all clone and unique clone data in which all duplications of N0375 clone were removed from the data did not show much differences in results of all studied traits (Table 4.1).

The results of descriptive statistics by traits indicated that, there are highly significant clone mean differences in studied enzyme activities. For example, in both SuSy and UGPase which are related to cellulose synthesis, mean values for SuSy specific activities (242.1 ± 69.2) were five times higher than UGPase activities (51.7 ± 12.1). Similar kind of pattern was also detected for lignin related enzymes. PAL, the

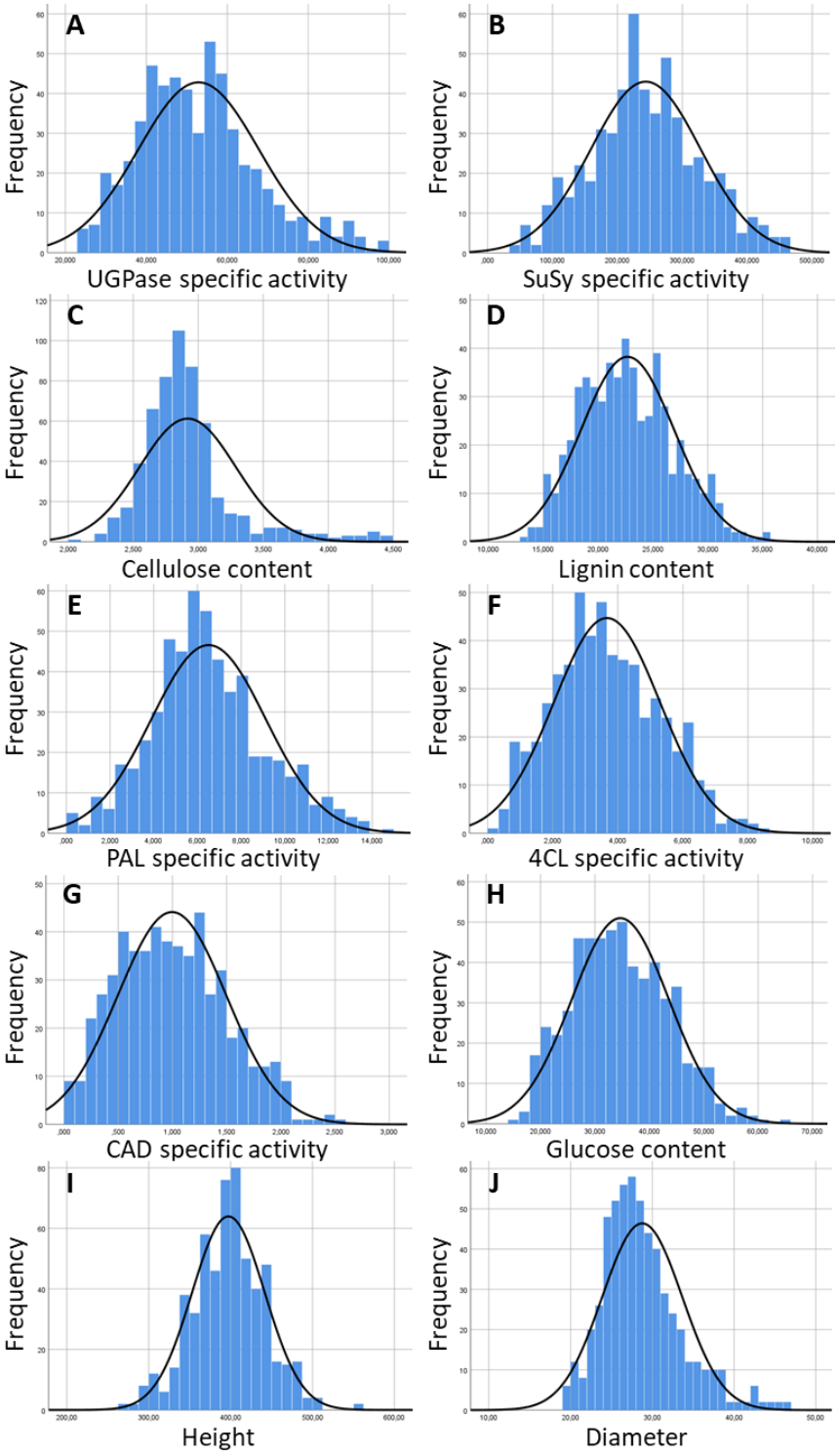


Figure 4.1. Frequency distribution of all traits in European black poplar clones: A. UGPase activity, B. SuSy activity, C. cellulose content, D. lignin content, E. PAL activity, F. 4CL activity, G. CAD activity, H. glucose content, I. height measurements, and J. diameter measurements

Table 4.1. *Descriptive statistics of all traits*

	All Clones		*Unique Clones*	
Variable	*N*	*Mean ± SD*	*N*	*Mean ± SD*
UGPASE	281	53.0 ± 12.2	196	51.7 ± 12.1
SuSy	275	243.5 ± 75.6	191	242.1 ± 69.2
Cellulose	285	20.3 ± 10.1	200	21.0 ± 11.2
PAL	279	6.5 ± 2.2	196	6.6 ± 2.2
4CL	279	3.7 ± 1.4	196	3.7 ± 1.3
CAD	281	1.0 ± 0.4	196	1.0 ± 0.4
Lignin	285	22.7 ± 3.5	200	22.4 ± 3.6
Glucose	285	34.7 ± 8.2	200	35.2 ± 8.1
Height	285	397.4 ± 44.5	200	399.3 ± 48.6
Diameter	285	28.8 ± 4.9	200	29.6 ± 5.3

first enzyme of the phenylpropanoid pathway had the highest enzyme activities (6.6 ± 2.2) while the smallest mean activity values were observed for CAD (1.00 ± 0.4), the last enzyme of monolignol biosynthesis. PAL activity was six times higher than CAD activity and 4CL mean value is between these two.

4.1. Genetic variations in cell wall related enzymes, cell wall components and growth traits

Mixed model ANOVA, variance component and broad sense heritability estimations were performed with all clone and unique clone data separately to detect the contribution of repeated clones to the total variance. The results were similar to each other in all studied traits. All traits varied significantly among clones within regions ($p<0.001$). Except PAL activity, there were significant variation also among regions for the other traits ($p \leq 0.05$). (Table 4.2).

According to the variance component estimation, the great portion of the total variance observed in UGPase (65.3%), PAL (55.9%), 4CL (62.9%), CAD (64.0%) and lignin content (57.9%) were due to within clones. The proportion of variation due to clones within regions were ranged from 33.0% (UGPase) to 44.1% (PAL) of the total variance. On the other hand, the great portion of variations observed in SuSy (53.2%), cellulose (60.0%) and glucose (64.1%) contents were due to clones within regions. Within clone variation of these traits was estimated as 43.1%, 40.0%, and 32.9%, respectively. Altogether, the repeat and region components of variance ranged from 0.8% to 3.8% (Table 4.2). Estimated broad sense heritabilities which give the magnitude about inheritance of the trait were high for SuSy (0.71), cellulose (0.75), and glucose contents (0.80) traits. For the remaining traits, broad sense heritabilities were moderate and estimated as 0.50 for UGPase, 0.61 for PAL, 0.54 for 4CL, 0.53 for CAD, and 0.57 for lignin content (Table 4.2).

Table 4.2. *Mean squares, variance components as percent of total variance (VC), and broad sense heritability estimates (h^2) for the studied traits*

Trait	*Rep. (df=1)*	*Region (df=9)*	*VC*	*Clones/regions (df=190)*	*VC*	*Error (df=199)*	*VC*	h^2
Cellulose	65.54^{ns}	456.37^{**}	0.00	241.00^{**}	60.00	54.03	40.00	0.75
SuSy	1322.69^{ns}	17597^{**}	3.75	9166.67^{**}	53.20	2651.02	43.05	0.71
UGPase	161.51^{ns}	411.62^{**}	1.67	284.20^{**}	33.02	141.52	65.32	0.50
Lignin	3.32^{ns}	47.14^{**}	3.16	24.57^{**}	38.99	10.46	57.85	0.57
PAL	21.44^{*}	4.26^{ns}	0.00	9.65^{**}	44.11	3.81	55.89	0.61
4CL	1.26^{ns}	3.99^{*}	0.81	3.34^{**}	36.29	1.56	62.90	0.54
CAD	0.1^{ns}	0.36^{*}	0.00	0.35^{**}	36.00	0.16	64.00	0.53
Glucose	76.89^{ns}	207.44^{**}	2.97	128.43^{**}	64.14	26.20	32.88	0.80

^{ns} Not significant at $p < 0.05$; * Significant at $p \leq 0.05$; ** Significant at $p \leq 0.01$

Among all regions, SuSy activity was the highest for clones originating from Marmara region (288.7) and the lowest in foreign clones (185.8) (Fig. 4.2). On average, clones from other regions yielded similar SuSy activity. Like SuSy, UGPase activity was also the highest for clones of Marmara region (58.4) and it was the lowest open pollinated (45.2) and foreign (48.3) clone populations (Fig. 4.3). Similarly, the lowest mean CAD activity values are detected for foreign (0.7) and open pollinated (0.8) clone populations (Fig 4.4). 4CL was also the lowest in open pollinated population (3.0), but it was calculated as the highest in foreign clones (4.2). On the other hand, PAL activity did not change among regions and mean of regions was reported as 6.5. (Fig. 4.5) Differences among regions based on studied enzyme activities were mainly due to the foreign and open pollinated populations. In general, the region component of the total variance was very low (lower than 4%) for all traits (Table 4.2 and 4.3).

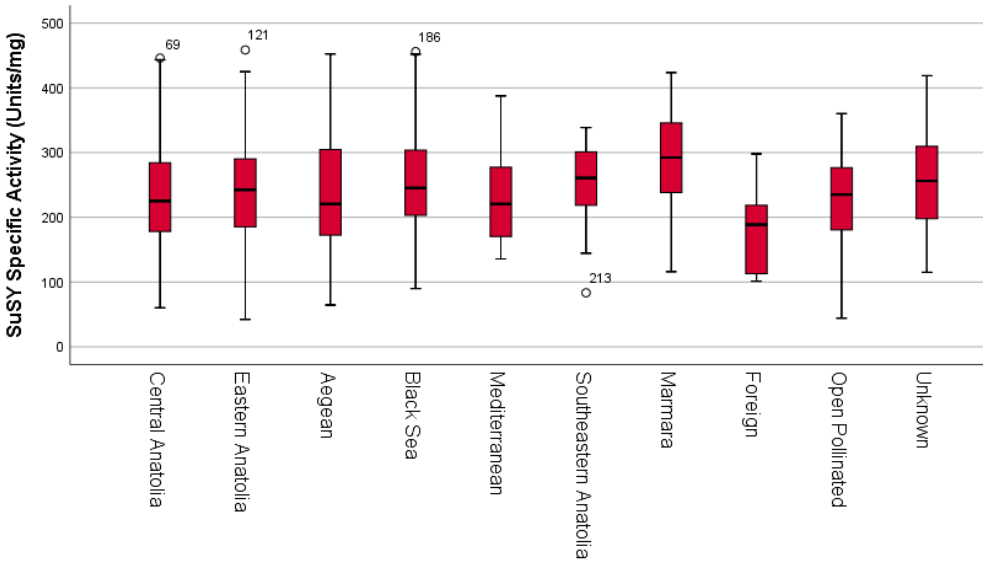


Figure 4.2. Boxplots of SuSy activity

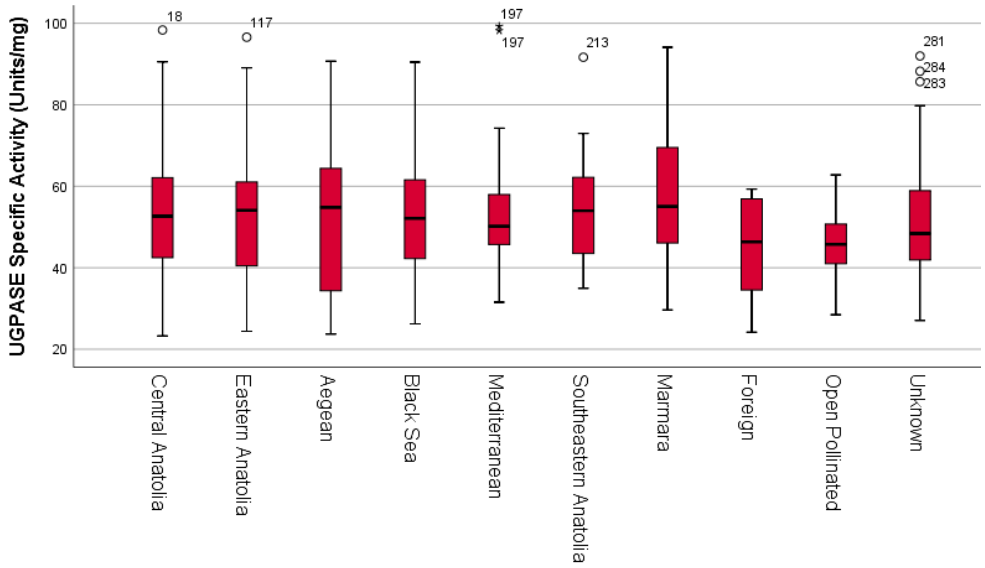


Figure 4.3. Boxplots of UGPase activity

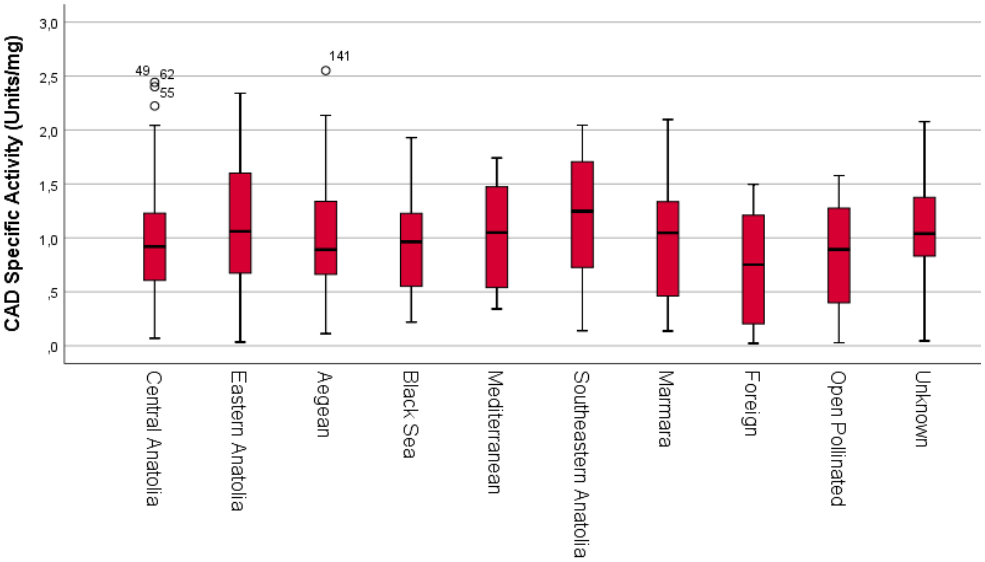


*Figure 4.4.*Boxplots of CAD activity

Table 4.3. *Population and overall means (± standart deviations) for the traits in the study*

Regions	*SuSy*	*UGPase*	*Cellulose*	*PAL*	*4CL*	*CAD*	*Lignin*	*Glucose*	*Height*	*Diameter*
C. Anatolia	234.2 ± 77.5	54.2 ± 14.3	21.3 ± 13.7	6.5 ± 2.6	3.6 ± 1.5	1.0 ± 0.5	21.8 ± 4.2	33.1 ± 8.7	409.9 ± 46.2	30.0 ± 5.7
E. Anatolia	243.6 ± 86.7	52.9 ± 14.3	19.2 ± 9.4	6.7 ± 2.8	3.8 ± 1.6	1.1 ± 0.5	23.4 ± 3.7	34.4 ± 7.3	390.1 ± 45.8	28.3 ± 4.2
Aegean	229.6 ± 98.6	53.4 ± 15.6	17.5 ± 6.0	5.9 ± 2.1	3.9 ± 1.4	1.0 ± 0.5	23.5 ± 4.4	35.9 ± 8.7	400.2 ± 36.4	27.5 ± 2.7
Black Sea	255.0 ± 81.3	53.6 ± 13.8	19.7 ± 6.4	7.0 ± 2.4	3.5 ± 1.6	0.9 ± 0.4	23.5 ± 3.8	34.2 ± 9.3	383.2 ± 48.7	26.4 ± 4.0
Mediterranean	239.3 ± 66.3	57.2 ± 16.5	27.1 ± 17.8	6.3 ± 2.9	3.8 ± 1.3	1.1 ± 0.5	24.6 ± 4.1	38.6 ± 8.6	427.5 ± 37.3	31.2 ± 6.5
S.Anatolia	247.6 ± 69.9	54.2 ± 11.9	20.5 ± 10.1	6.4 ± 2.2	3.6 ± 1.7	1.2 ± 0.6	22.2 ± 3.9	34.7 ± 8.7	399.2 ± 31.7	28.0 ± 3.3
Marmara	288.7 ± 73.3	58.4 ± 16.0	20.1 ± 9.7	6.0 ± 2.4	3.8 ± 1.6	1.0 ± 0.5	23.9 ± 4.7	33.8 ± 8.7	381.2 ± 36.3	26.6 ± 3.7
Foreign	185.8 ± 64.7	48.3 ± 11.0	35.8 ± 28.1	6.2 ± 2.9	4.2 ± 2.0	0.7 ± 0.6	21.3 ± 3.8	40.5 ± 6.3	394.7 ± 40.2	27.9 ± 4.0
Open Poll.	228.0 ± 80.8	45.2 ± 8.0	18.8 ± 5.8	6.5 ± 2.4	3.0 ± 1.5	0.8 ± 0.5	21.3 ± 3.1	33.9 ± 8.3	393.3 ± 28.5	31.5 ± 3.6
Unknown	261.5 ± 71.1	52.5 ± 15.4	19.1 ± 8.5	6.2 ± 2.3	3.7 ± 1.6	1.1 ± 0.5	23.9 ± 4.4	35.6 ± 10.9	394.6 ± 45.8	28.5 ± 4.9
Mean	243.3 ± 81.6	53.3 ± 14.3	20.5 ± 11.6	6.5 ± 2.5	3.6 ± 1.6	1.0 ± 0.5	22.8 ± 4.1	34.4 ± 8.7	397.4 ± 44.5	28.7 ± 4.9

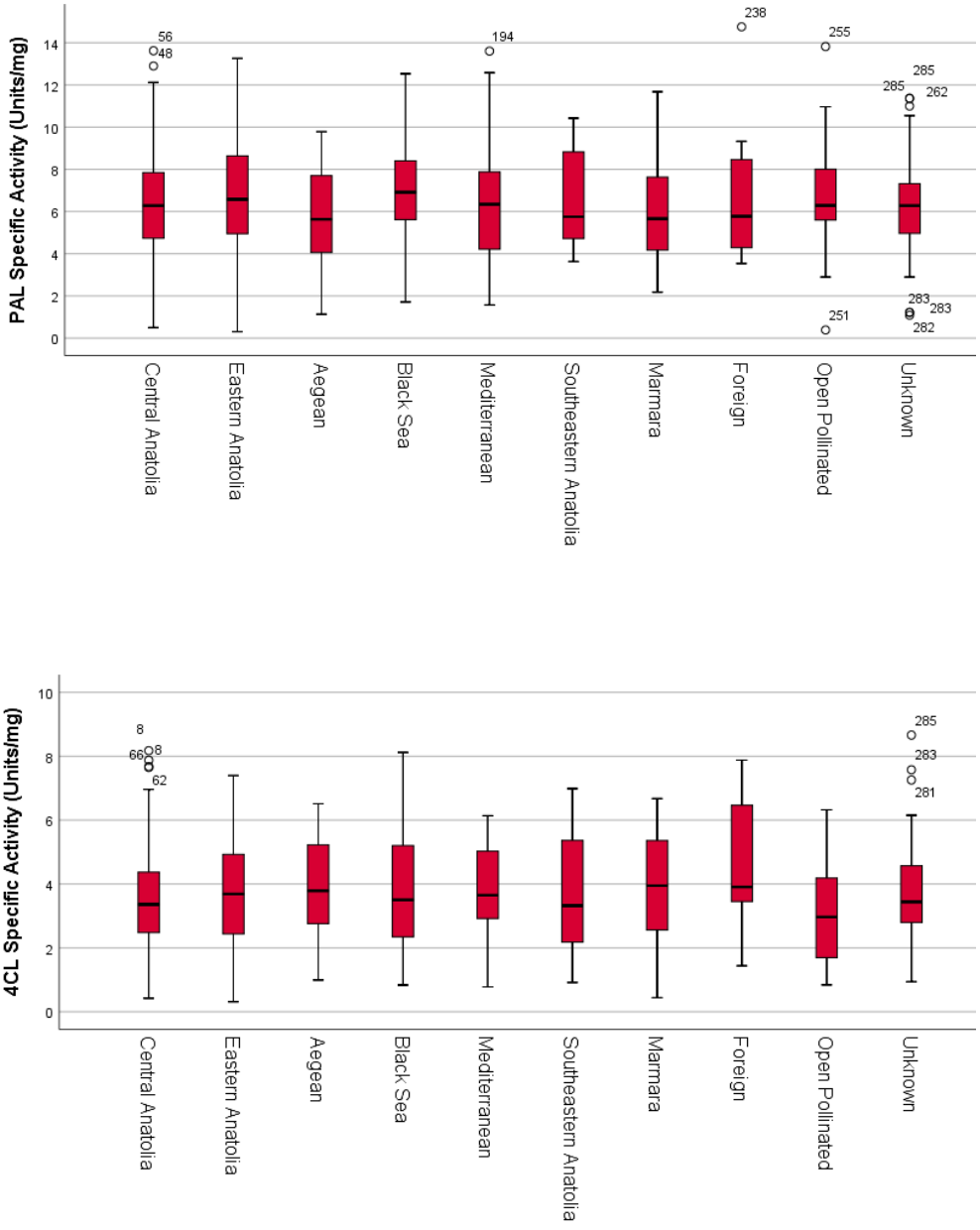


Figure 4.5. Boxplots of PAL and 4CL activities

4.2. Determination of the relationship between studied traits

In order to understand the relationships among the studied traits, both phenotypic and genetic correlations were estimated. Phenotypic correlations ($r_{p(x,y)}$) between pairwise traits were estimated as described by Kaya and Temerit (1994) and it showed very similar results with Pearson correlation approach. Based on phenotypic correlation results, it can be said that the result did not show any significant correlation between lignin content and lignin biosynthesis related enzymes. On the other hand, there were significant positive correlations between PAL, 4CL and CAD enzyme activities.

The phenotypic correlation coefficients (Table 4.4, Fig. 4.6) between PAL and 4CL, between PAL and CAD, and between 4CL and CAD were estimated as 0.218, 0.170, and 0.325, respectively. Also, PAL and CAD enzymes were slightly negatively correlated with UGPase enzyme (r_p = -0.11, $p < 0.05$; r_p = -0.122, $p < 0.05$, respectively). Interestingly, there were low positive correlations between SuSy and 4CL (r_p = 0.132, $p < 0.05$) as well as SuSy and CAD (r_p = 0.124, $p < 0.05$) enzymes. It was not surprising to detect positive correlation between cellulose content and SuSy and UGPase enzymes, but they were not as high as we expected (r_p = 0.184 $p < 0.01$, r_p = 0.160, $p < 0.05$, respectively). Cellulose content was also positively correlated with glucose content, height, and diameter in the same manner, but we could not detect any correlation between cellulose and lignin contents. These results were similar to the results of our previous study (Taşkıran, 2014). Just like cellulose content, lignin content was also positively correlated with glucose content (r_p = 0.250, $p < 0.01$), whereas it was slightly negatively correlated with diameter (r_p = -0.139, $p < 0.05$). Lastly, two important phenotypic growth parameters, height and diameter measurements were highly significantly correlated with each other (r_p = 0.777, $p < 0.01$) as expected.

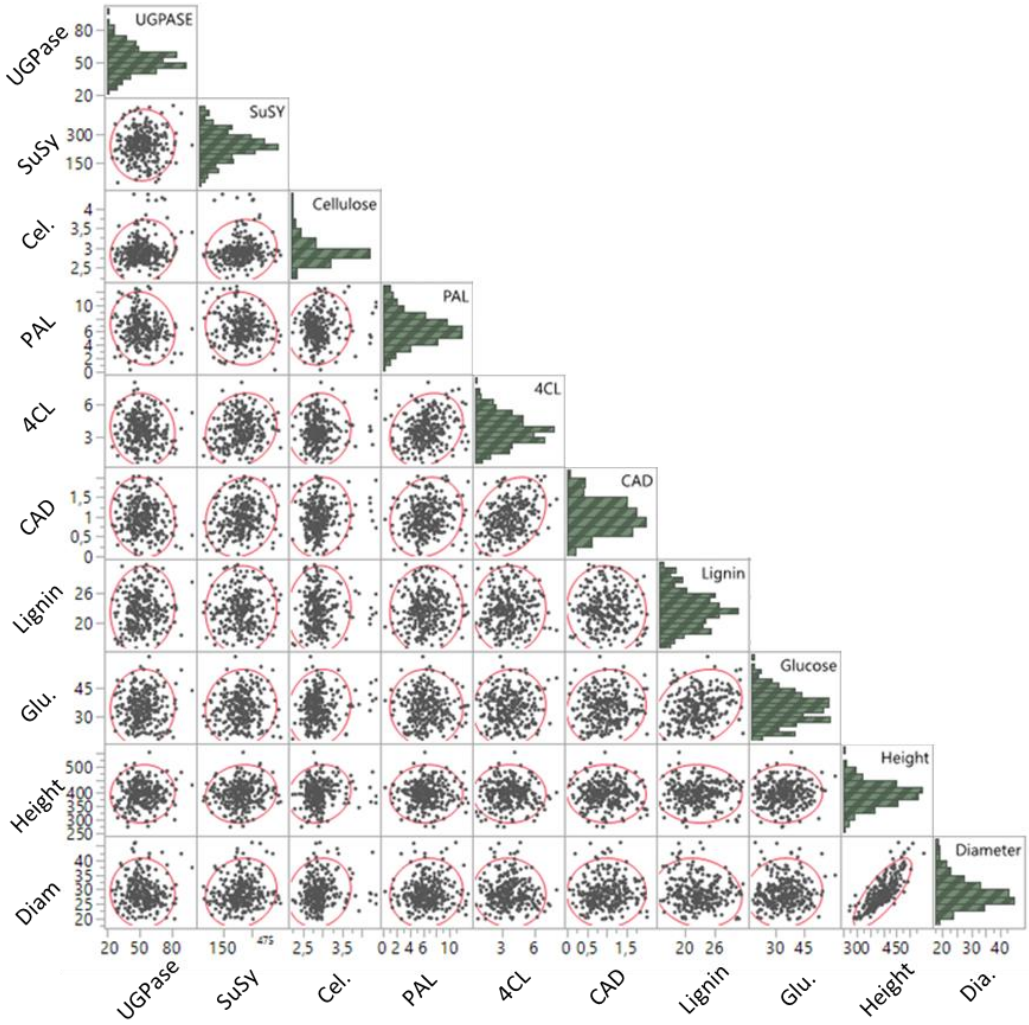


Figure 4.6. Scatterplot matrix of studied traits

In general, degree and sign of phenotypic and genetic correlations did not follow the same pattern (Table 4.4). UGPase and SuSy enzymes, slightly and negatively correlated with each other (r_g = -0.336). There were no genetic correlations between cellulose content and cellulose biosynthesis related enzymes (UGPase and SUSY) while there are positive phenotypic correlations between them. On the other hand, there are slightly positive genetic correlations between cellulose content and PAL, 4CL, CAD, Lignin and glucose contents. However surprisingly, there are negative

Table 4.4. *Phenotypic (above diagonal) and genetic (below diagonal) correlations (± standart errors) between studied traits*

r_p / r_g	*UGPase*	*SuSy*	*Cellulose*	*PAL*	*4CL*	*CAD*	*Lignin*	*Glucose*
UGPase	--	0.032±0.12	**0.160±0.11**	**-0.113±0.12**	**-0.105±0.13**	**-0.122±0.13**	0.090±0.14	0.066±0.11
SuSy	**-0.336±0.13**	--	**0.184±0.10**	0.082±0.10	**0.132±0.11**	**0.124±0.12**	0.022±0.12	0.074±0.10
Cellulose	-0.080±0.12	0.040±0.09	--	0.097±0.11	0.050±0.11	**0.143±0.11**	0.060±0.12	**0.126±0.10**
PAL	**0.111±0.13**	0.080±0.09	**0.270±0.09**	--	**0.218±0.11**	**0.170±0.11**	0.050±0.12	0.002±0.10
4CL	**0.156±0.15**	**0.368±0.11**	**0.196±0.11**	**0.101±0.11**	--	**0.325±0.11**	-0.010±0.13	0.050±0.10
CAD	**0.200±0.17**	**0.471±0.12**	**0.420±0.11**	0.014±0.11	**0.187±0.13**	--	-0.074±0.10	0.080±0.10
Lignin	**0.683±0.19**	**0.274±0.12**	**0.263±0.12**	**-0.293±0.13**	**-0.490±0.16**	**-0.750±0.20**	--	**0.250±0.10**
Glucose	**0.158±0.11**	**0.122±0.08**	**0.145±0.08**	0.020±0.09	0.030±0.09	0.045±0.10	**0.320±0.09**	--

genetic correlation between lignin content and PAL, 4CL, and CAD enzymes (r_g = -0.293, r_g = -0.490, and r_g = -0.750, respectively) though there is no phenotypic correlations among them. In addition to these interesting results, positive moderate correlation between lignin content and UGPase (r_g = 0.683) and low positive genetic correlation between lignin content and SuSy enzyme (r_g = 0.274) were detected.

4.3. Determination of potential lignosellulosic clones

To determine the clones with lignocellulosic potentials, average mean values of clones were sorted from minimum to maximum and vice versa for each variable. First 50 clones representing the highest and the lowest mean values were separately analyzed to examine possible changes on the relationships of the traits. Among these 50 clones, the first 10 clones were selected prior to analysis. But after realizing that particular clones had very close values with respect to compared traits, so the first 12 clones were used in evaluation of clones for studied traits.

To illustrate, when we analyzed 50 clones with maximum mean values for UGPase activity, the negative correlation coefficients between pairs of UGPase-PAL variables and UGPase-CAD variables increased from r_p = -0.113 to r_p = -0.251 and r_p = -0.122 to -0.261, respectively. Similarly, positive correlations between UGPase and cellulose content increased from r_p = 0.160 to 0.264. While all clones analyzed by phenotypic correlations, no relationship among UGPase and SuSy activities were observed. On the other hand, after selecting 50 clones with maximum UGPase activity, significant correlation was found between UGPase and SuSy traits (r_p = 0.243). The same pattern was also observed between UGPase and diameter measurements (r_p = 0.300). In addition to these results, the relationships between cellulose content and diameter (r_p = 0.462) and cellulose content and height (r_p = 0.330) were also improved (Fig. 4.7).

When we repeat the same analysis for SuSy activity with highest 50 clones, surprisingly we realized that lignin related enzymes PAL, 4CL and CAD were started

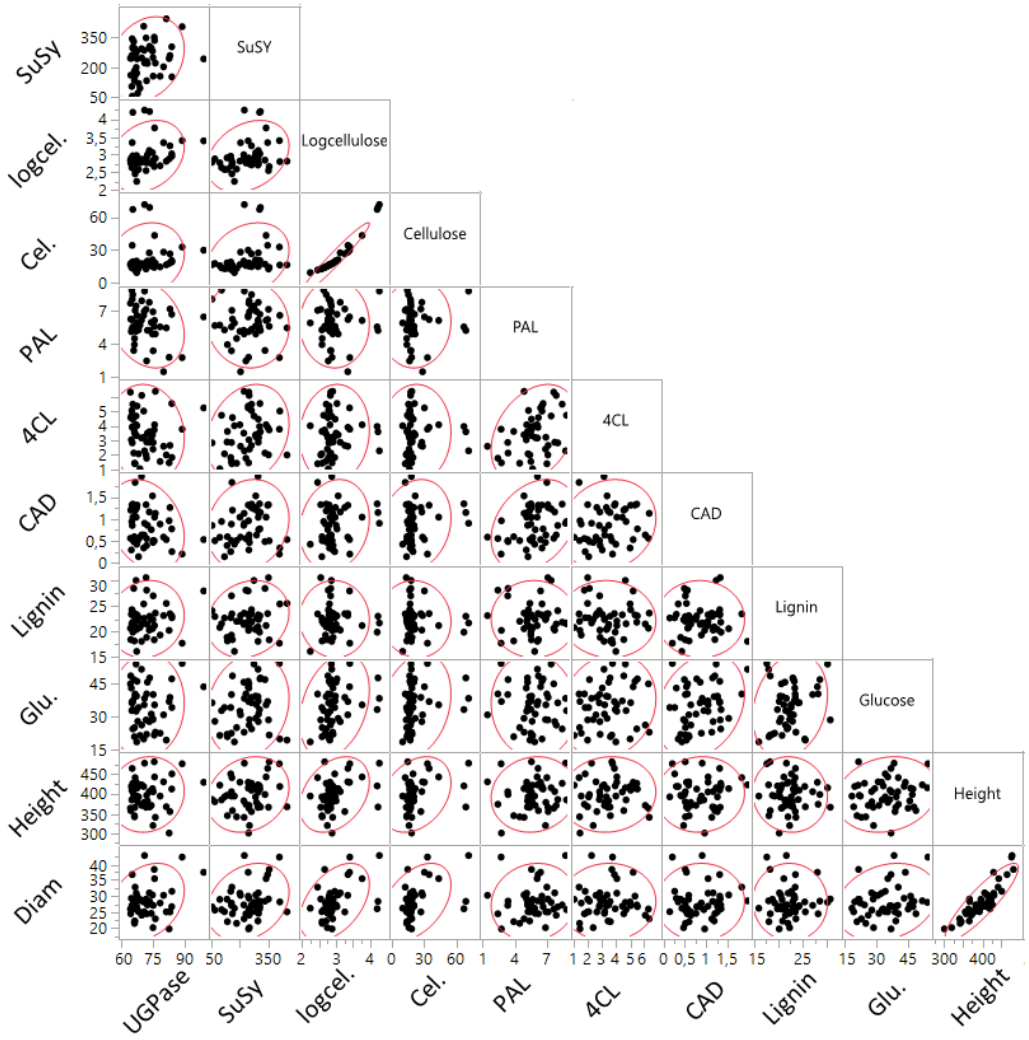


Figure 4.7. Scatterplot matrix of studied traits by using 50 clones with the highest UGPase activity.

to correlate negatively with diameter (r_p = -0.251, r_p = -0.125, and r_p = -0.121, respectively). Also, negative correlation among lignin content and diameter increased from r_p = -0.139 to r_p = -0.236. And for the first time, low but significant positive phenotypic correlation among lignin content and CAD activity was detected (r_p = 0.161).

Same kind of results were also observed when the test repeated for PAL, 4CL, and CAD enzymes with their highest 50 clones. The results were similar to SuSy results

given above. Again, 4CL was negatively correlated with diameter (r_p = - 0.237 for PAL 50 max. clones, r_p = -0.239 for CAD 50 max. clones, and r_p = - 0.216 for 4CL 50 max. clones). Moreover, based on the analysis with 50 maximum CAD values, CAD and diameter (r_p = -0.280) was negatively correlated, too. In this analysis, PAL and 4CL were slightly and positively correlated with lignin content (r_p = 0.180 and r_p = 0.130).

On contrary to the highest 50 clone analysis, we could not detect any particular pattern and relationships among traits with 50 clones representing the lowest measurements of each trait.

All these results led us to examine the highest 12 clones in each trait and their corresponding ranks in the maximum and minimum 50 clones of other traits. The chosen 12 clones of the trait were compared with the other related traits in terms of mean values (Figures 4.8-4.10). Five of the 12 clones representing the highest UGPase activity were also ranked among the highest 50 clones with their SuSy activities and cellulose contents. Especially, the clone labeled as 62160 (the second highest clone) was among the first 20 clones in terms of SuSy (8. Highest clone), cellulose content (18. highest), height (12. highest) and diameter (7. highest) measurements. In addition to these results, it was also among the 12 clones which had the lowest PAL and CAD activities (Fig. 4.8).

Similarly, clones N91021 and 821 were among the highest 50 for cellulose, SuSy, height and diameter, but also, they were among the lowest 50 in terms of lignin content (Fig. 4.8). In SuSy 12 highest clones rank, besides clone 62160, one more clone, N90102 had also lignocellulosic potential. It was among the highest 50 clones with respect to cellulose content and diameter measurement, but also among the 50 clones which had the lowest lignin contents. The clones labeled as N91120, N92217, and N033681 were among the highest fifty clones with respect to PAL, 4CL, and CAD activities. Also, clones N92160 had the highest lignin content (Fig. 4.9) and N91068

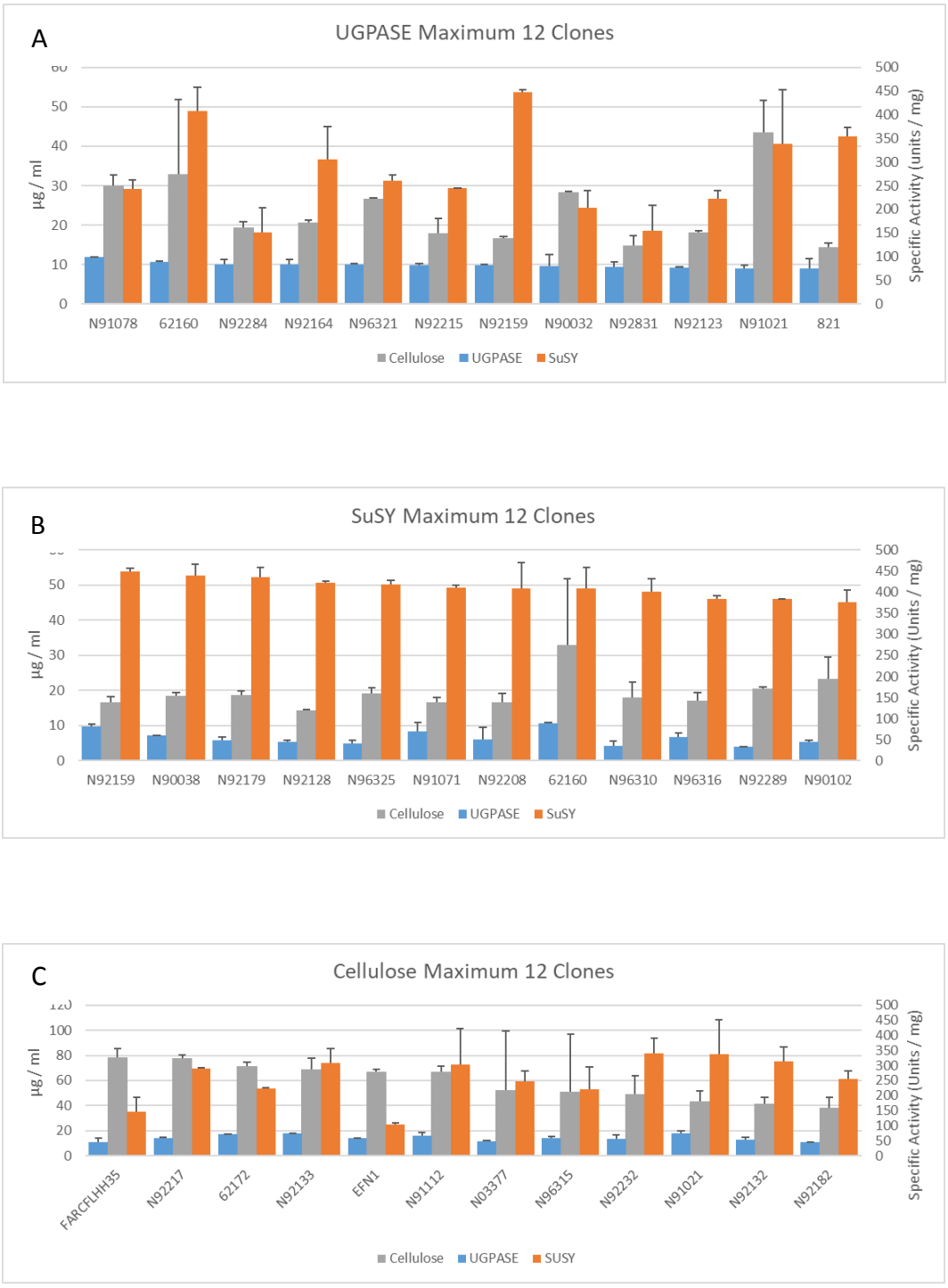


Figure 4.8. Twelve clones with maximum UGPase (A) and SuSy (B) activities and cellulose contents (C) (Error bars indicate standart deviation)

was among the highest 12 with its PAL activity (Fig. 4.10A). Our commercially registered clone Anadolu was the highest 7th clone in terms of PAL activity. Previously, it was also reported with highly up-regulated PAL expression (Yıldırım, 2013).

Based on the lowest 50 clones with respect to UGPase and SuSy activities, the clones 62160, N020506, N91073, and N92169 were recorded with the lowest PAL, 4CL, and CAD activities. N91073 and N92169 were also among the clones with the highest UGPase activity. N90032 had the lowest PAL and CAD activities, but high cellulose content and UGPase activity. Similarly, N92232 was observed with low PAL and 4CL activities, but with high cellulose content and SuSy activity. Almost twenty out of fifty clones with the lowest 4CL activity were also detected at among the clones with lowest CAD activity. Our commercially registered clones, were generally observed with average values in terms of enzyme activities except height and diameter measurements. Kocabey, Anadolu, Geyve, Ata, Gazi, and Çubuk 2 were among the highest 50 clones with their cellulose contents and SuSy activities. Also, these clones were observed with low 4CL and CAD activities.

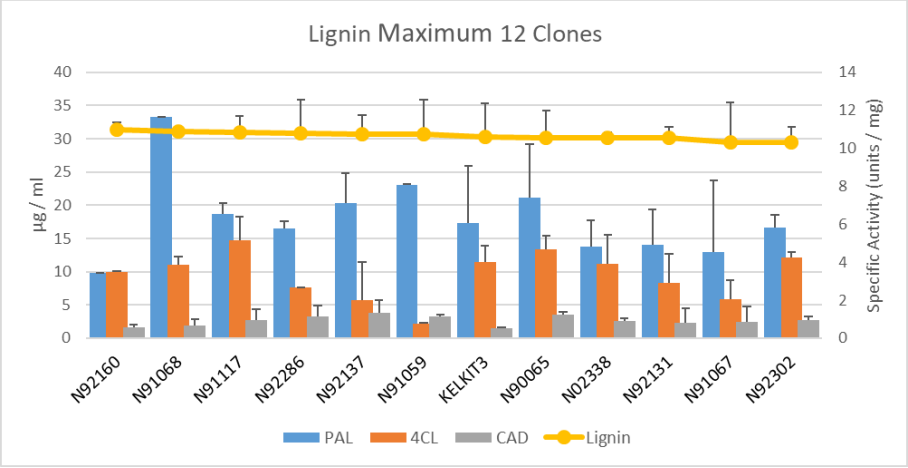


Figure 4.9. Twelve clones with maximum lignin contents and their PAL, 4CL, and CAD activities (Error bars indicate standart deviation)

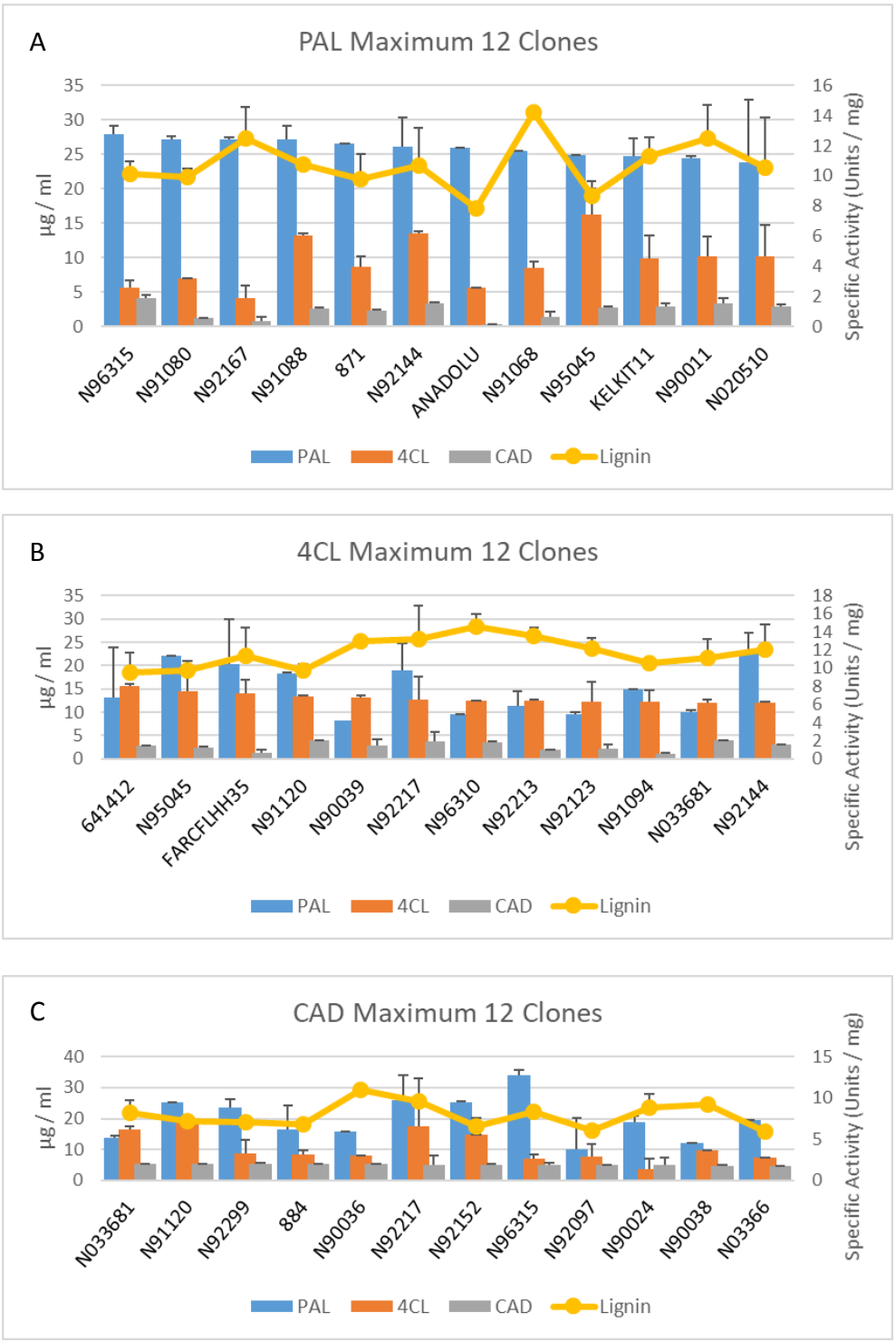


Figure 4.10. Twelve clones with maximum PAL (A), 4CL (B), and CAD (C) activities (Error bars indicate standart deviation)

Since lignin is an undesired polymer of lignocellulosic biomass, the lowest 12 clones with respect to lignin content and PAL, 4CL, and CAD activities were also determined. The clone 62160 was among the lowest 12 clones with its PAL and CAD activities (Figures 4.11A and 4.12A). Additionally, N92295, N03366 and 7740 were detected with the lowest lignin contents. The commercial clone ATA1 and Anadolu were detected with low lignin contents and CAD enzyme activities (Fig. 4.12B). Foreign clone Lvubaka showed the lowest CAD activity (Fig. 4.12A).

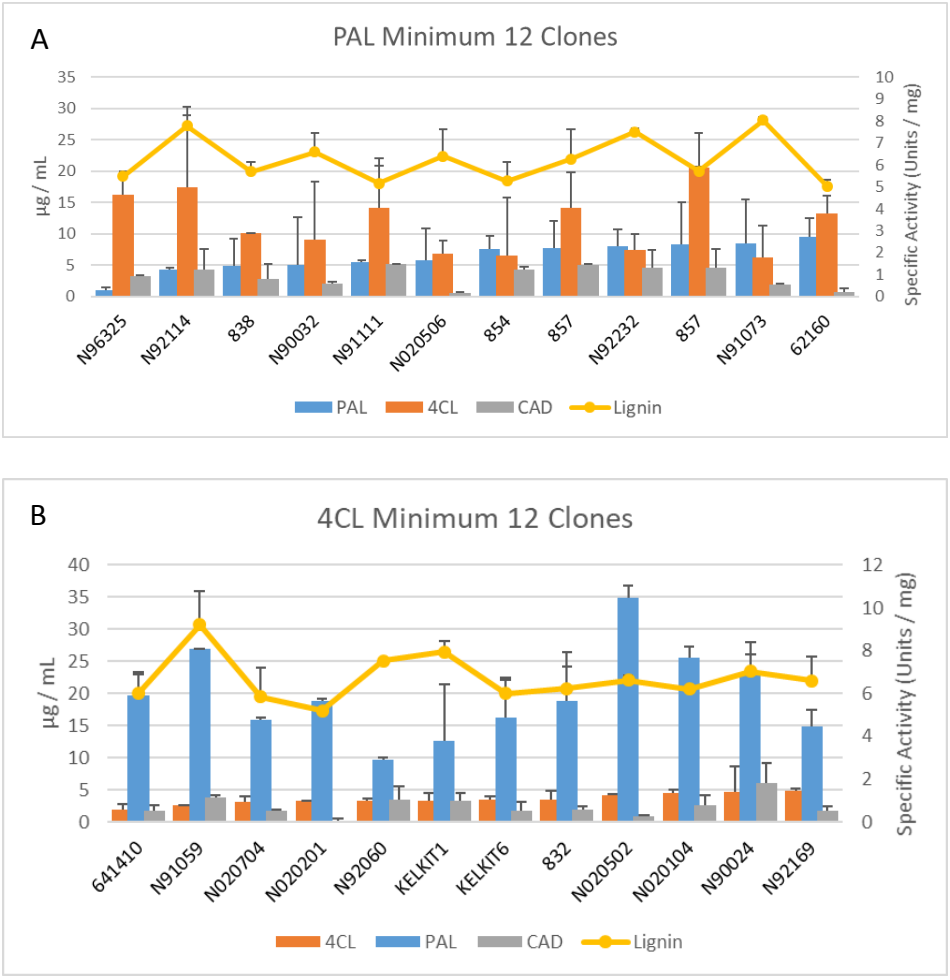


Figure 4.11. Twelve clones with minimum PAL (A) and 4CL (B) activities and their lignin contents (Error bars indicate standart deviation)

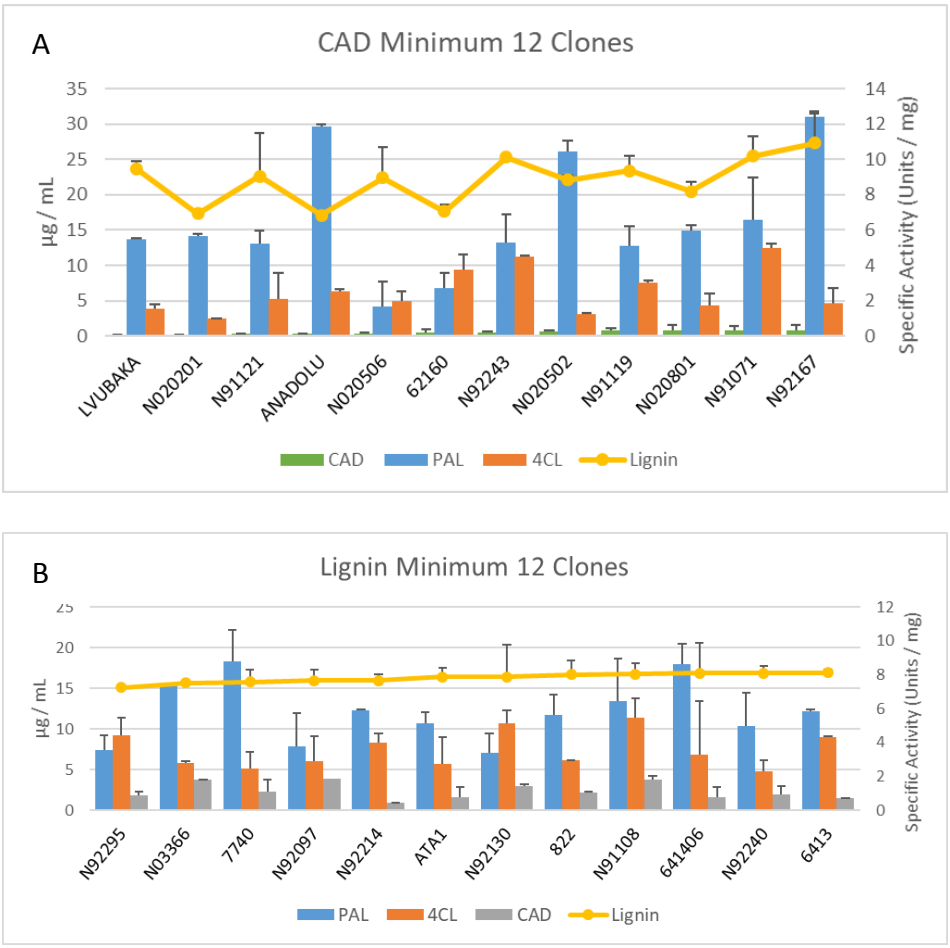


Figure 4.12. Twelve clones with minimum CAD activity (A) and lignin contents (B) (Error bars indicate standart deviation)

CHAPTER 5

DISCUSSION

5.1. Genetic variations in European black poplar populations

Previously, a study conducted by using the same European black poplar clones to assess their genetic diversity revealed that the clone N0375 was sampled 84 times and transferred to the collection with different clonal labelling (Ciftci *et al.*, 2017). Based on these results, two approaches were followed before and after removing all duplications of N0375 clone from the data. Interestingly, estimates of variance components and heritability in two analysis demonstrated great similarities to each other.

The results of the study on physiological and biochemical traits showed that there is high genotype effect on the phenotypic variation. This genotype effect accounted for more than half of the total variance for SuSy trait, cellulose and glucose contents, ranging from 53.2 in SuSy to 64.1% in glucose content. Similarly, the contribution of variation due to clones within region was also high for other cell wall related traits. It is clear that great portion of total variance ranging from 33.0 in UGPase to 44.1% in PAL was explained by clonal variation in UGPase, CAD, 4CL, Lignin, and PAL traits, respectively. In the study of Guerra *et al.* (2013), C6 sugars and lignin contents and S/G lignin ratio were measured from European black poplar. Variance component of clonal effects ranged from 45.2 to 70.2% for these traits that are compatible with the findings of our previous study (Taşkıran, 2014).

Biomass processing efficiency and fermentable sugar release in biofuel production is greatly affected from the genetic differences of plants (Davison *et al.,* 2006; Studer *et al.,* 2011). In the study of Wegrzyn *et al.* (2010), two-states-SNPs (homozygous AA and heterozygous AG) effects were reported as significant for lignin and C6 sugar

contents. The lignin content in heterozygous AG genotype was reported as 23.6%, whereas homozygous AA plants had lower lignin content (22%) which might affect sugar release in fermentation process.

Previous SNPs study indicated that small proportion of phenotypic variation can be explained by identified SNP markers associated with lignin and cellulose biosynthesis (1.1% to 3.7%) (Wegrzyn *et al.,* 2010). Rae *et al.* (2009) identified five biomass QTLs on chromosomes 3, 4, 10, 14, and 19 in poplar. Recently, 27 QTLs were identified in willow for wood chemical traits (cellulose, hemicellulose and lignin) on chromosome 14. These QTLs were explained 4.2 to 6.9% of total phenotypic variation (Pawar *et al.,* 2018). Similarly, in the study of Porth *et al*. (2013b), a phenotypic variance explained by all significant SNPs which were related to 17 cell wall and wood traits ranged between 5-26%. Therefore, based on these variation component results, selection at the clonal level could be practiced to improve lignocellulosic potential of the black poplar clonal collection for industrial plantations.

Not surprisingly, the effect of region was high in lignin content trait which explained 3.2% of the total phenotypic variation. As it is well known by numerous studies, lignin is a highly sensitive polymer to environmental conditions. Its composition and content can change based on different abiotic factors like drought, salt, and disease to strengthen cell wall (Moore *et al.,* 2008; Miedes *et al.,* 2014; Wildhagen *et al.,* 2018). The clones used in the study were originated mainly from three phytogeographic regions of Turkey which are Irano-Turanian, European-Siberian, and Mediterranean. These biogeographic regions reflect differences in climate, vegetation and endemism (Kavgacı *et al.,* 2015; Çoban and Willner, 2018). Our multiple comparisons by Duncan's multiple range tests displayed that the differences among regions for lignin content was mainly due to the clones originated from Mediterranean region and foreign populations. The highest lignin content (24.6 ± 4.1 µg/ml) observed in clones originated from Mediterranean region can be explained by climatic feautures of the region. Summers are very long, hot and arid, whereas winters are mild and wet in this region comparing to other regions. Changes in temperature, precipitation,

photoperiod, and biotic diversity all occur in different regions (Keller *et al.,* 2011). Therefore, it is not surprising to observe regional variation in lignin content which is more sensitive to environmental factors for local adaptation comparing to other quantitative lignocellulosic traits. To illustrate, it has been reported that drought brings about change in lignin content and increases lignin accumulation in cell wall to increase the mechanical strength (Mao *et al.,* 2004; Hu *et al.,* 2009; Le Gall *et al.,* 2015; Yıldırım and Kaya, 2017).

On the other hand, the highest SuSy and UGPase activities, and cellulose contents observed at clones originated from Marmara region can be explained by the effects of past demographic events besides environmental factors. Marmara region is one of the most preferred immigrant-receiving regions of Turkey. With rapid industrial development and high employment oppurtunities, the region received quite number of immigrants from other regions in the years following 1950 and 1980 (Doğan, 2013). This demographic history might be responsible for population differentiation of these quantitative traits for local adaptation by artificial selection rather than natural selection. Beside this, the region also has the lowest altitude ranges and mild climatic conditions comparing to other regions which provide suitable environment for growth of European black poplar clones and eventually lead to local adaptation.

Clonal heritability (h^2) of each trait was determined to estimate their inheritance. Quantitative genetic analysis of lignocellulosic traits showed moderate to high heritability estimates ranging from 0.50 in UGPase to 0.80 in glucose. The highest values which confirm the strong genetic influence on the trait were calculated for PAL activity (0.61), SuSy activity (0.71), cellulose content (0.75), and total glucose content (0.80). On the other hand, heritabilities for other traits (UGPase, CAD, lignin content and 4CL) were moderate and ranged from 0.50 in UGPase to 0.57 in lignin content. Previously, Guerra *et al.* (2013) reported heritability of black poplar lignin content as 0.58 by association genetics study which is highly compatible with our result. These results suggested that the variations observed in traits with moderate heritabilities were also affected by environmental and climate factors. 4CL as a key branch point

enzyme regulates the overall carbon flow to the phenylpropanoid pathway (Shigeto *et al.,* 2017). Recently, it has been reported that 4CL is sensitive to biotic and abiotic stresses and suggested to play important roles in stress response (Liu *et al.,* 2018; Sharma et al., 2019). As mentioned earlier, similar results were also reported for lignin content and CAD expressions (Trochet *et al*.,2010; Miedes *et al.,* 2014; Le Gall *et al*., 2015). The results of lignocellulosic traits before and after removal of the duplicates of N0375 strongly support our suggestion which states that beside genetic factors, heritability estimates for lignocellulosic traits are also affected by environmental factors.

5.2. Relationships among studied traits

At the early stages of our study, the possible regulatory enzymes of both cellulose and lignin metabolic pathways were selected expecting to see the effect of the activities of these enzymes on the amount of cellulose and lignin in European black poplar clones. As it is well known, wood quality and yield are directly associated with the composition and content of cell wall components. Therefore, the study of the relationships between lignocellulosic traits was the second objective of the study. However, in contrast to our initial expectations, the lignin content was not affected by the pathway enzymes namely PAL, 4CL, and CAD. According to our results, no significant correlation was observed between lignin content and pathway enzymes, though significant relationships were present between the enzyme pairs.

A phenotypic correlation between PAL and 4CL was estimated as $r_p = 0.218$. Also, for 4CL and CAD pair trait, it was $r_p = 0.325$, and for PAL and CAD pair trait, it was $r_p = 0.170$. In literature, there are no previous studies dealing with the effects of naturally found enzyme activities on lignin content. All previously reported studies are almost always based on gene expression or *in vitro* studies based on a relatively small set of data and sample size. In phenylpropanoid pathway, same enzymes can use different substrates. Not only 4CL and CAD, but also CCR and COMT have multiple

substrates (Tronchet *et al*. 2010; Vogt, 2010; Faraji *et al.,* 2018). As a result of this switch of substrates, different parallel pathways or interconnected pathways can be followed toward monolignol precursors (Valhomme *et al.,* 2019). Also, intermediates products of bypass pathway can become available for other branching pathways as substrates not directed to lignin biosynthesis (Chen *et al.,* 2014; Lin *et al.,* 2015; Lavhale *et al.,* 2018). So, there is multilevel regulation of the phenylpropanoid pathway and it is quite complicated.

Findings of positive correlations between lignin enzyme pairs clearly demonstrate the flux catalyzed by PAL and the following reactions catalyzed by 4CL and CAD. Several enzymes of the pathway are encoded by multigene families (Tuskan *et al.*, 2006; Barakat *et al.,* 2009; Shi *et al.,* 2010). As it is known, different isozymes of the same gene may have different activities, substrate affinities, and abundance in tissues and organs. Li *et al*. (2015) showed that 4CL1, 4CL2 and 4CL3 are all involved in p-coumaryl-CoA biosynthesis in *Arabidopsis*, but the product produced by the first two directed to lignin biosynthesis, while 4CL3 products are used to synthesize flavonoids. Likewise, only 4CL1 gene mutants showed reduced lignin content in the study in which three 4CL genes were editied by CRISPR methods (Zhou *et al.,* 2015).

In poplar, Van Acker *et al.* (2017) reported that, only CAD1 gene deficiency resulted in modified lignin content and structure. The results of Huang *et al*. (2010) showed 25% reduction in lignin content by using mutants for all PAL genes in *Arabidopsis*. In poplar, PAL isozymes are encoded by a small gene family with five genes (Tuskan *et al.*, 2006; Shi *et al*., 2010, 2013). An interesting study conducted by Wang *et al.* (2015) showed that severe reductions in the abundance of PAL, 4CL, and CAD enzymes are required to affect lignin deposition in *P. trichocarpa*. Similar to our results, the reductions in PAL and 4CL proteins did not show any effect on lignin content unless their abundances were below 10% in wild type plants. For the CAD enzyme, the reduction below to 50% in transgenic lines caused to decreases in lignin content and increases in S/G ratio (Wang *et al.,* 2015).

Contrary to the results obtained for the relationship between lignin content and phenylpropanoid pathway enzymes, cellulose content and SuSy activity (r_p = 0.184) and also cellulose content with UGPase activity (r_p = 0.160) showed positive phenotypic relationships. Negative phenotypic correlations were detected between UGPase and PAL, 4CL and CAD activities. These results can be evaluated as a competition for carbon allocation to cellulose and hemicellulose versus lignin because during the formation of cell wall, these polymers appears as the major carbon sinks (Sjostrom, 1993; Novaes *et al.*, 2010; Taşkıran, 2014).

Alternatively, glycosylation is a common process used in plant cells to stabilize highly toxic monolignols and it requires extra amount of carbon source (Anterola, 2002). As noted before, lignin content appears as a mechanical barrier to protect the cell and its accumulation increases during biotic and abiotic stress whereas an increase in diameter generally appears as an indicator of normal plant growth. The only way to compensate this carbon source requirement is shifting the carbon partitioning through lignin production as a normal consequence of photoassimilate partition (Winter and Huber, 2000; Koch, 2004; Coleman *et al.*, 2009; Zhou *et al.,* 2016; Stein and Granot, 2019). Slightly positive phenotypic correlations between cellulose content and SuSy activity and also between cellulose and UGPase activity agree with the results of Coleman *et al.* (2010) in which cellulose contents of the cells were increasing with UGPase and SUSY transgenic lines, though the overall impact was low. Similarly, Gerber *et al.* (2014) suggested that SuSy plays a role in total carbon allocation in wood cells, but it is not essentially needed for cellulose biosynthesis.

Generally, genetic correlations and phenotypic correlations for certain traits did not show similar results with respect to magnitude and sign. For example, in our study there was no phenotypic correlation observed between SuSy and UGPase activities, while their genetic correlation was estimated as r_g = -0.336. Even though they utilize UDP-glucose as subtrate, the preferred directions of reactions are not the same. According to our results, more than five times higher SuSy activity was observed compared to UGPase activity. SuSy as a bidirectional enzyme has a poor K_m for

sucrose (10-400 mmol/L) and cleaves sucrose at very high concentration to produce UDP-glucose for cellulose deposition. In its synthesis direction, it uses the same UDP-glucose pool as substrate like UGPase enzyme. Both SuSy and UGPase enzymes from *Arabidopsis* were reported with the same K_m values for UDP-glucose (0.05 mmol/L) in different studies (Meng *et al.,* 2008; Almagro *et al.,* 2012). These close substrate affinities may cause competition for the source. However, the regulations of various substrate pools in the source and sink tissues by increasing and decreasing their concentrations may change the reaction direction's of both enzymes. In addition to this, it has been reported that (Schrader and Sauter, 2002) abundance of SuSY enzyme in wood tissues increase sharply in the beginning of summer compared to other cellulose related enzymes like UGPase and Sucrose phosphate synthase (SPS)

As a summary, even though comparing studies with different sampling strategies, sample size and methods is very difficult, we try to compare our results with related studies. Our results, enlighted the relationships among lignocellulosic traits in natural black poplar clonal populations and their effect to environmental and genetic interactions.

5.3. Lignocellulosic clones

The wide clonal (genotypic) variation, moderate to high heritability and significant phenotypic correlation and in some cases extreme genetic correlations present in the studied traits suggested a possible clonal level selection to determine the clones with lignocellulosic potential for future breeding applications and industrial plantations. Therefore, the clones with the highest and the lowest values of cellulose and lignin contents were separately evaluated. One of the most remarkable results of this analysis was observation of increase in correlations between paired traits with top 50 clones with high lignocellulosic values. For instance, UGPase and cellulose content correlation increased from $r_p = 0.160$ to $r_p = 0.264$. Similarly, cellulose content and

diameter relationship was also increased to $r_p = 0.462$. Similar type of increases in correlation coefficients were also detected between other lignocellulosic traits.

Therefore, we decided to evaluate the highest twelve clones for each trait and also some specific clones with particularly high mean values for more than one trait. For example, the clone 62160 which was among the highest twenty clones in terms of UGPase, SuSy, cellulose content, height and diameter, but also it was among the lowest 12 clones with respect to PAL and CAD activities. Moreover, it showed the characteristics of lowest clones with its low lignin content. Similarly, N90102 took our attention not only by its high SuSy activity, cellulose content and diameter, but also with low lignin content.

Moreover, clones labeled as N91120, N92217, and N033681 were among the highest 50 clones with respect to PAL, 4CL, and CAD activities. Previously, N033681was reported as drought resistant clone and observed with highly significant up-regulated PAL gene expressions (Yıldırım, 2013). Also, clone N92160 which had the highest lignin content and N91068 which was among the highest 12 with its PAL activity were reported with the highest leaf abscission and least growth under drought conditions (Yıldırım, 2013). Again, in the same study, commercially registered clone Anadolu (the highest 7th Clone in terms of PAL activity in our study) was also reported with highly up-regulated PAL gene expression. In the same manner, N92179 which was the highest 3rd clones with its SuSy activity in our study was reported as cold resistant clones in a previous study (Zeybek, 2014).

The same clones mentioned above were also selected as extraordinary clones based on their mean cellulose, lignin contents and growth values in our previous study (Taşkıran, 2014). As we stated earlier, till now only seven European black poplar clones were registered commercially in Turkey by International Poplar Commission and they showed moderately high lignocellulosic potential in the current study. Therefore, selecting the superior clones in terms of lignocellulosic traits might be considered as an advantageous and useful approach to establish a new black poplar

breeding programme by molecular markers assisted tools to increase wood quality and biomass yield efficiency. In many poplar and willow breeding programmes, molecular marker-based selections were used widely in addition to phenotypic selection, especially for wood related traits (Wegrzyn *et al.,* 2010; Porth *et al.,* 2013b; Guerra *et al,* 2013; Schilling *et al.,* 2014; McKown *et al.,* 2014; He *et al.,* 2014; Allwright and Taylor, 2016; Zhou *et al.,* 2018).

Since the establishment of Behiçbey European black poplar clone bank, genetic diversity of these clones was determined by SSR markers and their drought and cold resistance were examined by numerous biochemical, physiological, and microarray studies as part of a moleculer black poplar breeding program supported by TUBITAK. (Zeybek, 2014; Ciftçi *et al.* 2017; Yıldırım and Kaya, 2017). Furthermore, their cellulose, lignin and glucose contents and also growth traits like diameter and height have been screened already (Taşkıran, 2014). By the contribution of the current study, their lignocellulosic potentials were also evaluated by the help of the activities of the enzymes of the important metabolic pathways.

Based on the results of all these previous studies, it is possible to identify new European black poplar clones to be register as a commercial clone suitable to different geographic regions of Turkey. However, these potential clones need to be subjected to field trials in different geographical regions of Turkey, regarding availability of the potential plantation sites.

CHAPTER 6

CONCLUSION

European black poplar is one of the most promising woody biomass crops and it has a great potential for lignocellulosic bioenergy production. It is not only ecologically, but also an economically important riparian zone fast-growing tree.

According to literature, the current study is the most comprehensive study conducted by biochemical and physiological approaches to examine natural genetic variation of lignocellulosic traits, their heritabilities and relationship of among these traits. Our sampling strategy and experimental design allowed us to determine the contribution of various factors to total variance of the studied traits. Not surprisingly, we did not observe any significant ramet and replications effects in our study. As expected, we concluded that the great portion of total phenotypic variation was caused by clonal variation (33.0% to 64.1%) which displayed the diverse genetic background of the clone bank for the studied traits.

In our study, moderate to high clonal heritabilities, ranging 0.50 (in UGPase) to 0.80 (in glucose) were estimated for lignocellulosic traits. Except for PAL activity, all lignin related traits and UGPase ativity displayed moderate heritability (0.50 - 0.57). However, high heritability estimates were found for PAL (0.61), cellulose (0.75) and SuSy (0.71) traits. These results clearly showed that lignin related traits were more susceptible to environmental factors than cellulose content and SuSy. Similar to our findings, various previous studies reported effects of environmental factors on lignin contents of poplar woods.

Contrary to our expectations, we could not detect any correlation between lignin content and the related pathway enzymes. Although PAL, 4CL and CAD were positively correlated with each other, and negatively correlated with UGPase enzyme,

it was observed that they may not be related to lignin content or, more complicated regulations in the pathway were taking place. The positive correlation among phenylpropanoid pathway enzymes clearly demonstrated the flux of the substrate into the pathway and the negative correlation with UGPase also showed competition for the same carbon source. Moreover, the positive relationship was detected between cellulose content and SuSy (r_p = 0.184) and UGPase (r_p = 0.160) activities. These correlation coefficients highly increased with the clones having maximum activities with respect to high cellulose and low lignin contents.

Moreover, the results with the highest mean values of clones aided us to select superior lignocellulosic clones for future studies. According to these results, various clones like 62160 were detected with specific mean values for more than one trait. This clone was among the highest twelve clones regarding UGPase, SuSy, cellulose, height, and diameter, but it was also among the lowest twelve clones with respect to PAL and CAD activities. Similarly, some clones like N033861 and N92160 with highest lignin related enzyme activities, were previously reported as drought and cold resistant clones with up-regulated gene expressions. Commercially registered clones (Anadolu, Ata, Gazi, Geyve, Kocabey, Çubuk 1, and Çubuk 2) showed moderate lignocellulosic potentials with respect to those clones determined in this study.

The results of this study indicated that, all lignocellulosic and wood related traits studied here show natural genetic variations. By selecting the lignocellulosic clones, new breeding and conservation programmes can be developed by using molecular markers assisted tools. The results of this study pave the way to perform clonal level selection. By combining several approaches, we are ready to select and register new commercial clones to increase biomass yield efficiency. In our study S/G lignin ratio, hemicellulose and xylose contents were not studied. Since most of the recent transgenic mutant studies for these enzymes reported with structural changes in lignin composition rather than lignin content, it would be very beneficial to study these traits to clarify relationship between studied lignin and related traits.

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APPENDIX A

Table A. 1. *The Origin of European Black Poplar Trees in Behiçbey Clone Bank*

Clone Identity	Region	City
N.92.282	Aegean	Afyon
62/160	Central Anatolia	Ankara
62/172	Central Anatolia	Ankara
62/191	Central Anatolia	Ankara
64/13	Central Anatolia	Ankara
64/14	Central Anatolia	Ankara
64/14.06.1	Central Anatolia	Ankara
64/14.10	Central Anatolia	Ankara
64/14.12	Central Anatolia	Ankara
77/40	Central Anatolia	Ankara
82/1	Central Anatolia	Kırşehir
82/2	Central Anatolia	Kırşehir
82/3	Central Anatolia	Kırşehir
82/4	Central Anatolia	Kırşehir
83/1	Central Anatolia	Yozgat
83/10	Central Anatolia	Kayseri
83/12	Central Anatolia	Kayseri
83/13	Central Anatolia	Kayseri
83/3	Central Anatolia	Kayseri
83/5	Central Anatolia	Kırşehir
83/6	Central Anatolia	Kırşehir
83/8	Central Anatolia	Kırşehir
83/9	Central Anatolia	Kayseri
85/1	Eastern Anatolia	Ağrı
85/11	Eastern Anatolia	Erzurum
85/14	Eastern Anatolia	Erzurum

Table A.1 *(Continued)*

85/15	Eastern Anatolia	Erzurum
85/16	Eastern Anatolia	Ağrı
85/4	Eastern Anatolia	Ağrı
85/6	Eastern Anatolia	Ağrı
85/7	Eastern Anatolia	Ağrı
87/1	Central Anatolia	Kırşehir
88/1	Central Anatolia	Ankara
88/3	Central Anatolia	Ankara
88/4	Aegean	Aydin
88/5	Aegean	Denizli
88/6	Aegean	Denizli
88/8	Aegean	Denizli
ALTERRA	Foreign	Foreign
ANADOLU	Central Anatolia	Ankara
ATA 1	Central Anatolia	Ankara
ÇUBUK 1	Central Anatolia	Ankara
ÇUBUK 2	Central Anatolia	Ankara
EFN.1	Foreign	Foreign
EFN.2	Foreign	Foreign
FARCFLHHZ 35	Foreign	Foreign
GAZİ	Central Anatolia	Ankara
GEYVE	Central Anatolia	Ankara
KAE N.92	Unknown	Unknown
KELKİT 1	Blacksea	Gümüşhane
KELKİT 11	Blacksea	Gümüşhane
KELKİT 3	Blacksea	Gümüşhane
KELKİT 4	Blacksea	Gümüşhane
KELKİT 6	Blacksea	Gümüşhane
KELKİT 7	Blacksea	Gümüşhane
KELKİT 8	Blacksea	Gümüşhane
KELKİT 9	Blacksea	Gümüşhane
KOCABEY	Mediterranean	Adana
LVUBAKA	Foreign	Foreign

Table A.1 *(Continued)*

N.02.01.02	Open pollination	Open pollination
N.02.01.04	Open pollination	Open pollination
N.02.01.05	Open pollination	Open pollination
N.02.02.01	Open pollination	Open pollination
N.02.02.03	Open pollination	Open pollination
N.02.02.06	Open pollination	Open pollination
N.02.05.01	Open pollination	Open pollination
N.02.05.013	Open pollination	Open pollination
N.02.05.02	Open pollination	Open pollination
N.02.05.03	Open pollination	Open pollination
N.02.05.06	Open pollination	Open pollination
N.02.05.07	Open pollination	Open pollination
N.02.05.08	Open pollination	Open pollination
N.02.05.09	Open pollination	Open pollination
N.02.05.10	Open pollination	Open pollination
N.02.05.12	Open pollination	Open pollination
N.02.07.02	Open pollination	Open pollination
N.02.07.03	Open pollination	Open pollination
N.02.07.04	Open pollination	Open pollination
N.02.07.05	Open pollination	Open pollination
N.02.08.01	Open pollination	Open pollination
N.02.338	Unknown	Unknown
N.03.324	Unknown	Unknown
N.03.333	Unknown	Unknown
N.03.355	Eastern Anatolia	Erzincan
N.03.356	Eastern Anatolia	Erzincan
N.03.357	Eastern Anatolia	Erzincan
N.03.358	Eastern Anatolia	Erzincan
N.03.364	Eastern Anatolia	Erzurum
N.03.365	Eastern Anatolia	Erzurum
N.03.365	Eastern Anatolia	Erzurum
N.03.366	Eastern Anatolia	Erzurum
N.03.367	Eastern Anatolia	Erzurum

Table A.1 *(Continued)*

N.03.368	Eastern Anatolia	Erzurum
N.03.368.1	Eastern Anatolia	Erzurum
N.03.368.A	Eastern Anatolia	Erzurum
N.03.371	Eastern Anatolia	Elazığ
N.03.372	Eastern Anatolia	Elazığ
N.03.373	Central Anatolia	Kırşehir
N.03.375	Central Anatolia	Kırşehir
N.03.376	Central Anatolia	Kırşehir
N.03.377	Central Anatolia	Ankara
N.03.378	Central Anatolia	Ankara
N.03.399	Unknown	Unknown
N.62.164	Unknown	Unknown
N.82.008	Unknown	Unknown
N.82.166	Unknown	Unknown
N.85.010	Unknown	Unknown
N.85.018	Unknown	Unknown
N.90.008	Eastern Anatolia	Kars
N.90.010	Eastern Anatolia	Van
N.90.011	Eastern Anatolia	Van
N.90.012	Eastern Anatolia	Van
N.90.013	Eastern Anatolia	Muş
N.90.014	Eastern Anatolia	Bingol
N.90.016	Eastern Anatolia	Malatya
N.90.020	Eastern Anatolia	Sivas
N.90.024	Eastern Anatolia	Sivas
N.90.027	Eastern Anatolia	Sivas
N.90.028	Eastern Anatolia	Sivas
N.90.030	Eastern Anatolia	Sivas
N.90.032	Eastern Anatolia	Erzurum
N.90.034	Eastern Anatolia	Erzurum
N.90.035	Eastern Anatolia	Erzurum
N.90.036	Eastern Anatolia	Erzurum

Table A.1 *(Continued)*

N.90.038	Eastern Anatolia	Erzincan
N.90.039	Eastern Anatolia	Erzincan
N.90.045	Eastern Anatolia	Erzurum
N.90.046	Eastern Anatolia	Erzurum
N.90.050	Central Anatolia	Ankara
N.90.062	Unknown	Unknown
N.90.065	Unknown	Unknown
N.90.102	Unknown	Unknown
N.91.002	Unknown	Unknown
N.91.021	Unknown	Unknown
N.91.052	Blacksea	corum
N.91.054	Unknown	Unknown
N.91.058	Central Anatolia	Yozgat
N.91.059	Central Anatolia	Yozgat
N.91.063	Central Anatolia	Yozgat
N.91.067	Eastern Anatolia	Sivas
N.91.068	Eastern Anatolia	Malatya
N.91.071	Eastern Anatolia	Malatya
N.91.073	Eastern Anatolia	Malatya
N.91.074	Central Anatolia	Ankara
N.91.075	Mediterranean	Kahramanmaraş
N.91.076	Mediterranean	Kahramanmaraş
N.91.077	Mediterranean	Kahramanmaraş
N.91.078	Mediterranean	Kahramanmaraş
N.91.080	Mediterranean	Kahramanmaraş
N.91.081	Central Anatolia	Kayseri
N.91.083	Central Anatolia	Kayseri
N.91.084	Central Anatolia	Kayseri
N.91.085	Central Anatolia	Kayseri
N.91.088	Central Anatolia	Niğde
N.91.089	Central Anatolia	Niğde
N.91.090	Central Anatolia	Niğde
N.91.091	Central Anatolia	Aksaray

Table A.1 *(Continued)*

N.91.092	Central Anatolia	Aksaray
N.91.094	Eastern Anatolia	Malatya
N.91.095	Southeastern	Adiyaman
N.91.101	Southeastern	Gaziantep
N.91.102	Southeastern	Gaziantep
N.91.103	Southeastern	Gaziantep
N.91.105	Southeastern	Gaziantep
N.91.108	Southeastern	Gaziantep
N.91.109	Mediterranean	Osmaniye
N.91.110	Mediterranean	Osmaniye
N.91.111	Mediterranean	Osmaniye
N.91.112	Mediterranean	Hatay
N.91.118	Mediterranean	Antalya
N.91.119	Southeastern	Gaziantep
N.91.120	Southeastern	Gaziantep
N.91.122	Marmara	Sakarya
N.91.212	Unknown	Unknown
N.92.058	Unknown	Unknown
N.92.060	Unknown	Unknown
N.92.073	Unknown	Unknown
N.92.097	Unknown	Unknown
N.92.114	Unknown	Unknown
N.92.123	Marmara	Sakarya
N.92.124	Marmara	Sakarya
N.92.126	Marmara	Bilecik
N.92.128	Marmara	Bilecik
N.92.130	Marmara	Bilecik
N.92.131	Marmara	Bilecik
N.92.132	Marmara	Bilecik
N.92.133	Central Anatolia	Eskişehir
N.92.134	Central Anatolia	Eskişehir
N.92.137	Marmara	Yalova
N.92.138	Marmara	Yalova

Table A.1 *(Continued)*

N.92.140	Marmara	Kocaeli
N.92.142	Central Anatolia	Çankırı
N.92.144	Central Anatolia	Çankırı
N.92.148	Central Anatolia	Çankırı
N.92.149	Blacksea	Kastamonu
N.92.152	Blacksea	Kastamonu
N.92.153	Blacksea	Kastamonu
N.92.154	Blacksea	Kastamonu
N.92.156	Blacksea	Kastamonu
N.92.159	Blacksea	Sinop
N.92.160	Blacksea	Samsun
N.92.162	Blacksea	Samsun
N.92.164	Blacksea	Amasya
N.92.165	Blacksea	Amasya
N.92.166	Blacksea	Tokat
N.92.167	Blacksea	Tokat
N.92.168	Blacksea	Tokat
N.92.169	Blacksea	Tokat
N.92.170	Blacksea	Amasya
N.92.171	Blacksea	Amasya
N.92.176	Blacksea	Tokat
N.92.179	Blacksea	Tokat
N.92.182	Blacksea	Tokat
N.92.185	Blacksea	Amasya
N.92.187	Blacksea	Amasya
N.92.195	Blacksea	Amasya
N.92.200	Blacksea	Çorum
N.92.202	Central Anatolia	Çankırı
N.92.204	Central Anatolia	Çankırı
N.92.206	Aegean	Kütahya
N.92.208	Aegean	Kütahya
N.92.209	Aegean	Afyon
N.92.211	Aegean	Afyon

Table A.1 *(Continued)*

N.92.213	Aegean	Afyon
N.92.214	Central Anatolia	Konya
N.92.215	Central Anatolia	Konya
N.92.217	Central Anatolia	Konya
N.92.218	Central Anatolia	Konya
N.92.219	Central Anatolia	Konya
N.92.223	Central Anatolia	Ankara
N.92.224	Central Anatolia	Konya
N.92.230	Central Anatolia	Konya
N.92.232	Central Anatolia	Konya
N.92.233	Central Anatolia	Karaman
N.92.236	Central Anatolia	Konya
N.92.237	Central Anatolia	Konya
N.92.239	Central Anatolia	Niğde
N.92.240	Central Anatolia	Niğde
N.92.243	Mediterranean	Adana
N.92.245	Marmara	İçel
N.92.247	Central Anatolia	Karaman
N.92.250	Central Anatolia	Konya
N.92.252	Central Anatolia	Konya
N.92.254	Aegean	Afyon
N.92.255	Aegean	Afyon
N.92.256	Central Anatolia	Eskişehir
N.92.258	Central Anatolia	Eskişehir
N.92.260	Mediterranean	Adana
N.92.269	Blacksea	Zonguldak
N.92.271	Aegean	Kütahya
N.92.276	Aegean	Uşak
N.92.278	Aegean	Uşak
N.92.284	Aegean	Denizli
N.92.286	Aegean	Denizli
N.92.289	Aegean	Denizli
N.92.292	Marmara	Isparta

Table A.1 *(Continued)*

N.92.293	Marmara	Isparta
N.92.295	Marmara	Isparta
N.92.297	Marmara	Isparta
N.92.298	Marmara	Isparta
N.92.299	Marmara	Isparta
N.92.301	Aegean	Afyon
N.92.302	Aegean	Afyon
N.92.831	Unknown	Unknown
N.93.304	Southeastern	Gaziantep
N.93.306	Marmara	Bursa
N.93.309	Central Anatolia	Sivas
N.95.045	Unknown	Unknown
N.96.310	Eastern Anatolia	Malatya
N.96.315	Eastern Anatolia	Malatya
N.96.316	Eastern Anatolia	Malatya
N.96.317	Eastern Anatolia	Malatya
N.96.319	Eastern Anatolia	Elazığ
N.96.320	Eastern Anatolia	Bitlis
N.96.321	Eastern Anatolia	Van
N.96.322	Eastern Anatolia	Van
N.96.323	Eastern Anatolia	Van
N.96.325	Eastern Anatolia	Van
URIFFMH	Foreign	Foreign

APPENDIX B

Table B.1. *Mean squares, variance components as percent of total variance (VC), and broad sense heritability estimates (h^2) for the studied traits of all clones without removing duplicated clones*

Trait	*Repeat (df=1)*	*Region (df=9)*	*VC*	*Clones/regions (df=275)*	*VC*	*Error (df=283)*	*VC*	h^2
Cellulose	5.28^{ns}	455.18^{**}	0.92	196.21^{**}	54.46	53.30	44.62	0.71
SuSy	82.75^{ns}	19769.0^{**}	2.23	11108.00^{**}	58.18	2835.55	39.59	0.75
UGPase	771.48^{*}	498.83^{**}	2.81	287.55^{**}	31.75	146.34	65.44	0.49
Lignin	1.50^{ns}	59.51^{**}	3.59	23.17^{**}	31.85	11.67	64.56	0.50
PAL	37.91^{**}	5.55^{ns}	0.49	10.11^{**}	48.35	3.53	51.16	0.65
4CL	4.40^{ns}	3.4^{**}	0.13	3.71^{**}	39.43	1.62	60.44	0.57
CAD	0.0^{ns}	0.54^{**}	1.39	0.33^{**}	32.41	0.17	66.20	0.49
Glucose	40.71^{ns}	152.97^{**}	0.57	132.04^{**}	66.95	25.82	32.49	0.80

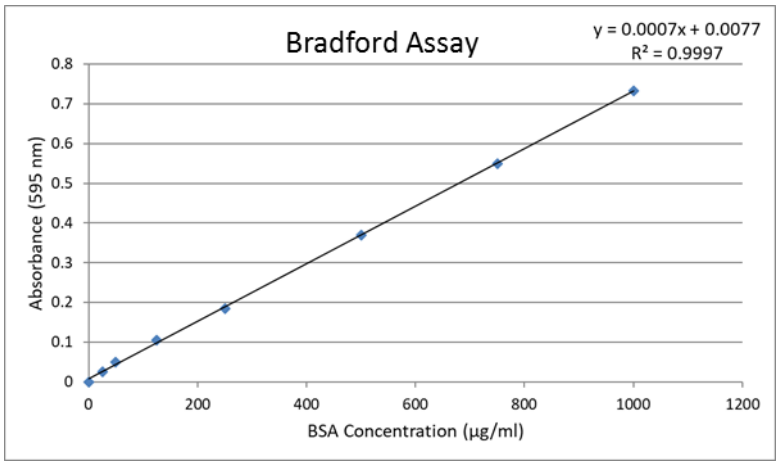
Table B.2. *Pearson correlations coefficients between studied traits*

	SuSy	*Cell.*	*PAL*	*4CL*	*CAD*	*Lign.*	*Glu.*	*Height*	*Diam.*
UGPase	0.032	**0.130***	**-0.128***	-0.082	**-0.120***	0.082	0.038	0.053	-0.025
SUSY		**0.133***	-0.082	**0.150***	**0.157****	0.043	0.070	0.101	**0.125***
Cell.			0.106	0.047	0.105	0.044	**0.125***	**0.133***	**0.132***
PAL				**0.219****	**0.153***	0.049	-0.019	0.037	0.069
4CL					**0.342****	0.028	0.062	-0.006	-0.051
CAD						-0.029	0.088	-0.023	0.019
Lign.							**0.267****	-0.040	**-0.139***
Glucose								0.048	0.046
Height									**0.777****

* Correlation is significant at $P \leq 0.05$ level (2-tailed).
** Correlation is significant at $P \leq 0.01$ level (2-tailed).

APPENDIX C

C.1. Total Protein Content Determination



BSA Conc. (µg/ml)	Abs. (595 nm)
1000	0.733
750	0.55
500	0.369
250	0.185
125	0.1055
50	0.05
25	0.025
0	0

C.2. PAL Specific Activity Calculation

- Specific activity of PAL : Units / mg = µmol of trans-cinnamate mg^{-1} protein min^{-1}

$$\text{Units / mg} = \frac{(\Delta A_{290/min}\text{sample} - \Delta A_{290/min}\text{control}) \times V_{tot} \times \iota \times DF}{\varepsilon_{transcinnamate}(mM) \times V_{pro} \times \text{mg protein}}$$

- V_{tot} = Total volume of reaction mixture
- V_{pro} = Volume of protein inside of reaction mixture
- ι = Light path = 0.725 cm (for 250 µL well)
- **DF** = Dilution factor
- $\Delta A_{290/min}$**sample** = Absorbance after an hour extracted from initial absorbance and calculated for minute
- $\varepsilon_{transcinnamate}$ = Molar extinction coefficient of transcinnamate at 290 nm as µM ($1x10^4\ M^{-1}cm^{-1}$)

C.3. Determination of Cellulose Content

Cellulose content of the branches was determined from 0.125 g freeze-dried samples according to the standard procedure described by Uppdegraf (1969). All chemicals were obtained from Sigma-Aldrich (Germany). The powders were digested by 750 µl acetic acid/nitric acid reagent to remove lignin, hemicellulose and xylosans inside of 15 ml falcon tubes. Acetic/nitric reagent was prepared by mixing 150 ml 80% acetic acid and 15 ml concentrated nitric acid. After stirring 2 minutes, loosely capped tubes were placed in a boiling water bath for 30 minutes. Following that they were centrifuged for 10 minutes at 5000 rpm. Supernatants were discarded carefully to prevent disruption of the solid cellulose found at the bottom of the tube. The remaining residue was washed with distilled water to remove acetic/nitric reagent completely and centrifuged again for 5 minutes at 5000 rpm. After discarding supernatant, 2.5 ml 67% sulphuric acid was added to the remaining residue and allowed to stand for an hour at room temperature. During this time, tubes were vortexed twice to assure complete degradation of cellulose. From these tubes, 250 µl of solutions were taken and transferred to 50 ml falcon tubes which include 25 ml distilled water and they vortexed for a minute. Inside of 15 ml falcon tubes, 250 µl of this diluted solution, 1 ml distilled water, and 2.5 ml cool anthrone reagent were mixed well. Anthrone reagent was prepared by solving 0.2 g anthrone with 100 ml concentrated H_2SO_4. It was chilled 2 hours in refrigerator prior to use so that it could be utilized freshly. Finally, loosely capped tubes were heated in boiling water bath for 10 minutes and cooled rapidly. By using 96 well Epoch Elisa Micro Plate Reader (Biotech, France), the color range was measured at 630 nm in triplicate by using cold anthrone reagent and distilled water as blanks.

C.4. Preparation of Cellulose Standard

To prepare the stock standard, 25 mg pure cellulose (Sigma-Aldrich, Germany) was added into 2.5 ml 67% sulphuric acid and allowed to stand for an hour. Tubes were vortexed twice during this time. 250 µl of solution was diluted to 25 ml with distilled

water to contain 100 µg cellulose / ml. Volume of series of 25 µl, 50 µl, 100 µl, 200 µl, 400 µl, 600 µl, 800 µl, 1000 µl, and 1250 µl were taken from this diluted stock solution. Final volumes were brought to 1250 µl with distilled water. Finally, 2.5 ml cool anthrone reagent was added and vortexed for a minute. The tubes were placed in a boling water bath for 10 minutes. After cooling rapidly, the color range was measured at 630 nm in in triplicate. Anthrone reagent was used as a blank.

C.5. Determination of Acid-Soluble Lignin Content

Acid soluble lignin content was determined from 0.2 g oven-dried samples according to the standard TAPPI Test Method T250 "Acid-Soluble Lignin in Wood and Pulp" which is a modification of the classic "Klason Lignin" determination (Dence, 1992). All chemicals were obtained from Sigma-Aldrich (Germany). Samples were first treated with 3 ml 72% cold H_2SO_4, added gradually. After stirring the test tubes a minute, they were allowed to stand for 2 hours at room temperature and vortexed once an hour. Having transferred the content of the test tubes to baby jars which include 50 ml distilled water, the remaining residue at the test tube was rinsed with extra distilled water and transferred to baby jar. Final volume of solution was brought to 115 ml to dilute the acid 3%. Baby jars were loosely capped and autoclaved for 30 minutes to hydrolyze the polysaccharides to soluble fragments, later allowed cooling until near room temperature before removing the caps and settle the supernatant until it is clear. The supernatant solution which includes acid soluble lignin was filtered through Whatman number three filter paper to 100 ml Erlenmeyer flask. To obtain the absorbance range between 0.2 and 0.7, a sample of the clear filtrate was diluted as needed. Inside of 96 well plate 30 µl sample and 240 µl 3% H_2SO_4 were mixed by pipetting. The absorbance at 205 nm was measured in triplicate by using the same solvent, 3 % H_2SO_4, as a blank.

C.6. Determination of D-Glucose Content

The determination of D-Glucose content was carried out by some modification and combination of TAPPI Test Method T250 “Acid-Soluble Lignin in Wood and Pulp” and the standard procedure which was described by Hedge and Hofreiter (1962). All chemicals were obtained from Sigma-Aldrich (Germany). The supernatant obtained from 0.2 g sample by hydrolysis with 3 ml 72% H_2SO_4, dilution to 115 ml with distilled water, autoclaving for 30 minutes, and filtering through Whatman number three filter paper, respectively was used to measure total carbohydrate content. Exactly one hour later after autoclave stage, above 1 ml of supernatant solution, 4 ml cold anthrone reagent was added and vortexed for a minute inside of 15 ml falcon tube. Afterwards, falcon tubes were placed inside of boiling water bath for 8 minutes. Having cooled rapidly, the color range was measured at 630 nm in triplicate by using 96 well Epoch Elisa Micro Reader plate by using cold anthrone reagent as blanks.

C.7. Preparation of D-Glucose Standard

To prepare the stock standard, 0.2 g D-glucose was dissolved inside of 115 ml distilled water. Next, to prepare working standard, 10 ml of stock solution was completed to 100 ml with distilled water to contain 174 µg glucose / ml. Volume of series of 50 µl, 100 µl, 200 µl, 400 µl, 600 µl, 800 µl, and 1000 µl were taken from this solution. Final volumes were brought to 1000 µl with distilled water. Finally, 4 ml cool anthrone reagent was added and vortexed for a minute. The tubes were placed in a boling water bath for 10 minutes. After cooling rapidly, the color range was measured at 630 nm in triplicate. Anthrone reagent was used as a blank.

CURRICULUM VITAE

PERSONAL INFORMATION

Surname, Name	: TAŞKIRAN, Bircan
Nationality	: Turkish (TC)
Date and Place of Birth	: 19 May 1978 / KARABÜK
Phone	: +90 312 210 51 60
E-mail	: taskirans78@gmail.com

EDUCATION

Degree	Institution	Year of Graduation
PhD	METU Biology	2020
MS	METU Biology	2014
BS	Abant İzzet Baysal University, Biology	2009
High School	Karabük Fevzi Çakmak High School	1994

WORK EXPERIENCE

Year	Place	Enrollment
2018-now	METU, Biology	Research Assistant
2017-2018	Cornell University, SIPS, Horticulture Department	Visiting Scholar
2011-2017	METU, Biology	Research Assistant
2010-2011	Çankırı Karatekin University, Biology	Research Assistant

FOREIGN LANGUAGES

Advanced English

PUBLICATIONS

Acar P., Taşkıran B., Özdeğirmenci F., and Kaya Z. (2019) Turkish *Salix* L. Species: Molecular Phylogeny and Morphology *Forestist in press*

HOBBIES

Travelling, Arts, Gardening

INTERNATIONAL CONGRESS

Toth J., Cala A., Taskiran B., Stack G., Wilk R., Crawford J., Yeats T., Viands D., Smart C.D., Rose J.K., and. Smart L.B. (2019) Characterization of Cannabinoid Profiles and THCA/CBDA Gene Sequences in Industrial Hemp Cultivars in New York State. Plant and Animal Genome Conference XXVII (PAG) San Diego, CA, US January 12-16, 2019 (Poster Presentation, PO1087)

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