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# "*Populus spp.* dormant bud production and chemical characterization of bioactive phenolic compounds from bud extracts and sprout exudates"

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*Dedicated to my lovable "Parents"*

*and to my best friend "Hussain1017"*

**Abstract**

The global forestry industry, after having experienced a market recession in the last ten years, has now turned its vision towards integrated biorefinery. New models and business strategies are constantly being explored to reinvent the global wood, pulp/paper, bioenergy, food, and pharmaceutical industry through the sustainable exploitation of resources. In this way, poplars are important forest species due to their noble characteristics to fulfill the current needs of a growing population. Poplars are considered as the fastest growing tree under temperate zones. Short rotation coppice (SRC) plantations are well known during the XX century, which will be perfectly suitable to poplar species due to their fast juvenile growth. The main aim of this work is to characterize and identify, among poplar species, those genotypes that will couple bud production with a higher concentration of pharmacologically bioactive phenolic compounds. In this way, the use of short rotation coppice system can be beneficial to produce buds and other raw materials for the pharmaceutical industry and biomass for energy. In this study 48 different poplar genotypeswere selected for preliminary chemical characterization by using Near Infra-Red (NIR) spectroscopy. Four *Populus nigra* genotypes ("Poli", "58-861", "810-93" and"74.00") were used for the estimation of total biomass and bud production that, in average, ranged from 3.82 to 10.91 ton $ha^{-1}$ and 21.96 to 145.29 Kg $ha^{-1}$, respectively. ANOVA showedlow significant variance for average biomass productionand medium significant variance for average bud production between the four analysed genotypes. EtOH:$H_2O$ (70:30) solution was used for the extraction of bioactive phenolic compounds from *P. nigra* and *P. deltoides* genotypes. $^1$H NMR spectra results showed similar results for *P. nigra* bud extracts and sprout exudates. Total Phenolic Content (TPC) results showed significant variance between the four *P. nigra* genotypes but there was no significant variance between the four *P. deltoides* genotypes analyzed. Genotype "Poli" (smaller buds) has a high concentration of phenolic content (107.64 mg (GAE)/g) whereas genotype "810-93" (maximum bud length) has the minimum phenolic content (61.64 mg (GAE)/g). According to Devappa et al., 2015, more than 160 phytochemicals from *Populus* species have been identified. However, their natural role in plants has yet to be discovered. Most identified phytochemicals have potential pharmaceutical applications compared to other industrial uses. The symbiotic existence between the forestry and bio-refinery pharmaceutical industries could open new trade routes for the development of a sustainable green bio-economy.

**Keywords:** *Populus* species, Poplar bud production, NIR spectroscopy, $^1$H NMR spectra, TPC analysis.

**Index**

## 1. Introduction

Poplars (*Populus* spp.), known as "the trees of the people" (Gordon 2001), are incredibly fast growth species and captured people's interest around the World. People and poplars coevolved for many years. Poplars have highly diverse uses: timber, pulp, and paper, biofuels/bioenergy, protection of river banks, windbreaks, shelterbelts, improve soil quality, phytoremediation, watershed protection, and carbon sequestration (Berguson et al. 2010). As *Arabidopsis thaliana*, poplars are also considered a model plant for perennial woody plants because they are among the fastest-growing species, widespread all over the World, with a small genome, great natural genetic diversity within and between the species; they can be quite easily propagated by sexual and vegetative propagation in huge quantity and can be also genetically transformed. (Jansson & Douglas, 2007; Bradshaw et al., 2000; Dickmann, 2001). *Populus nigra* and *Populus deltoides* are two widespread species in Europe, and Northern America, respectively (Stettler & Bradshaw,1996; FAO, 2015). There is a higher number of hybrids selected crossing these two species which are known as euramerican poplar hybrids (*Populus* ×*canadensis*). These hybrids are the planting material used for the establishment of traditional poplar culture and, more recently, short rotation forestry (SRF), mainly for the production of timber and biomass (Wullschleger et al., 2002).

Currently, we are assisting to an increasing interest of the chemical industry on the economic potential of chemical products of natural origin from the main commercial tree species. Despite the apparent abundance of information on metabolite structure and activity, there is a relative dearth of data on the yield or variability in yield of metabolites for tree species upon which to base an assessment of economic potential. In the short-term, basic research is required to quantify the impact of tree genetics and environmental factors on yield and hence the final quality of the tree product (https://secure.fera.defra.gov.uk/treechemicals/review/index.cfm). Phytochemicals are secondary metabolites of plants that are not primarily essential for their growth, but are important for protecting plants by pathogens and insects, and for maintaining plant health (Pandey and Rizvi, 2009). In poplar trees, bark and other tree residues (leaves, buds and branches and exudates) are rich sources of bioactive phenolic compounds of pharmaceutical importance. Poplars are a natural library of various highly useful chemicals, still little explored for their potential uses. These compounds can be harvested as extracts (heterogeneous mixtures of chemicals) or as pure chemicals through isolation and purification procedures. Plant-based natural products are playing vital role in food, agriculture, pharmaceuticals, and cosmetics industries. Especially phenols caught the first place in food and pharmaceutical industries due to their antioxidant, anticancer, anti-inflammatory, and antimicrobial activity (Devappa et al., 2015).

## 2. State of the art

### 2.1 The scientific interest of the *Populus* genus

Trees are used to produce a variety of wood-based products including timber, pulp, and paper; More recently their use as a source of renewable energy has been highlighted. Relative to food crops, the domestication of trees has only just begun; the long generation time and the complex nature of juvenile and mature growth forms are contributory factors(Taylor, 2002). To accelerate domestication, and to understand some of the unique processes that occur in perennial woody plants a "model tree" is needed to complement the genetic resources being developed in *Arabidopsis*.In this role, *Arabidopsis thaliana* has gained a supreme acceptance amongst plant scientists. The reasons for this are quite clear because the species is widely distributed around the globe, completesitslife cycle very rapidlyandcanbe easilygenetically transformed (Taylor, 2002). The wide range of genetic and trait variation of *Arabidopsis thaliana* may be exploited to produce crosses to follow segregation of specific traits and,in the creation of genetic maps, recombinant inbred lines (RILs) and near isogeneic lines (NILs). Researchers sequenced the complete genome (AGI,2000) of *Arabidopsis thaliana.* Approximately 7000 researchers are using this model for experimental systems,butitis not suitable for crop plants like rice, wheat, and maize because these are monocotyledonous plants with different physiology, biochemistry, and development compared to dicotyledon weed systems(Huala et al., 2001).

However, this model system doesn't fit with woody plants for traits related to perennial species. Some unique characteristics of tree growth, anatomy, physiology, and biochemistry canbe investigated in woody trees. A large number of these unique characters are related to their perennial growth (Bradshaw et al., 2000). As trees grow long periods, they form secondary xylem which completely differs from vascular cambium(Taylor, 2002).Trees have a complex crown architecture to compete with other plants for light, showing different signal transduction pathways based on seasonality(dormancy, types of bud release, nutrients storage in harsh conditions, etc.) with respect to the environment. Similarly, the beginning of flowering varies in woody plants depending on the species (willows1-year-old, poplars4-6-year-old, andoaks30-year-old). Despite this, several genes of *Arabidopsis* are similar to those of woody species, such as in the case of CO/FT regulatory module that controls timing of flowering and seasonal growth cessation in poplar (Bohlenius et al., 2006; Weigel and Nilsson, 1998).

The power of genetics, molecular biology, and genomics make poplars attractive as a model plant. In general, these species are widespread geographically in different altitude and latitude

range(Brunner et al., 2004), which shows large intra-specific variability and high phenotypic plasticity in different environments (Jansson et al., 2007).Besides the great genetic variation among natural populations, there are other important characteristics that made poplars a good model species for research such as aptitude to sexual and asexual reproduction to attain interspecific hybridization, rapid and adaptivephysiological response to the environment, close coupling of physiological traits and biomass productivity, well characterized molecular physiology and finally rapid growth to maintain sustainable bio-economy (Bradshaw et al., 2000). *Populus* species has 19 haploid chromosomes (2n=38) with a small genome size of about 450-550 million base pairs (Bradshaw et al., 2000; Taylor, 2002). The *Populus* genome is just 4 times larger than *Arabidopsis* and 40 times smaller than the size of the *Pinus* genome (about 22 billion base pairs), which makes it simple the work in many molecular genetic techniques like gene cloning.

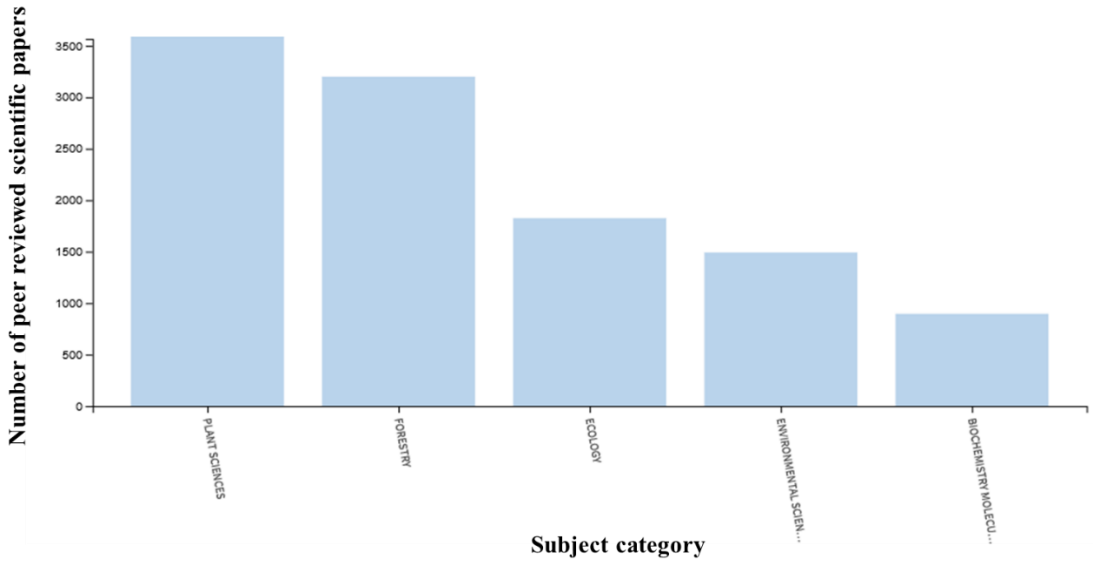



Figure2.1 - Peer-reviewed scientific papers (13,206) on *Populus* ssp. on the period 1990-2019 (Source: WOS, September 2019)

Well-established collaborations among poplar scientists have produced more than 13,000 papers in the recent years (Fig. 2.1),leading to share genetic materials, genetically informative two- and three-generation pedigrees, DNA-based genetic markers, common field measurement protocols, and clonal plantation trials(Klopfenstein et al., 1997; Stettler et al.,1996).

Since past decades, there is a great improvement in genomics, bioinformatics, and molecular biology, public access to the DNA sequence, experimental sequence tag (EST) system, DNA microassay, bacterial artificial chromosome (BAC) library, gene ontology (GO), high throughput plant transformations, and regeneration.

Table 2.1 - Comparison of genome size of *Arabidopsis thaliana*, *Pinus* and *Populus*.

| Species | Genome size (Mbp) |
|---|---|
| *Arabidopsis* | 100–150 |
| *Pinus* | 20 000 |
| *Populus/Salix* | 450–550 |

From September 2004, the entire genome sequence of *P. trichocarpa* Torr. & Gray (Tuskan et al., 2006) is available on the website of the Joint Genome Institute (http://jgi.doe.gov/). The sequence of the *P. trichocarpa* genome provides a powerful tool for exploring the structure and function of the genome in the poplar and similar genus using comparative mapping.

Strengths of *Populus* to considered as a model system are well-established collaboration among poplar biologists, abundant genetic variation in natural populations, ease of sexual propagation and interspecific hybridization, physiologic responses to environmental variables are rapid and pronounced, close coupling of physiological traits and biomass productivity, well-characterized molecular physiology, dramatic patterns of nitrogen allocation, use, and storage. We have observed healthy poplar leaves with nitrogen concentrations exceeding 8% of dry weight (J.Cooke, K. Brown, J. Davis, unpublished), compared with the 1–1.5% maximum levels found even in fertilized conifer needles. Nitrogen levels fluctuate dynamically among organs within the same tree (roots, stem, and leaves) in concordance with seasonal rhythms of active growth and dormancy), physiological process models for poplar growth and development, cloning of individual tree genotypes, closely related to other angiosperm model plants (unlike the pines and other gymnosperms, poplars diverged relatively recently from other angiosperms, such as Arabidopsis, which serve as models for integrating genetics into the study of plant biology), small genome size, basic molecular genetics toolkit, and facile transformation and regeneration to create a transgenic poplars.

### 2.2 *Populus* genus, sections, and species: *Populus nigra and Populus deltoides*

Poplars as members of the *Salicaceae* family are trees with many valuable characteristics which have led to multiple beneficial uses for society and the environment since the dawn of history. The accumulated global knowledge and information on poplars could fill many volumes. The characteristics which have made them so attractive and useful include fast growth, ease of propagation, propensity to hybridize, pleasing appearance and many uses. As well as providing

wood, fiber, fuelwood, and other forest products. Poplars benefit society in the rehabilitation of degraded land, restoration of forest landscapes and mitigation of climate change. All of these benefits support rural livelihoods and contribute to sustainable development, particularly in developing countries. (Isebrands and Richardson, 2014).

### 2.2.1 Evolution and migration history of *Populus*

The genus *Populus* has a great history and was one of the oldest genera of existing angiosperms originating in China and Japan during the Triassic, although these fossil records are now associated with other taxa. While the closest relatives in the *Flacourtiaceae* come from tropical Asia, the fossil record now indicates that the genus *Populus* had tropical origins in North America during the late Paleocene, about 58 million years ago (Collinson 1992). These first leaf fossils are very similar to the current *P. mexicana* in the *Abaso* section (Eckenwalder 1996). At the end of the Eocene, the first Eurasian relatives of other sections appeared, with those of the *Turanga*sectionconfinedin the old World and an ancestor of the *Leucoides* section invaded the temperate region. During the Oligocene precursors of *Tacamahaca* and *Aigeiros*sectionsappearedand thesewould not have become distinct sections until the Miocene; at that time also members of the *Populus* section appeared (Collinson 1992, Eckenwalder 1996a).

The evolution of the advanced sections of *Populus* has been characterized by rapid speciation during allopatry cycles but influenced by a widespread introgression, both within and between sections (Eckenwalder 1984b, 1996; Smith and Symata 1990;Kaul 1995). This rapid sequence of eventswith very conflicting evidence and the confusion that characterized species identification made it difficult to keep track of the recent evolutionary history of poplars in the more advanced sections (Eckenwalder 1996). Although there is evidence of evolutionary divergences between the sections, these are widely distributed in the northern hemisphere. The species within the sections are highly correlated and many of them are the most widely distributed among the tree species.

Poplars are pioneer species and migrate rapidly. Pollen studies have shown that *Populus* species often dominated the first forest communities after the glaciations (Cwynar 1988, Keenan and Cwynar 1992). In Europe, *P. tremula* is the first pioneer species. *P. nigra* occurs along the rivers along with *Salix alba*. It is believed that the large stands of *P. tremuloides* in North America were born immediately after the withdrawal of the Pleistocene ice sheet and have since been kept asexually by root suckers, making them some of the largest and oldest organisms in the World

(Dickson &Barnes 1975, Kemperman and Barnes 1976, Mitton and Grant 1980, Cheliak and Dancik 1982).

### 2.2.2 Taxonomy and natural distribution of *Populus*

Poplar species are members of the *Magnoliophyta* division, class *Magnoliopsida* (dicot), subclass *Dilleniidae*, order *Salicales*andincludes the family genera *Populus*(Isebrands, 2014). The genus is taxonomically divided into sections. Sixof these sections are widely recognized: *Turanga, Leucoides, Aigeiros, Tacamahaca,Populus, and Abaso* (Dickmann and Kuzovkina, 2014).
Periodically, taxonomists were intended to add further sections of individual species to solve classification problems. For example, Browicz (1966) proposed the *Tsavo* section, to include the East African species *P. ilicifolia*, a species not even recognized by some taxonomists and included by others in the *Turanga* section. The *Ciliates* section was proposed to include the Himalayan species *P. ciliata*, previously included inthe*Leucoides* (Khosla and Khurana 1982), an obvious mistake that others have suggested to be resolved by classifying the species under *Tacamahaca*. Yet another section, *Abaso*, has been proposed and recently accepted to host *P. mexicana* which appears to be weakly related to other species of the *Aigeiros* section (Eckenwalder 1996).

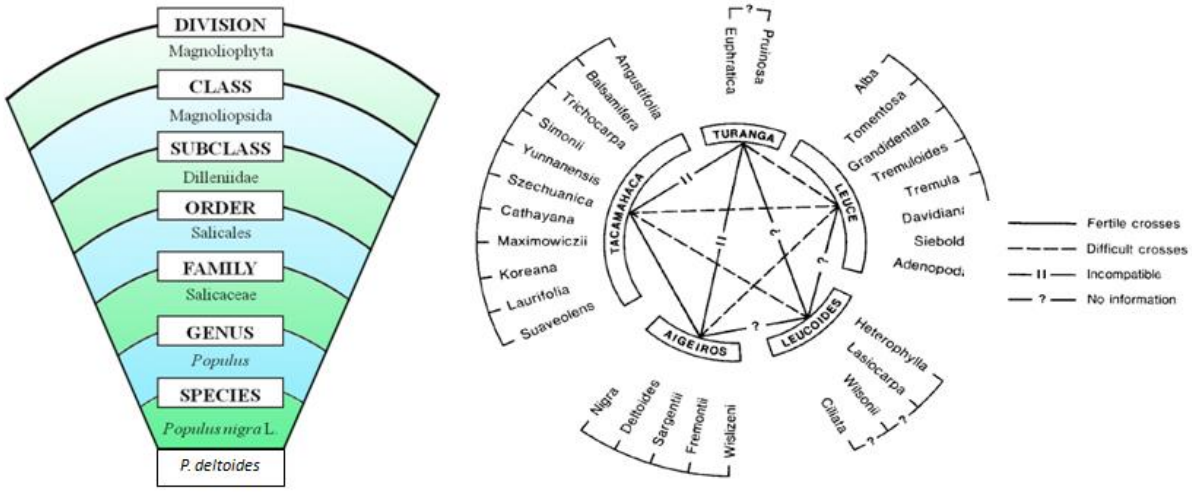



Figure 2.2 -Taxonomy hierarchy of *Populus nigra and P. deltoides* (on the left). Scheme of possible hybridization among sections of the genus *Populus* (on the right), (modified from Zsuffa, 1975).

Table 2.2 - Botanical characteristics of *Populus* and *Salix* (Modified from FAO, 2014)

| Character | Genus *Populus* (poplars, cottonwoods and aspens) | Genus *Salix* (willows, sallows and osiers) |
|---|---|---|
| Genome | $2n = 38$ chromosomes; rarely triploid; 485 ± 10 million DNA base pairs, 45,555 nuclear genes, 153 chloroplast and mitochondrial genes | $2n = 38$ chromosomes; diploid to dodecaploid (12×); genome has not been sequenced |
| Flowers | Appear before leaves, catkins pendulous, wind pollinated. Flowers with oblique perianth, cup-shaped disk without nectaries; bracts irregularly denticulate, shed rapidly; stamens numerous, 5–50, usually with reddish anthers; pollen thin-walled and non-aperturate; stigmas with 4 or more lobes; ovaries with 2, 3 or (rarely) 4 carpels | Appear before, with, or after leaves; catkins mostly erect; insect or wind pollinated. Perianth and disk usually absent but with 1 or 2 nectaries; bracts entire, pubescent, usually persistent; stamens few, 2–12, usually with yellow anthers; pollen thick-walled and tricolpate; stigmas 2-lobed; ovaries with 2 carpels |
| Fruit | 2-, 3- or 4-valved capsule | 2-valved capsule |
| Leaves | Variable in shape – deltoid to cordate to ovate to lancelotate, occasionally palmately lobed; venation palmatopinnate; margins serrate or dentate and glandular. Indeterminate shoots heterophyllous; heteroblasty occurs in some taxa | Never lobed or deltoid, almost always elongate in shape – obovate, oval, ovate-lanceolate, lanceolate or lanceolate-linear; venation pinnate; margins finely serrate or entire, occasionally glandular. Indeterminate shoots homophyllous |
| Stipules | Not persistent | Sometimes persistent and prominent |
| Petioles | Long, sometimes flattened transversely; glands may occur at junction of petiole and leaf blade | Short, round in cross section |
| Buds | Elongated, often pointed, covered by several overlapping scales, sometimes resinous and fragrant; usually divergent from twig; mostly monopodial with prominent terminal bud | Enveloped by a single scale; closely appressed to twig; mostly sympodial and lacking a true terminal bud |
| Shoots | Moderately stout; brown, purple or red in colour; circular or angular in cross section; lenticels prominent; pith pentagonal in cross section; heterophyllous (dwarf shoots may be present). Many taxa produce root suckers | Slender; green, brown, yellow, orange, purple or red in colour; circular in cross section; pith circular in cross section; homophyllous (does not form brachyblasts). Rarely develop root suckers |
| Wood | Light (specific gravity 0.31–0.40), straight grained, soft, pale, not durable, often with a disagreeable odour when wet; rays homocellular | Light (specific gravity 0.30–0.42), uniform, straight grained, soft, pale, not durable, tough and shock resistant, odourless; rays heterocellular |
| Habit | Medium to large trees, rarely shrubs | Extremely variable; can be procumbent plants, multi-stemmed shrubs and medium to large trees |
| Habitat | Mostly warm and cold temperate regions; common in wetlands, riparian corridors or uplands; few taxa found in tundra and alpine zones | Mostly cold temperate regions; common in wetlands, peatlands, riparian corridors, but uncommon in uplands; abundant in tundra and alpine zones |
| Number of taxa | 22–45 | 330–500 |

Table 2.3 -Suggested classification, nomenclature, and occurrence of *Populus* species, and synonyms in square brackets ( ModifiedZsuffa 1975 and Eckenwalder 1996)

| Section | Scientific name & synonyms | Common names | Occurrence |
|---|---|---|---|
| *Abaso* Ecken. | | | |
| | ***P. mexicana*** Wesmael | | Mexico |
| *Turanga* Bge. | | | |
| | ***P. euphratica*** Oliv. | **Euphrates poplar**, bahan | Spain, NE Africa, Asia |
| | ***P. ilicifolia*** (Engler) Rouleau | | E. Africa |
| | ***P. pruinosa*** Schrenk | | E. Eurasia |
| *Leucoides* Spach | | **large-leaved poplars** | |
| | ***P. lasiocarpa*** Oliv. | **Chinese necklace poplar** | China |
| | ***P. glauca*** Haines [*P. wilsonii* Schneid.] | | China |
| | ***P. heterophylla*** L. | **swamp cottonwood**, swamp poplar, | USA |
| *Tacamahaca* Spach | | **balsam poplars** | |
| | ***P. angustifolia*** James | **narrowleaf cottonwood**, narrowleaf balsam poplar | southern Sask. And Alberta to southwestern US |
| | ***P. balsamifera*** L. | **balsam poplar** | North America |
| | ***P. ciliata*** Royle | | Himalayas |
| | ***P. laurifolia*** Ledeb. | **laurel poplar** | eastern Asia |
| | ***P. simonii*** Carr. | **Simon poplar** | eastern Asia |
| | ***P. suaveolens*** Fish. [*P. cathayana* Rehd. *P. koreana* Rehd *P. maximowiczii* A.Henry ] | **doronoki**, Japanese poplar | NE China, Japan |
| | ***P. szechuanica*** Schneid. | | E. Eurasia |
| | ***P. trichocarpa*** Torr. & A.Gray | **black cottonwood**, western balsam poplar | western Canada and US |
| | ***P. yunnanensis*** Dode | | E. Eurasia |
| *Aigeiros* Duby | | **Cottonwoods and Black Poplars** | |
| | ***P. deltoides*** Marsh. [*P. sargentii* Dode, *P. wislizenii* Sarg. ] | **eastern cottonwood** (ssp. *deltoides*), **plains cottonwood** (ssp. *monilifera*), **Rio Grande cottonwood** (ssp. *wislizenii* | Quebec, Ontario Prairie Provinces to Texas SW USA |
| | ***P. fremontii*** S.Wats. | **Fremont cottonwood** | SW USA |
| | ***P. nigra*** L. | **black poplar**, European black poplar | Europe, western Asia |
| *Populus* L. [*Leuce* Duby] | | **aspens** | |
| | ***P. adenopoda*** Maxim. | | |
| | ***P. alba*** L. | **white poplar**, silver poplar | central and southern Europe to N. Africa, central Asia |
| | ***P. gamblei*** Haines | | E. Eurasia |
| | ***P. grandidentata*** Michx. | **largetooth aspen**, bigtooth aspen, aspen, poplar, popple | eastern North America |
| | ***P. guzmanantlensis*** Vasq. & Cue. | | Mexico |
| | ***P. monticola*** Brand | | Mexico |
| | ***P. sieboldii*** Miq. | **Siebold aspen**, Japanese aspen | Japan |
| | ***P. simaroa*** Rzed. | | Mexico |
| | ***P. tremula*** L. [*P. davidiana* (Dode) Schneid.] | **European aspen**, tremble, Zitterpappel | Europe, northern Africa, north-eastern Asia |
| | ***P. tremuloides*** Michx. | **trembling aspen**, quaking aspen | North America |



*Populus nigra* (black poplar) is widespread in Europe (except Scandinavia) and distributed to North Africa and Western Asia (FAO, 2015). *P.nigra* shows close characteristics to the *Aigeiros*

section in some traits and DNA of chloroplast has been reported to be similar to that of *P. alba* (Smith and Sytama, 1990). Black poplars are large trees, reach around 40 m of height, 2 m diameter and live a maximum of 200 to 300 years (Weisgerber, 1999).

section in some traits and DNA of chloroplast has been reported to be similar to that of *P. alba* (Smith and Sytama, 1990). Black poplars are large trees, reach around 40 m of height, 2 m diameter and live a maximum of 200 to 300 years(Weisgerber, 1999). It shows different shapes of crown architecture depending on the altitude and latitude of provenance, varietyandtype of management. It can be easily regenerated by vegetative propagation as well as sexual propagation. Black poplar is a pioneer species that colonizes river floodplains, wastelands, and other nudesites, with the wet and sandy soil exposed after seasonal floods that provide the optimal seedbed. It is a characteristic species of riparian ecosystems, which are among the most biologically diverse in the area of its natural range (FAO, 2015).

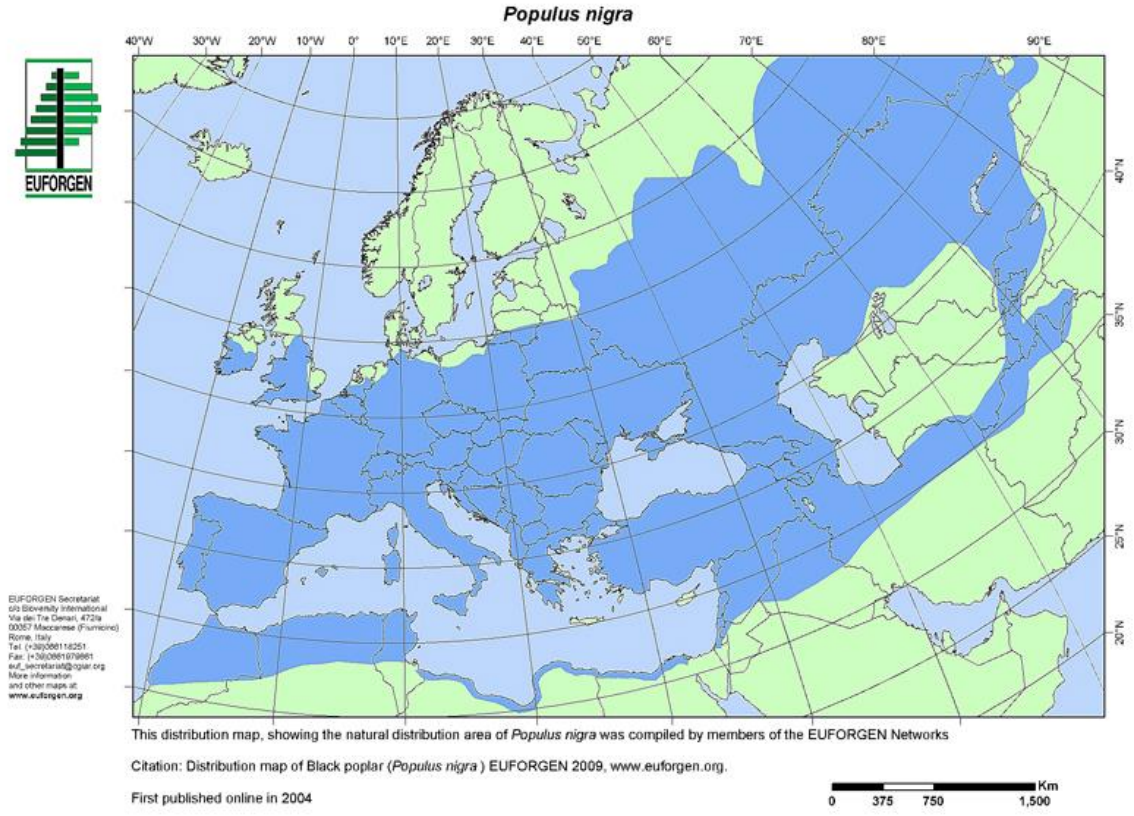



Figure 2.3 -Distribution map of black poplar (*Populus nigra* L.), EUFORGEN 2009 (URL: http://www.euforgen.org/distribution_maps.html)

Black poplars wood is not of high quality,and *P.* ×*canadensis* hybrids (*P. deltoides* × *P. nigra*) are mainly used in many countries for poplar plantations. As it is a widespread species with a highgeneticdiversity, it is used as the male parent in genetic improvement programmes all over the World to produce euramericana hybrids (FAO, 2015). More recently, the intersectional

hybrids between *P. nigra* and othertaxa in the *Tacamahaca* section, including the Asians *P. maximowiczii*, *P. laurifolia, P. simonii, P. cathayana* and the North American, *P. trichocarpa* has been successful. In China, *P. simonii* × *P. nigra* was a favorite combination for a long time (Weisgerber, 1999).

*Populus deltoides* Marshall (eastern cottonwood) is also one of the silviculturally important poplar and it is distributed natively over the Eastern, Southern and mid-western of USA and Southern Canada. It is also spreading tremendously to the entire temperate World (Stettler and Bradshaw, 1996; FAO,2 015). It is a medium to large tree, reaching around 45 m of height and a diameter of about 3 m or more. Cottonwood shows different crown structures depending on the type of forest, in a closed forest it shows small and columnar crowns, whereas in open places it shows highly forked branches to form a round massive crown.The twigs are stout, angular to ribbed in cross-section and produce slightly resinous outcurved buds. It is widespread in Northern America in lower lands and riparian corridors.

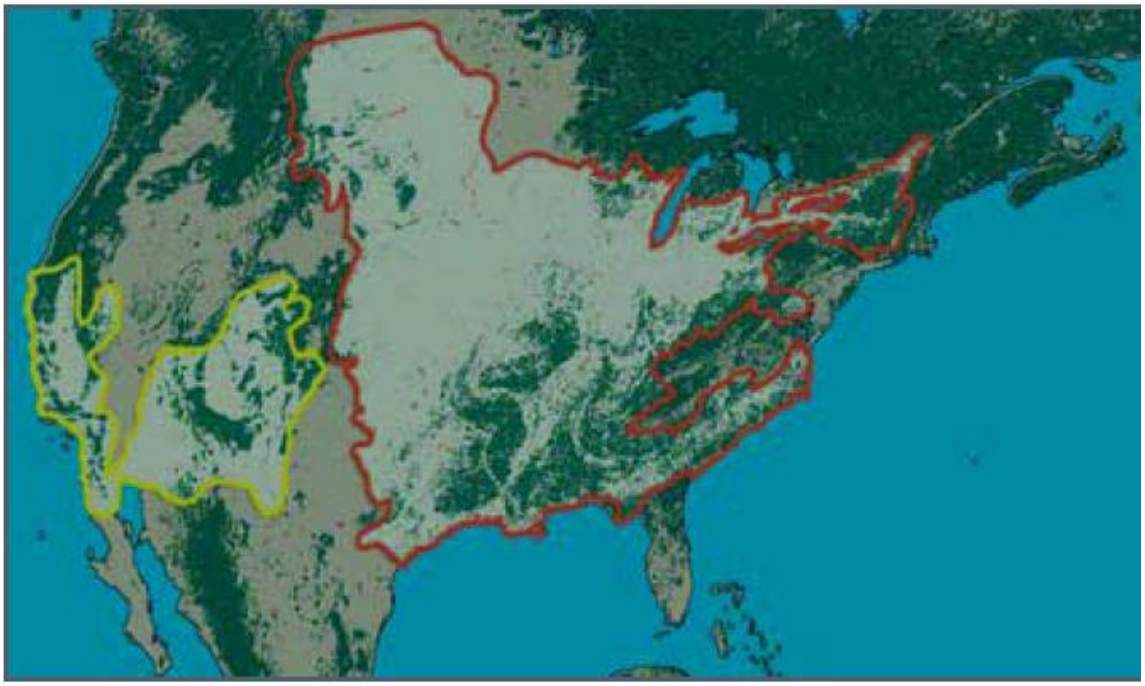


Figure 2.4 -Natural range of *P.deltoides* in the red color circle, whereas *P.fremontii* in the yellow circles (modified from FAO, 2015)

*P. deltoides* is considered as the fastest-growing species in Northern America and in the entire World. On the fertile soils of the Mississippi river delta, it reaches 4 m of height per year during the first 5 years (Knew et al., 1970). It can also produce approximatelyan average of 145 $m^3$ pulpwood in 12 years rotation (McKnight, 1970).

### 2.2.3 Biology and ecology of *Populus*

In general poplar species have a short life span (on average 100 years) compared to other broadleaves, but some individuals can reach 300 years (Weisgerbar, 1999). Some populations present a branchy and irregular crown, some others produce stands with a straight stem and a well formed crown that have been propagated vegetatively for commercial use. Until now there were 40 *P. nigra* clones registered in the IPC register of clones (FAO, 2015). At the juvenile stage, the bark appears white yellow and turns to dark with deep cracks along the bark.

Figure 2.5 - *P.nigra* leaves, vegetative buds, flowering buds, and seeds (on the right). *P.nigra* tree in the river bank (picture source Wikimedia) and *P. nigra*("58-861" genotype) buds (Photo: A.B. Uppara).

Typically, there are two kind of leaves (dimorphic) that can be observed in *P.nigra*, one is small with dark green color and has long flattened petioles. Another kind of leaves (neoformed leaves) are longer, broader, ovate or deltoid in shape (Dickmann and Kuzovkina, 2008).

*P. nigra* produces two different kind of branches, named as sylleptic and proleptic branches. Typically proleptic branches are originated from winter dormant bud whereas sylleptic buds grow from the buds during the same growing season. Black poplar is a dioecious tree species that must pass through female and male flowers on separate single-sex individuals. Both flowers are pendulous catkins, about 10 cm long, male reddish-purple and female greenish in color (Barsoum, 2001). Black poplars can also show hermaphroditic flowers, it means male and female

flowers on the same catkin or both male and female flowers on one tree on separate catkins (Wyckoff and Zasada, 2007).

*Populus deltoides* is also a fast growing species, native to northern America. Generally, trees grow about 45 m in height and can have a diameter up to 3 m or more. Even *P. deltoides* can produce two kind of leaves. Preformed leaves are with a deltoides shape with prominently toothed margins, whereas, neo-formed leaves are long and with fine toothed edges. This species produces slightly resinous, out curved buds. In young plants, the bark is smooth and light greenish-yellow to dark color, while in mature trees it is deeply furrowed and ashy-grey in color (Cooper and Van Haverbeke, 1990).

Figure 2.6 -*Populus deltoides* leaves, fruits, and seeds (on the left side). *P. deltoides* male catkins (on the right side). (Sources: https://www.minnesotawildflowers.info;https://plants.ces.ncsu.edu)

*Populus deltoides* produce male and female flowers during the spring season. Female flowers are from light yellowish to green color, whereas male flowers have a reddish-purple color. The fruit is formed by green and ovate shape capsules on the long pendulous catkins. The capsulesat maturity split into three or four carpels and release cottony seeds in the environment. *P. nigra* and *P. deltoides* are able to propagate by asexual and sexual reproduction.

Seed maturation and dispersion happens within 5-6 weeks after fertilization. Mature catkins consist of many capsules, about 20-50, each developed from a single flower each capsule contains about 4-5 small seeds (2 mm) with small or absent endosperm (Braatne et al., 1996). Seeds are viable for a very short period (about 2 weeks) and have a quick germination capacity. Seed germination doesn't depend on the season, but on moisture content of the soils. These seeds

are covered by hydrophobic cotton like structures, which are helpful to carry them around for long distances by wind or water during flood seasons (Wyckoff and Zasada 2007 and FAO, 2015). For these characteristics *P. nigra* and *P. deltoides* are pioneer species able to occupy flood plains and other wetlands.

Figure 2.7 - Riparian forest with black poplar along the Paglia river, VT – Italy (Photo: M. Sabatti).

After flooding the soil has a sufficient quantity of water, which is a perfect condition for the germination of the poplar seeds. The seeds travel through a high-speed wind as well as through the water. Poplar species don't tolerate the anoxic condition of the soil for a long time. They have the ability to reproduce vegetatively by root suckers, from broken branches, root collar sprouts, stool shoots, stem, branch, and root cuttings.

Poplars are widely distributed in theriparianecosystemsfromlowlandsto mountain areas in subtropical, temperateand boreal regions. They form large populations throughout the riparian banks as single species or mixed with *Salix alba* and other riparian species. In general, all poplars are heliophilous pioneer species colonizing nude virgin soilsafter floods, harvesting, forest fire, and land clearing. These species are very exigent of water and nutrients, and are verycompetitivewith the neighboring species (Heilman, 1996).

Figure 2.8 - Regeneration of *P.nigra* by various parts of plants (vegetative regeneration) along the Paglia river, VT – Italy (Photo: M. Sabatti).

Compared to the other sections of the *Populus* genus, the *Ageiros* section is distributed at lower elevations (Braatne et al. 1996).

### 2.2.4 Ecological importance of *Populus*

Poplars are friendly to the environment, so they were planted for an environmental purpose fromalongtime. Nowadays, in many places poplars are used for shelter, protecting crops, linear plantations in the cities torefreshtheenvironment and are also used for aesthetic purposes (IsebrandsandKarnosky, 2001; FAO,2015).However, in the 20$^{th}$ century poplars began to be selected and primarily used for the wood and pulp industry, but in recent years theuse for the environmental improvement and the ecosystem services they can provide is increasing(Constanza et al.,1997; USDA Forest Service, 2011). Ecosystem services are goods and services produced by the ecosystem to the society, which includes protection services, carbon storage,

phytoremediation, biodiversity, nutrient cycle, watershed services, and wildlife habitats (Zalesny, 2011).

Poplars were mainly used as windbreaks and shelterbelts since ancient days. In general, windbreaks and shelterbelts protect farmlands, homes, feedlots, orchards, and agricultural fields from high wind flow and to create a microclimate (Ford-Robertson, 1971; Helms, 1998). The design of the windbreaks and shelterbelts may vary depending upon the crops to protect or purposesto pursue (Rosenberg, 1974; Hagen, 1976; Tabler, 1980; Heisler and Dewalle, 1988; Vezina, 1994; Fan, 2010).

Figure 2.9 –*Populus nigra* planted in line as a windbreak to protect from strong wind. (Source: https://3fatpigs.co.uk/)

Black poplars and cottonwoods are used to control soil erosion and as riparian buffers since the 18th century in Mississippi alluvial valley (FAO, 2015). The main aims of this poplar plantations are to protect river banks and control the leaching of agricultural chemicals and waste into the river stream. Poplar riparian belts purify river water by control leaching of a higher amount of nitrogen, phosphorus, heavy metals and carcinogenic agents. They also enhance surface runoff, infiltration and maintain the quality of groundwater (Thornton, et al., 1998). There are many co-benefits from the government (ecosystem service payments in Northern America at the Mississippi River) on the qualified agricultural area (www.fsa.usda.gov). During the flood time, these keystone species (poplars) protect from soil erosion, which maintains the accumulation of sediment particles or fine particles and organic matter to protect the soil. Sometimes poplars contribute to form small islands within the rivers. Even though they have some problems (broken

due to flood stream), poplars have a great capacity to regenerate easily in many different ways (Corenblit et al., 2014).

Figure 2.10 - Development of a pioneer island within the active stretch of the Tagliamento river (Italy). Here the young *P.nigra*plants made an island structure during the flood by an accumulation of sediment or fine particles along with coarse gravels. (Gurnell et al., 2001). URL: https://onlinelibrary.wiley.com/doi/

Great appreciation for vegetative propagation is given to *P. nigra*, because it provides bio-engineering plant materials with low cost to restore the riparian river banks. Another important aspect of the contribution of poplars to the riparian habitat complexity is theincrease of biodiversity, with a big chance to invite a different kind of bird species (woodpecker, etc), animals(beaver, porcupine, and castles), insects and different fungi species, (Cooper and Van Haverbeke, 1990; Perala, 1990). Poplar natural stands mainly spread in Europe, especially in Spain, Romania, Hungary, and France account for about 131’400 hectares (Coaloa & Nervo 2011).

The land restoration plays also a great ecological role, because poplars as pioneer species, are used for the restoration of mine spoil lands in the northern hemisphere (Brenner et al., 1984). There are huge piece sof land that have been spoiled by farmer mine (Knabe, 1964; Rockwood et al., 2006). Poplars are fast growing species well adapted to different climate conditions that are quite suitable for the restoration of mine spoil lands (Davidson, 1979; Lumme & Tormala,1988; Rockwood et al., 2006). One of the most recent applications is the use of poplars for reclamation

and recovery of coal and oil sands region of Western North America (Isebrands & Richardson, 2014).

Figure 2.11 - Oilsands strip mine(on the left); Revegetation with poplar (on the right). Both pictures are from a site near Fort Mcmurray, Canada (FAO, 2015).

Poplars helping to nature unlimitedly by phytoremediation. Phytoremediation term, well known from the 1990s, means cleaning up or remediate the contaminated soils. Phytoremediation is "a technology that utilizes plants and then the associated rhizosphere microorganisms to remove, transform, or contain toxic chemicals located in soils, sediments, groundwater, surface water, and even the atmosphere" (Susarla et al., 2002). Typically, there are some sequenced processes in phytoremediation, which are phyto-extraction (extraction and translocation of contaminants in plant tissue), phyto-volatilazation (transform and convert into different form and sent to the air), rhizosphere degradation(degradation of pollutants at rhizosphere by a microorganism), phyto-degradation(degradation of pollutants within the plant cells), phyto-stabilization(maintenance of pollutants in soil and groundwater at certain level by absorption and accumulation in root system), hydraulic control(absorption and transfer of large quantities of water to translocate the pollutants from one place to another place.

Nowadays, pollution (air pollution, water pollution, soil pollution, etc.) is increasing gradually due to anthropogenic activities, so currently, there is a major interest on phytoremediation in the World. Phytoremediation brings a clear solution to reduce pollutant concentration released in the environment by various human activities such as: mining extraction, agricultural fertilizers, pesticides, chemical industry, sewage treatment, landfills, and urbanization.

Currently, phytoremediation is quite interesting to standardize many classes of pollutants, named as heavy metals, pesticides, chlorinated solvents, hydrocarbons, radionuclides, explosives, and other carcinogenic agents.

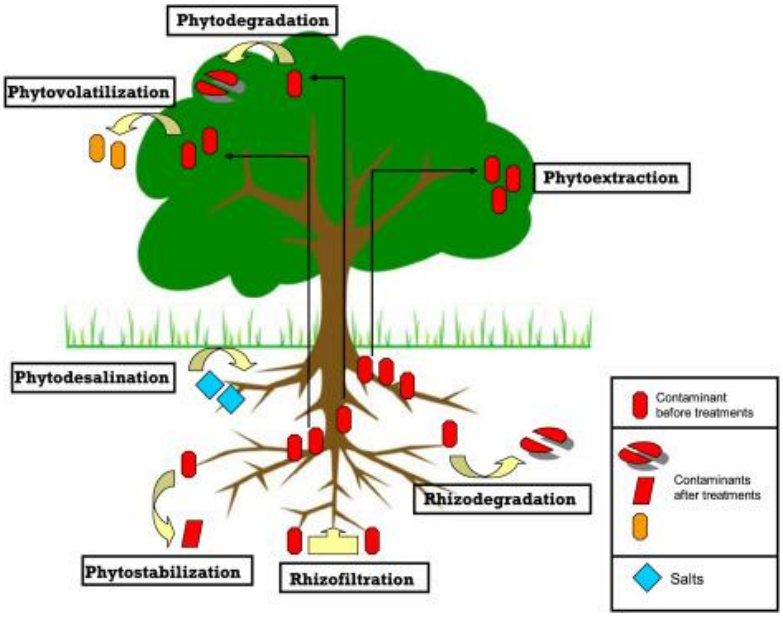



Figure 2.12 - The overall different physiological processes of Phytoremediation above ground, below ground, and alternative destinies of different forms of pollutants. (Oliver & Oliver, 2017). Source: https://www.sciencedirect.com/science/article/pii/S2352186417300330#dfig1

Poplars and willows are stood in first place for phytoremediation because they are fast growing species and have the deep root system to take large quantities of water and nutrients (Isebrands and Karnosky, 2001; Licht andIsebrands, 2005). Poplars not only take a large amount of water and also facilitate the growth of micro-organisms in the rhizosphere to enhance the plant and microorganism interaction(Robinson et al., 2000).

*Populus nigra* and *Populus deltoides* are considered among the best species for phytoremediation as they have all the suitable characteristics for this activity. Researches are looking not only to ecological constraints of the use of poplar species, but they are also focusing on the use of more productive plant materials. In fact, they are currently looking towards the use of poplar hybrids*P.*×*canadensis*in phytoremediation activities. The bioaccumulation capacity of *P.*×*canadensis*and *P. nigra* was tested for many different pollutants: the suspected 1,4-dioxane carcinogen (Aitchison et al., 2000), for cadmium (Gaudet et al., 2011; Zacchini et al., 2011, Robinson et al., 2000), for the high explosive 2, 4,6-trinitrotoluene (TNT) (Thompson et al., 1998), for atrazine used in the control of parasites (Chang et al., 2005) and many volatile compounds (Burken and Schnoor, 1999).

However, poplar has ultimate advantages in many directions and, due to these reasons, there is huge pressure on *P.nigra* and *P.deltoides* natural stands in riparian habitat. The decline of these pioneer species causes the reduction of the genetic variability within the species. Many forest biologists consider *P. nigra* one of the most threatened tree species in Europe (Dickmann and Kuzovkina, 2008) and, as a consequence, the riparian forests of *Populus* and *Salix* are designated as priority habitats in the Natura 2000 conservation strategy in Europe (Directive 92/43 / EEC). In order to protect the geographical distribution of poplars and their genetic pool, the inventory and protection of the existing natural populations of *P. nigra* is strongly recommended. Since 2008, the European Forest Genetic Resources Program (EUFORGEN) coordinates these efforts in the European Union.

### 2.2.5 Economic interest, aboveground biomass production and allocation of poplars

Poplars are important species, thanks to their economic value in terms of wood production and other wood by-products (Devappa et al., 2015). From poplar bark and residual wastes generated after poplar harvesting, it is possible to produce many useful industrial and pharmacologically active chemical compounds, and by-products used in the food industry and agricultural industry (FAO, 2015; Devappa et al., 2015).

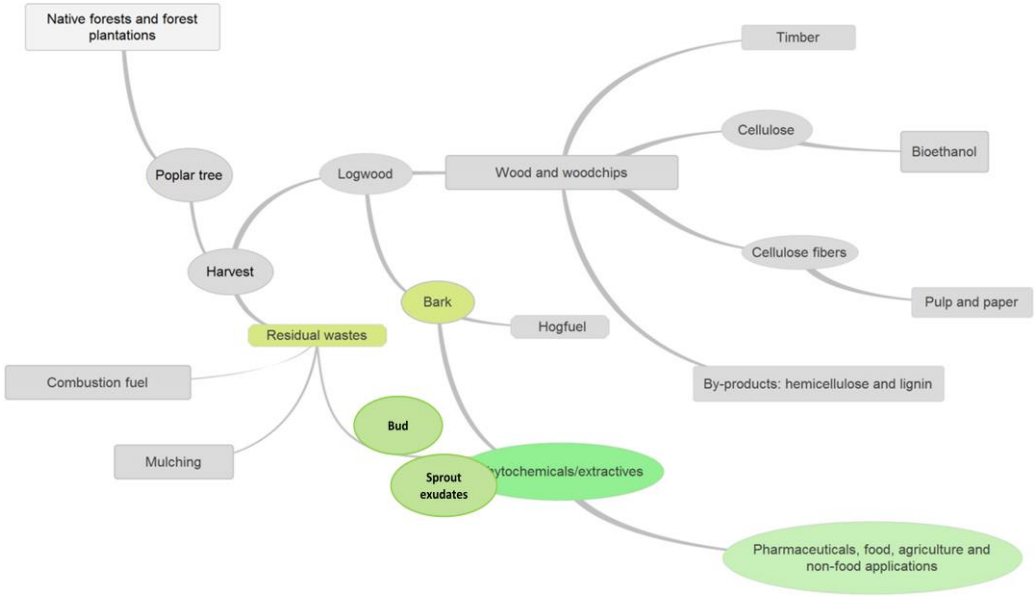



Figure 2.13- Integrated forest biorefinery concept: Potential products from poplar trees including phytochemicals from bud (Modified from. Devappa et al., 2015)

The goal is to produce more value from a given volume of harvested trees using material previously considered residual waste, and this can be obtainedby producing a wide range of

diversified product lines. This makes companies more resilient to market demand or price changes for each commodity (Devappa et al., 2015).

Since the seventies, the World is focusing on climate change and natural resource management to attain sustainability. Many countries are showing interest in afforestation activities with pure poplar stands or hybrid poplar plantations and agroforestry. The World wide poplar area is about 87,282,108 ha. In which, Russian Federation has highest area (24,757,000 ha), then USA (17,698,000 ha) and then China (12,900,000 ha) (IPC 2012). Currently, there is a huge poplar afforestation program contributing to China's three north shelterbelt program (SFA, 2010), which is the biggest poplar plantation project in the World, with 5'255'000 hectares of plantations, 3'867'000 hectares of agroforestry, and environmental plantations (FAO, 2008).

Wood production remains the main purpose of poplar cultivation in the European Union, where they cover an area of about 855'000 hectares .Approximately 80,000 hectares (8%) of poplars have been used for various protection systems. The poplar round wood production is about804'5000 cubic meters per year, destined for the production of plywood and veneers (40%), sawn wood (31%), pulp and paper (15%), reconstituted wood (9 %), bioenergy (4%) and other uses (1%) (Coaloa & Nervo, 2011).

In Italy, 118'500 hectares are occupied by poplar plantations that are 12% of total European area planted with poplars. In the same country, 20% of the poplar plantations (23'700 hectares) have a protective purpose, while the other 80% (94'800 hectares) have a productive purpose (Coaloa & Nervo, 2011). "The National Inventory of Forests and Carbon Forest Tanks" (INFC, 2007) provides lower values, with only 66'269 hectares used in poplar arboriculture. A surface of 940'200 hectares addressed to poplar's plantations is estimated, equal to 4% of the total plantations present in Europe.

In general, poplars give great support to reduce the pressure on nonrenewable resources. Many types of research are focusing on bioenergy/biofuels from poplar to maintain the sustainability of natural resources and forests. European Union introduced a policy to support renewable resources with the directive "20-20-20" scheme. This policy main motto is to achieve 20% of the increment in renewable resources by 2020. The main objectives of the 20-20-20 scheme are: The reduction of greenhouse gas emissions up to 20% with respect to the baseline of 1990; ii. The 20% increment in renewable resource usage; iii. The reduction of non-renewable resources (fossil fuels) up to 20% (Wyckoff and Zasada, 2007).

### 2.2.5.1 Short rotation forestry(SRF)

A short rotation coppice forest is the best choice to produce biomass in a short time. This kind of system is perfectly fit for stakeholders, who have small scale agricultural land (Verlinden et al., 2015). Fast growing broadleaves species have high resprouting capacity and are mainly used for short-rotation systems. Poplar is the best species because it has fast juvenile growth and incredible resprouting capacity (Sabatti et al., 2014). Short rotation forestry (SRF) is a process that began in the ’80 of the century in Sweden , in which plants are coppiced at their basis to resprout. This process is at the basis of poplar forest resilience after floods,and any other natural disturbance, where trees resproutvegetatively (Blake et al., 1983). It is a very easy and cheap process to producethemaximum quantity of biomass in a short period of time (Sennerby-Forsse et al., 1994; Verwijst, 1996). SFR system has socio-economic as well as ecological benefits (Sabatti et al., 2014).

SRF systems have multiple uses, as to produce highvolumes of biomass in agricultural land, protect soil from erosion, transfer and allocation of nutrients, great carbon sink contributing to the reduction of atmospheric $CO_2$, release of high amount of oxygen into the atmosphere, purification of underground water and also phytoremediation; all these benefits are directly or indirectly connectedto improve the biodiversityof the system (Perlack&Ranneyet al., 1987; Hansen, 1991; Hall & House, 1994; Zacchini et al., 2009; Tognetti et al., 2013; Perttu,1995; Isebrands and Karnosky, 2001; Immerzeel et al., 2014).*Populus* hybrids are the broad-leaved trees used for the production of biomass in SRF (Rae et al. 2004), which produce yields up to 35 tons oven-dried weight per hectare per year (ton $ha^{-1}$ $year^{-1}$) (ScarasciaMugnozza et al. 1997). Within the genus *Populus*, there is a large phenotypic and genetic variation in growth performance, canopy architecture and physiology (Ceulemans et al., 1990; Cervera et al., 2005), with remarkable plasticity for plant productivity estimated in different environments (Marron et al., 2010a). ,

The total estimation of the short rotation forest system in Europe is about 50-70,000 ha (Weitz et al., 2014). Mainly there are three kinds of SRF systems:i. SRF – Swedish system, this is an annual rotation system, with high plant density, of about 14,000 trees per hectare, mainly intended with double line planting; ii. SRF – European system, this is a biennial rotation system, with medium to high-density of plants, about 5000-6000 plants per hectares, and mainly planted in a single row;iii. SRF – American system, this is five years rotation period, density about 1500 to 1700 trees per hectare, in single row. Both Swedish and European systems are mainly suitable for the production of biomass for energy and biofuels, while the American system ismainly used for production of chips for the pulp and paper industry (Mareschi et al., 2005).

### 2.2.6 Secondary metabolites in poplars: phenolic compounds from vegetative buds and from sprout exudates

Biomass of fast growing trees and shrubs is used to produce a wide range of bio-based products, including polymers, lubricants, building materials, pharmaceuticals, as well as bioenergy and fuels (Parajulia et al., 2015). Bioactive compounds from plants are classified according to various criteria including functional, pharmacological or toxicological effects. However, Bernhoft (2010) classified the phenolic compounds according to biochemical pathways and chemical classes.

The main groups of bioactive compounds include (1) phenolic compounds, (2) glucosides, (3) terpenoids, (4) resins, (5) carotenoids, (6) tocopherols and tocotrienols, (7) phytosterols, (8) alkaloids, (9) furocoumarins and naphthodianthrones, (10) proteins, and peptides, (11) l-ascorbic acid and others (Barba et al. 2014; Bernhoft, 2010; Blomhoff, 2010; Devappa et al., 2015).

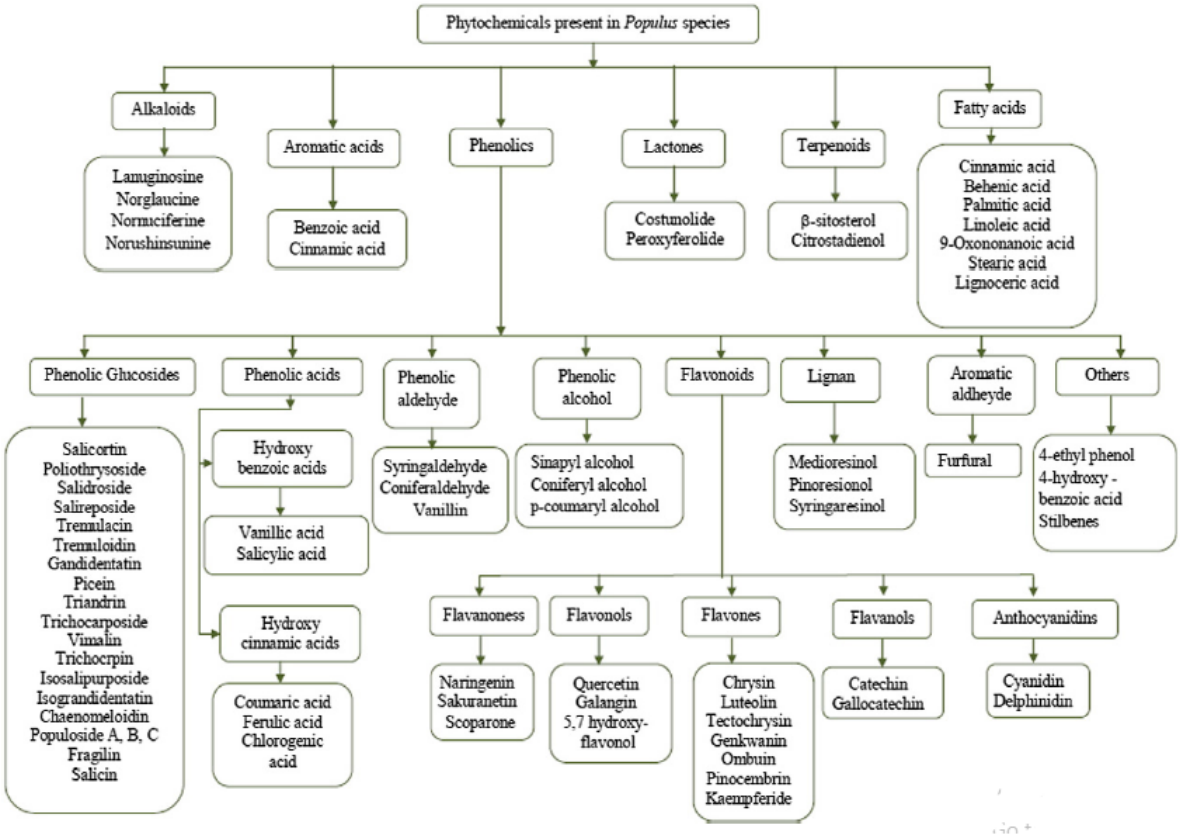



Figure 2.14 - Major phytochemicals in poplar species (Modified from Devappa et al., 2015)

The biomass of trees and shrubs, including short-rotation woody crops, contains secondary metabolites with proven biological activity (Mahdi, 2010; Sulima et al., 2017; Valette et al., 2017). Phenolic compounds are one of the most important and most commonly present groups of bioactive substances in woody plant tissues. Phenols are important in plant physiology with their

role in pigmentation, flavor, and resistance to pathogens and parasites (Cheynier et al., 2013; Heiska et al., 2007). Most phenols have antioxidant properties due to the hydrogen donating properties of the phenolic hydroxyl group and are the most abundant antioxidants in the human diet, also used in chemotherapeutic drugs (Blomhoff 2010).

The five main classes of bioactive phenolic compounds are phenolic acids, flavonoids, stilbenes, lignans and tannins (Blomhoff, 2010; Falcone Ferreyra et al.,2012). So far over 8000 compaunds with flavonoids structure have been identified. The flavonoid group is divided into six subgroups, such as flavones, flavonols, flavanones, isoflavones, and anthocyanins (Blomhoff, 2010; Koirala et al., 2016; Zhang et al., 2015; Devappa et al., 2015).

Several species of *Populus* have traditionally been used in medicine, due to their multiple medicinal properties (Wang,2011). Poplar buds are coated with a viscous substance, an exudate, which has been reported to contain several varieties of phenolic compounds, depending on the species studied, such as: terpenoids, flavonoid aglycones, chalcones, phenolic acids, and their esters. Black poplar (*Populus nigra*) is, among the poplar species, one of the most interesting species for its unexplored variability in bud phenolic content, being widely distributed in Europe (Ryu, 2003, Zhang,2006).

The chemical characterization of its exudates has: the flavonoid aglycones, such as pinocembrin and pinostrobin; some flavonols, such as galangin, quercetin, kaempferol, chrysin, and apigenin; some esters of phenolic acids (Park, 2002; Falcao, 2010). In recent years, functional food (nutraceutical) have had more attention due to the increase in consumer's concerns about their health, which stimulated more research effort in such food. Another important effect of nutraceutical foodisitsanti-aging activity by preventing the formation of free radicals in oxidative - reductive reaction in the human body (Dudonneet, al., 2011).

In general, poplar bud extracts contain hundreds of lipophilic compounds of different volatility. In the literature there is a limited number of articles available to identify the chemical constituents present in poplar buds exudates (Rubioloet al., 2013). Figure 2.15 shows the presence of biologically active phenolic compounds in *Populus nigra* that should be necessary to better identify and test for their biological uses for human health.

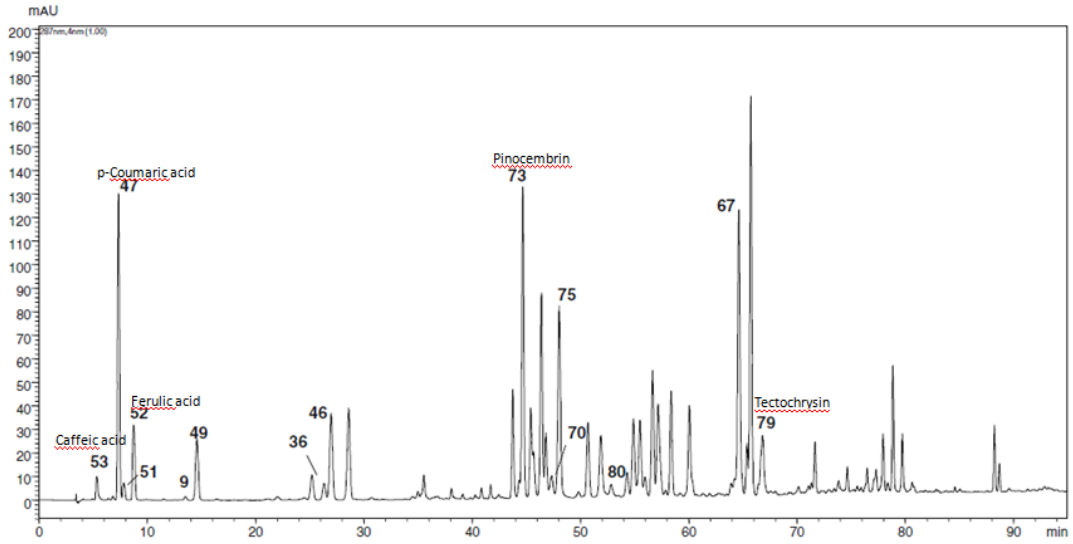



Figure 2.15 - HPLC-PDA profile of poplar bud extracts, the chromatogram run at 280 nm. The peaks illustrate some compounds in *Populus nigra* species. (Modified from Rubiolo et al., 2013)

As poplar bud resinous exudates are rich in phenols, mainly flavonoids, phenyl propanoids, and their esters, they are the main source used by bees to produce propolis. It has numerous biological activities, for example, antimicrobial, antioxidant, anti-inflammatory, immunomodulatory, anticancer, antitumor, antiulcer, hepato protective and cardioprotective (Kasetal., 2017).

Many different chemical compounds have been shown to exhibit inhibitory effects on melanogenesis through inhibition of the enzymatic activity of tyrosinase, like, hydroquinone, derivatives of ascorbic acid, kojicacid, azelaic acid, corticosteroids, retinoids andarbutin. However, most of these compounds are forbidden in cosmetics and skincare products. Furthermore, the long-term effects of these chemicals on the skin are not optimal and can cause serious damage to the skin. Therefore, people look for natural and harmless compounds of natural origin. Plants are one of the main sources of bioactive molecules and, in particular, poplar bud exudates are rich in natural and organic compounds. According to Maack & Pegard (2016), poplar bud extract showed the best results on the inhibition of tyrosinase, which is mainly responsible for aging.

In recent decades, propolis has been widely accepted and used commercially in the form of home remedies, toothpaste, creams, ointments, drops, and food supplements. Once bees collect poplar bud resinous exudates, propolis is obtained by enriching it with the saliva and enzymatic secretions of bees. Propolis is used by bees as a natural sealant in hives and as a protective barrier against intruders. Propolis is believed to play an important role in the immunity of honey bees by reducing the spread of bacteria and parasites in hives and eliminating biological contamination in a colony. As a natural product, propolis has a complex chemical composition (Maseket, al.,

2018). The most studied type of propolis, poplar propolis, represents over 300 identified compounds divided into: esters of aromatic acids, terpenoids, aromatic acids, long-chain aliphatic fatty acids and their esters, aliphatic hydrocarbons and wax esters, amino acids, flavones and flavonols, aliphatic acids (short-chain), glycerol derivatives, flavanones, aldehydes, alcohols, aliphatic acid esters, chalcones, sugar and sugar alcohols, acetophenones and other ketones, dihydrochalcones, steroids and other ingredients (Finstromeet et al., 2010).

More than 160 different types of compounds have been reported from *Populus* species with the majority belonging to the groups of phenolic glycosides, triterpene esters, steryl esters, sterols, fatty acids, flavonoids, alkaloids, lactones, lignans, and resins. In poplar species, several phytochemicals with free radical scavenging or antioxidant properties have been identified and their medicinal effects include the inhibition of oxidative mechanisms leading to degenerative diseases. Some examples of phytochemicals include populoside, trichocarposide, grandidentatin, picein, aromadendrene, lignans, among others (Devappa et al., 2015)

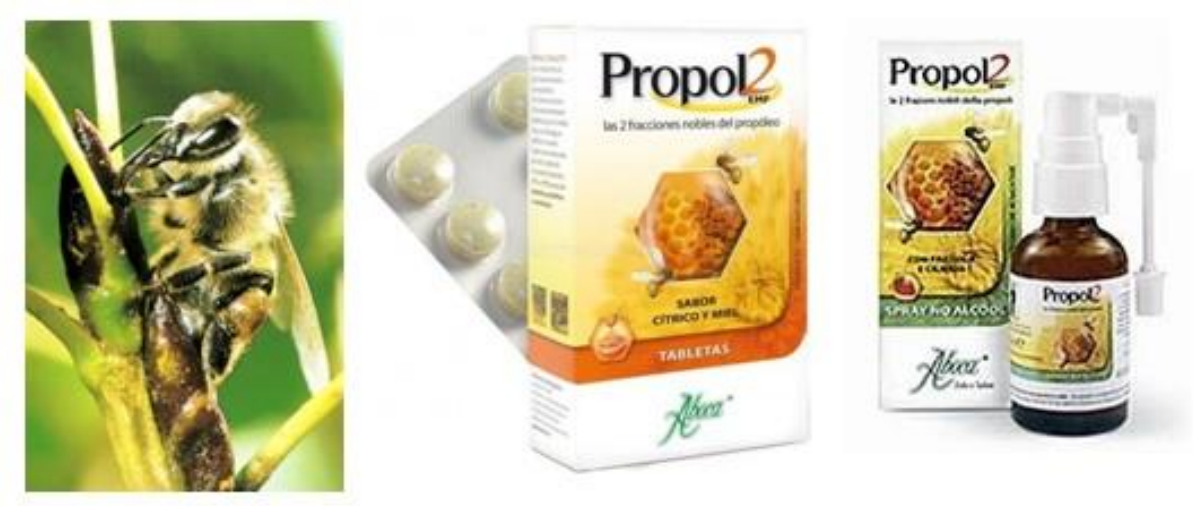



Figure 2.16 - Honey bee propolis in solid, liquid and aerosol dosage forms (Sorce: https://it.aboca.com)

Currently, many pharmaceutical companies are focusing on the formulation of plant-based propolis to reduce pressure on honey bees as well as to increase the production of simil propolis products on a large scale basis. Recently, Aboca pharmaceutical company succeeded to formulate poplar bud based propolis indifferent formulations (Fig. 2.17).


Figure2.17 - Poplar bud propolis in solid and liquid dosage forms(Sorce: https://it.aboca.com)

The chemical composition of propolis is variable, depending on the regional plant ecology. For example: according to Masek (2018), in temperate climate regions, propolis resin is collected mainly from buds and cracks in the bark of *Populus* species. The resulting propolis of poplar is characterized by flavonoids without substituents of ring B, such as pinocembrin, pinobanksin, galangin, and chrysin and phenylpropanoid acids and their esters, e.g. phenyl ester of caffeic acid.

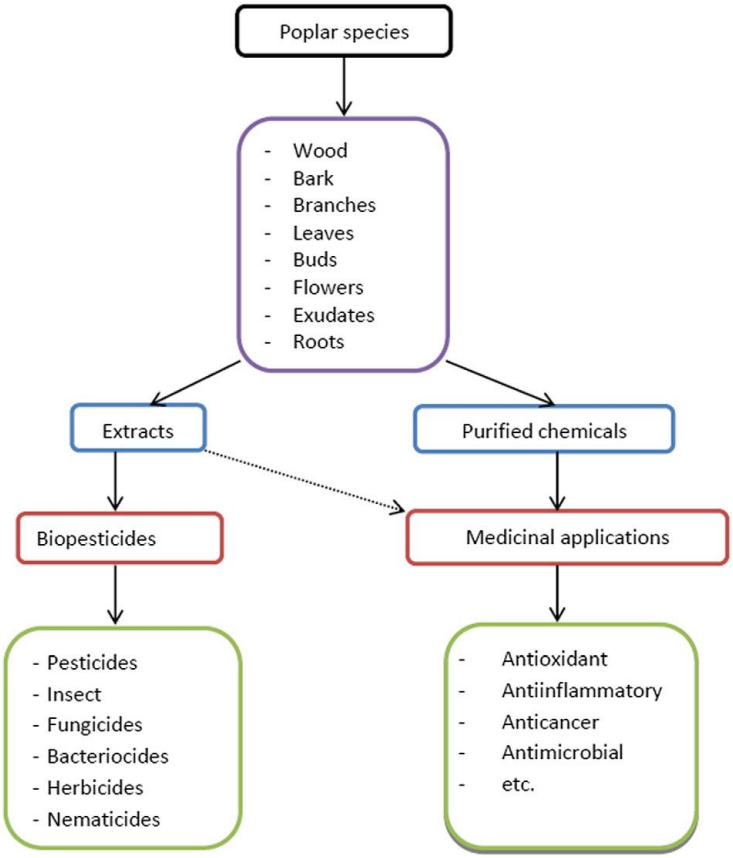



Figure 2.18 - Potential use of poplar phytochemicals from various useful parts of the plant. (Modified from Devappa et al., 2015)

Raw propolis cannot be used without a previous solvent extraction that removes waxes and other redundant ingredients and preserves bioactive compounds. The solvents normally used in the extraction procedure are methanol, ethanol, water or their mixtures (Maseket et al., 2018).

The poplars of the *Tacamahaca* section, such as *P. balsamifera L.* (*P. tacamahaca*), have bud exudates that differ from the typical poplars of the *Aigeiros* section, such as *P. deltoides* and *P. nigra,* in the type of compounds that they contain. *P. balsamifera* bud exudates contain diidrochalcones, which lack in*P. deltoides and P. nigra*. On the other hand, the bud exudates of these species contain a certain number of flavanones that are usually not present in *P. balsamifera* exudates (Greenway & Whatley, 1990; Greenway & Whatley, 1991).

Looking at the great diversity of species and hybrids within the *Populus* genus, there is a certain interest by pharmaceutical and food industries to focus on poplar plantations, particularly using SRF system, to produce buds, bark, and sprout exudates to be used for the formulation of pharmacologically active compounds.

## 3. Aims and research objectives

There are many interacting factors that will influence the success of adding value to trees, ranging from the yield of the tree metabolites to the chances of displacing competitor products in the market. If undertaken in isolation, lengthy searches for exotic metabolites, or elegant schemes for isolation, or screening for possible uses for the isolated substance, are likely to yield good science but zero utilization (https://secure.fera.defra.gov.uk/treechemicals/review/index.cfm).

Nowadays, environmental issues became a great challenge to human life. The awareness about climate issues is not only from today, but it started since the seventies (Sgroi et al.,2014). Day by day, the global population is increasing, so it is also increasing the pressure on the environment and natural resources. As we know, every year the warming potential is increasing up to 2ppm of $CO_2$ (World meteorological organization, 2013). To face these running problems, trending research is focussing on natural resource management by innovative techniques, especially to maintain sustainable forest management.

As reviewed in the literature, poplar species can contribute to satisfying the needs of a growing population in terms of biomass, wood, pulp and pharmaceutical industry. The region of Siyang in China is a good example of how poplar plantations have improved the environment and the people's livelihoods (https://www.youtube.com/watch?v=NGt-VSZkYHs&pbjreload=10). Traditional poplar culture and short rotation coppice systems are trending practices that can find more differentiated and suitable uses of the timber and the raw biomass produced. Nevertheless, poplars have raised recently a certain interest for their economic value in terms of biochemical and pharmaceutical importance for the industry.

**The aim of this work** is to characterize and identify, among poplar species, those genotypes that will couple bud production with a higher concentration of pharmacologically bioactive phenolic compounds. In this way, the use of short rotation coppice system can be beneficial to produce buds and other raw materials for the pharmaceutical industry and biomass for energy. The evaluation and comparison of the chemical characteristics of bud extracts and of sprout exudates in *Populus* species is also an important objective to fill the research gap.

**Research objectives:**

A) Calibration studies of Near-Infrared (NIR) Spectroscopy for rapid assessment of bud chemical traits in a large sample of *Populus* species and hybrids.

B) Estimation and characterization of bud production in *Populus nigra (*short rotation coppice system) genotypes in relation to biomass production and crown architecture.

Fresh and dry weight bud production from different *P. nigra* genotypes will be estimated in relation to biomass components (stem and branches) of crown architecture.

C) Comparison of phenolic compound content in vegetative dormant buds of poplar species.

The activity will consist of sampling, extraction, and identification (by NMR spectrophotometer) of phenolic chemical compounds of interest for pharmaceutical use present in the following species:

1. *Populus nigra*
2. *Populus deltoides*

D) Chemical characterization and comparison between bud extracts and sprout exudates of *Populus nigra* and *Populus deltoides*.

## 4. Materials and methods

### 4.1 Site description and plantation characteristics

The experimental farm of the University of Tuscia hosts a huge collection of poplar germplasm from different provenances in Italy. The germplasm collection of poplar is planted in two zones of the farm, named zone A and zone B. Zone B hosts the oldest experimental trials such as the *Populus alba* common garden study, the *P. alba*, and *P. nigra* $F_1$ progeny and a collection of poplar species from different areas of the World planted about 25 years ago. Zone A is mainly used to maintain stool bed cuttings that are coppiced every year the *P. nigra* $F_1$ progeny and the *P. nigra* germplasm collection belonging to five different populations from Southern, Central and Northern Italy. $F_1$ progeny represents a short rotation coppice forest system with $F_4$ generation. In the stool bed cuttings are also maintained several genotypes of *P. deltoides* and *P. trichocarpa* species.

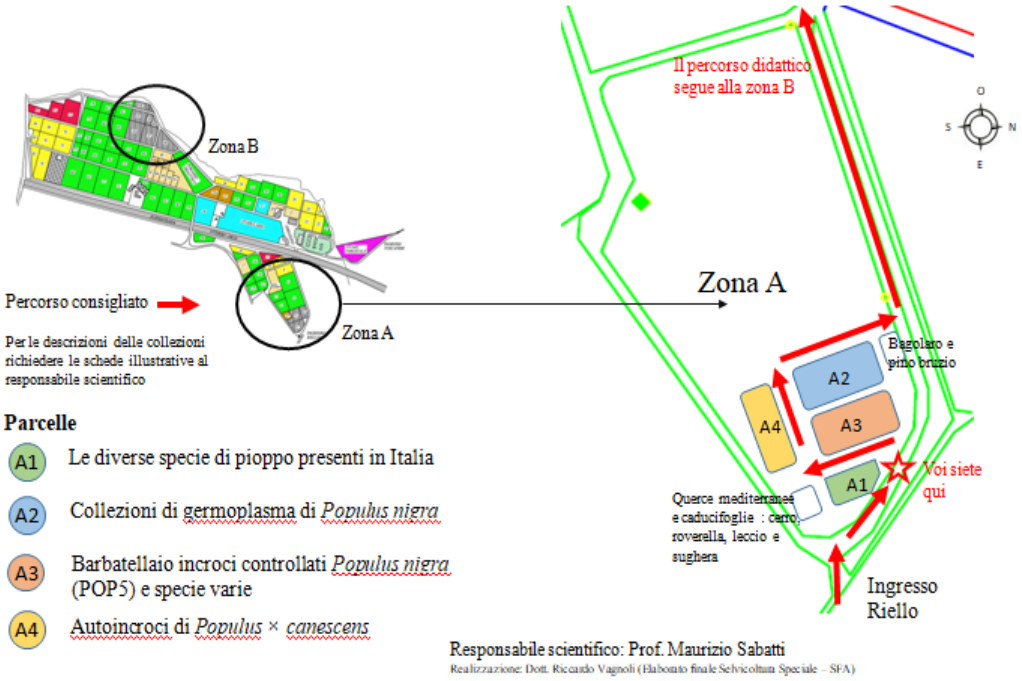
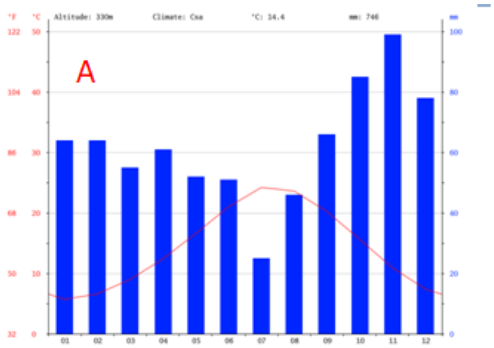
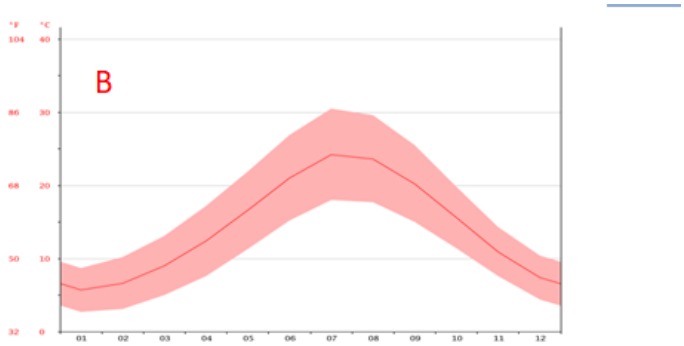



Figure 4.1 - Schematic representation of poplar germplasm trials at the University of Tuscia experimental farm. Zone A hosts the *P. nigra* Italian germplasm collection (A2) and the *P. nigra* $F_1$ progeny (A3) (From Vagnoli - University of Tuscia, 2017)

The experimental farm is located in Viterbo (VT) (42º25´36´´N, 12º05´02´´E), Central Italy, at an altitude of about 310m above sea level. The experimental site is 0.8 ha of flat agriculturalland with sandy silt soil with the possibility to provide drip irrigation. The soil pH is slightly acidic due to the volcanic origin and has good physical and chemical properties. The climate in Viterbo is Mediterranean temperate, with a dry summer period. This position is classified as Csa by

Köppen and Geiger. The average annual temperature in Viterbo is 14.4 ° C and the average annual rainfall is 746 mm.The driest month is July with 25 mm of monthly mean precipitation and an average temperature of about 24.2 ºC. Most rainfall occurs in November, with a monthly average of 99 mm. The difference in precipitation between the driest month and the wettest month is 74 mm (Climate data org).




C

| | January | February | March | April | May | June | July | August | September | October | November | December |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Avg. Temperature (°C) | 5.7 | 6.6 | 9 | 12.4 | 16.6 | 21 | 24.2 | 23.6 | 20.2 | 15.6 | 10.9 | 7.4 |
| Min. Temperature (°C) | 2.7 | 3.1 | 5 | 7.6 | 11.3 | 15.2 | 18 | 17.7 | 15 | 11.4 | 7.6 | 4.4 |
| Max. Temperature (°C) | 8.7 | 10.2 | 13.1 | 17.2 | 21.9 | 26.9 | 30.5 | 29.6 | 25.5 | 19.8 | 14.3 | 10.4 |
| Avg. Temperature (°F) | 42.3 | 43.9 | 48.2 | 54.3 | 61.9 | 69.8 | 75.6 | 74.5 | 68.4 | 60.1 | 51.6 | 45.3 |
| Min. Temperature (°F) | 36.9 | 37.6 | 41.0 | 45.7 | 52.3 | 59.4 | 64.4 | 63.9 | 59.0 | 52.5 | 45.7 | 39.9 |
| Max. Temperature (°F) | 47.7 | 50.4 | 55.6 | 63.0 | 71.4 | 80.4 | 86.9 | 85.3 | 77.9 | 67.6 | 57.7 | 50.7 |
| Precipitation / Rainfall (mm) | 64 | 64 | 55 | 61 | 52 | 51 | 25 | 46 | 66 | 85 | 99 | 78 |

Figure 4.2 - Meteorological data of Viterbo. A. Mean annual precipitation and temperature. B. Average temperature by month. C. Viterbo weather by month. (Modified from: CLIMATE DATA ORG)
URL: (https://en.climate-data.org/europe/italy/lazio/viterbo-1187/)

The *P. nigra* stool bed cuttings were planted in April 2015 in Viterbo (VT) at the experimental farm of Tuscia University. Most of the genotypes of the *P. nigra* germplasm collection were represented in the plantation by a single plot with five cuttings. The four *P. nigra* genotypes ("Poli", "58-861", "810-93"and"74.00") used for the biomass collection in this experiment and to estimate the bud production were planted randomly in the poplar collection with three replicates, each one including five plants. Hardwood cuttings (30 cm) were planted at a distance of 2 m × 0.30 m and between the groups of five cuttings, a distance along the row of 0.6 m was released. The density of the stool bed cuttings is approximately 11,905 plants $ha^{-1}$.

The plantation was drip irrigatedas necessary during the dry period in summer, weeded and was not treated with pesticides and fungicides in 2019. The plantation was pollarded each year during

winter leaving the height of the stump of about 30 cm. After each coppicing,the number of shoots on each stump was reduced to a few stems (2-3) in mid-June.

### 4.2 Plant material

The selected 48 genotypes (Table 4.1) from 3 sections of poplars, named *Aigeiros, Tacamahaca, Populus* and poplar hybrids were used to collect buds from the stem and the branches. Four *P. nigra* genotypes were used in this experiment for the estimation of the above-ground biomass and bud production.

Table 4.1 - List of genotypes used for the collection of buds from the following poplar genotypes.

| Progr | Section | Species | Pop | Code | Genotype | Zone |
|---|---|---|---|---|---|---|
| 1 | Aigeiros | P. nigra | Paglia | PG | 2 PG | Zone A |
| 2 | Aigeiros | P. nigra | Paglia | PG | 15 PG | Zone A |
| 3 | Aigeiros | P. nigra | Paglia | PG | 20 PG | Zone A |
| 4 | Aigeiros | P. nigra | Paglia | PG | 21 PG | Zone A |
| 5 | Aigeiros | P. nigra | Paglia | PG | 23 PG | Zone A |
| 6 | Aigeiros | P. nigra | Paglia | PG | 51 PG | Zone A |
| 7 | Aigeiros | P. nigra | Basento | BS | 10 BS | Zone A |
| 8 | Aigeiros | P. nigra | Basento | BS | 11 BS | Zone A |
| 9 | Aigeiros | P. nigra | Basento | BS | 17 BS | Zone A |
| 10 | Aigeiros | P. nigra | Basento | BS | 32 BS | Zone A |
| 11 | Aigeiros | P. nigra | Basento | BS | 33 BS | Zone A |
| 12 | Aigeiros | P. nigra | Stura | ST | 7 ST | Zone A |
| 13 | Aigeiros | P. nigra | Stura | ST | 8 ST | Zone A |
| 14 | Aigeiros | P. nigra | Stura | ST | 17 ST | Zone A |
| 15 | Aigeiros | P. nigra | Stura | ST | 25 ST | Zone A |
| 16 | Aigeiros | P. nigra | Ticino Sud | SN | 12 SN | Zone A |
| 17 | Aigeiros | P. nigra | Ticino Sud | SN | 18 SN | Zone A |
| 18 | Aigeiros | P. nigra | Ticino Sud | SN | 20 SN | Zone A |
| 19 | Aigeiros | P. nigra | Ticino Sud | SN | 24 SN | Zone A |
| 20 | Aigeiros | P. nigra | Ticino Sud | SN | 37 SN | Zone A |
| 21 | Aigeiros | P. nigra | Ticino Nord | N | 7 N | Zone A |
| 22 | Aigeiros | P. nigra | Ticino Nord | N | 8 N | Zone A |
| 23 | Aigeiros | P. nigra | Ticino Nord | N | 10 N | Zone A |
| 24 | Aigeiros | P. nigra | Ticino Nord | N | 38 N | Zone A |
| 25 | Aigeiros | P. nigra | Ticino Nord | N | 43 N | Zone A |

| | Section | Species | Pop | Code | Genotype | Zone |
|---|---|---|---|---|---|---|
| 26 | Aigeiros | P. nigra | Registered | | JP | Zone A |
| 27 | Aigeiros | P. nigra | Sinni | P | Poli | Zone A |
| 28 | Aigeiros | P. nigra | Dora Riparia | V | 58-861 | Zone A |
| 29 | Aigeiros | P. nigra | Full sib | | 810-93 | Zone A |
| 30 | Aigeiros | P. nigra | Full sib | | 74-00 | Zone A |
| | Section | Species | Pop | Code | Genotype | |
| 31 | Aigeiros | P. deltoides | | | 928-05 | Zone A |
| 32 | Aigeiros | P. deltoides | | | 330-98 | Zone A |
| 33 | Aigeiros | P. deltoides | | | 22--02 | Zone A |
| 34 | Aigeiros | P. deltoides | | | 66-98 | Zone A |
| | Section | Species | Pop | Code | Genotype | |
| 35 | Tacamahaca | P. trichocarpa | | | Nisqually | Zone B |
| 36 | Tacamahaca | P. trichocarpa | | | CH2 | Zone B |
| 37 | Tacamahaca | P. maximoviczii | | | D1 | Zone B |
| 38 | Tacamahaca | P. maximoviczii | | | D3 | Zone B |
| 39 | Tacamahaca | P. simonii | | | PST | Zone B |
| | Section | Species | Pop | Code | Genotype | |
| 40 | Populus | P. alba | | | 6K10 | Zone A |
| 41 | Populus | P. tremula | | | VV | Zone A |
| 42 | Populus | P. tomentosa | | | PT | Zone B |
| | Section | Species | Pop | Code | Genotype | |
| 43 | AxA | P. x canadensis | | | I-214 | Zone A |
| 44 | AxA | P. xcanadensis | | | L. Avanzo | Zone B |
| 45 | AxA | P. xcanadensis | | | Robusta | Zone B |
| 46 | AxT | P. xpopularis | | | Pxp | Zone B |
| 47 | AxT | P. xgenerosa | | | Dec-91 | Zone B |
| 48 | (AxT)xA | P. xgenerosa | x P. nigra | | 860-91 | Zone B |

Their origin is from different locations in Italy or was obtained from controlled crosses. The *P. nigra* genotype "58-861"(Fig. 4.3)was collected in Northern Italy (45° 09′ N, 7° 01′ E), near the Dora Riparian river close to the Italian Alps, at 597 m above sea level. It is adapted to the fresh and humid conditions of the area of origin and its phenotype presents large leaves and a few branches; it can be considered "sensitive to drought" (Regier et al., 2009, Cocozza et al., 2010). The *P. nigra* genotype "Poli" (Fig. 4.3) was collected in southern Italy (40° 09′ N, 16° 41′ E), close to the mouth of the Sinni river, at 7 m of altitude above sea level. It is adapted to the dry and hot climatic conditions of the areaand its phenotype presents small leaves and a lot of branches; itcanbe considered "drought-tolerant" (Regier et al., 2009, Cocozza et al., 2010). The two genotypes were used as parents of an $F_1$ generation and this progeny originated by parents

from contrasting environments showed highly variable phenotypes forphenological and biometric traits (Fabbrini et al. 2012, Ludovisi, 2014).

"810-93" and "74.00" are $F_1$ progenies of *P. nigra* originated by controlled crosses of a private nursery (Alasia Franco Vivai). They have a phenotype similar to the genotype "58-861" of northern origin, with good growth, large leaves, and a limited number of branches at the end of the first year.

Figure 4.3 - Morphological differences between female "58-861" (on the left) and male "Poli" (on the right), parents of $F_1$ *P.nigra* intra-specific cross. (From: Ludovisi, 2014)

### 4.3 Bud collection from 48 poplar genotypes in the stool bed cuttings

Each one of the selected 48 genotypes (Table 4.1) we relabelled with tags including the specific code for each genotype. The labels were stapled on all the selected plants at 15 cm of height on the stump on the beginning of February. On 04 of March, poplar buds were collected from 48 selected genotypes, to evaluate chemical characteristics in terms of bioactive phenolic compounds and stored in a cold chamber at 4°C for NIR measurement and grinding.

### 4.4 Above-ground biomass collection, estimation of biomass and bud production

Six plants of the 4 *P. nigra* genotypes selected for biomass collection, two per each replicate,

were labelled on the beginning of February. On 27 of February 2019, the labelled trees were harvested using a chain saw and the following measurements were collected on each tree to characterize crown architecture:

1. The complete measurement of total height (H, m), mid-height (H/2) was marked in red on the stem, stem circumference (Circum, mm) at 1 m, height of the last branch, total number of sylleptic branches andstool height (Fig. 4.4);

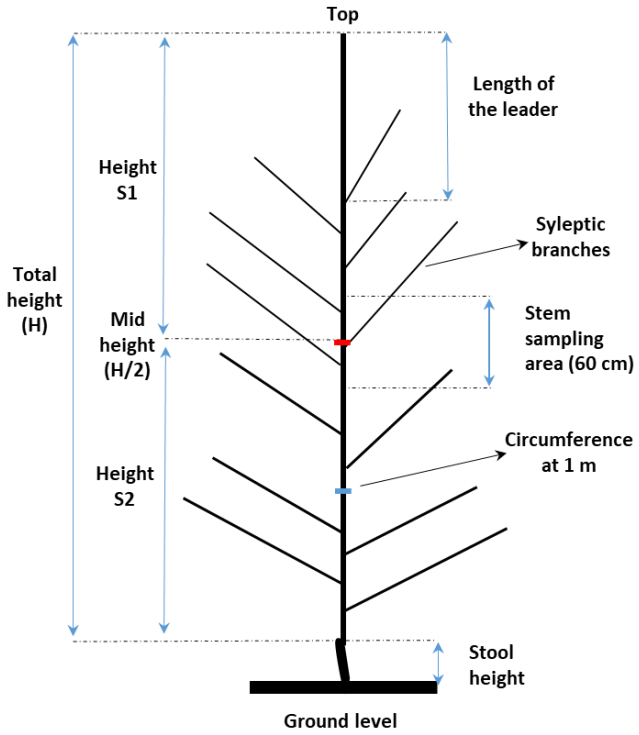



Figure 4.4 - *P. nigra* crown architecture withtotal height (H) and mid-height (H/2, in red). H/2 is indicated to define two compartments (height S1, height S2) to characterize branch distribution along the stem. The stem sampling area (60 cm) is centered on H/2 and was used to calculate mean internode length. Other parameters such as circumference, sylleptic branches, stool height and length of the leader are also indicated.

2. The stem sampling area (60 cm) was centered on H/2. Bud and branches present in this area were counted to calculate mean internode length(Fig. 4.4);

3. The calculation of the total plant dry biomass was done on the basis of the direct calculation of the total fresh weight of stem and branches. The measure of the fresh weight of the total plant was done with a dynamometer. After this operation, the branches were removed and only the stem fresh weight was obtained to calculate for difference the branch fresh weight.

4. All the stem buds were collected separately from the two stem sections S1 and S2 (Fig. 4.4) and conserved in plastic bags.


Figure 4.5 - Schematic representation of process flow of biomass collection, bud and biomass estimation. A. Biomass collection steps in University experimental farm, Viterbo. B. Separation of buds from stem and branches. C. Weighing of fresh weight(FW) and dry weight (DW). D. The drying process with paper bags in a tray dryer.

5. At this point, two stem samples were randomly collected from S1 and S2 sections (Fig. 4.4) and put in separated plastic bags. Branches randomly collected by all the plants were chipped in a dustbin and two random samples of branch chips were also collected and put in plastic bags. All the material collected in the field was stored in the cold chamber at 4°C.

Table 4.2 - List of acronyms used to represent the crown architecture and its description.

| **Acronym** | **Description** |
|---|---|
| $Circum_{100}$ (mm) | Circumference at 100 cm height from stool height |
| H (cm) | Total height |
| HBr (cm) | Height of the last branch |
| $L_{arrow}$ (cm) | Length of the leader |
| $N_{syll}$ Br Tot | Total number of sylleptic branches |
| $I_{br}$ cm/br | Branch index |
| NBr60 | Number of branches at stem sampling area |
| Nbud60 | Number of buds at stem sampling area |
| $Nb_{nodes}$ | Total number of nodes |
| Int L cm/nodes | Inter node length index |

6. After a few days, the samples of stem, branches, and stem buds were weighted in the lab to measure their fresh weight (FW) and transferred from polythene bags to paper bags to be dried in the oven for 2 days at 60°C. After two days their dry weight was measured to determine fresh weight/dry weight ratio (Fig. 4.5);

7. A replicated sub-sample of the branch chip was also used to calculate the bud dry mass of the branches using the same procedure described above (Fig. 4.5).

Collected data were organized in an Excel file to be processed and obtain the following traits:

Figure 4.6 – Sprout exudates: In the month of May sprout exudates were collected from the four *Populus nigra* genotypes ("Poli", "58-861", "74-00", and "810-93").

### 4.5 Statistical analysis

Statistical analysis was performed by analysis of variance (ANOVA) using the general linear model (GLM) procedure of the statistical analysis system. The data set was tested for normality and homoscedasticity of the residues. The data were analyzed according to the following ANOVA linear model

$$\mathbf{Y_{ij} = \mu + R_i + G_j + \varepsilon_{ij}}$$

Where:

$Y_{ij}$: observation for the $j^{th}$ genotype in the $i^{th}$ replicate,

$\mu$: general mean,

$B_i$: effect of the $i^{th}$ replicate,

$G_j$: effect of the $j^{th}$ genotype (random),

$\varepsilon_{ij}$: residual error.

$B_i$ is the effect of the replicate i, and $G_j$ is the effect of the genotype. The replicate effect was not significant ($P \geq 0.05$) for all traits.

### 4.6 Near Infra-Red (NIR) Spectroscopy principle and methodology

**Principle/Theory**

NIR spectroscopy uses the spectral range from 780 to 2500 nm (12.500 to 4,000 cm-1) and provides much more complex structural information related to the vibration behaviour of bonding combinations. The recording of the NIR region of the electromagnetic spectrum involves the response of the molecular bonds O - H, C - H, C - O and N - H. These bonds are subject to changes in vibration energy when irradiated by the NIR frequencies, and in these bonds exist two vibration patterns including stretching vibration and bending vibration. The energy absorption of organic molecules in the NIR region occurs when the molecules vibrate or are translated into an absorption spectrum within the NIR spectrometer (H.Cen & Y.He, 2007).

**Methodology**

The first experimental trial done on poplar buds by using NIR spectroscopy. There were some experimental data done with poplar wood cellulosic and hemicellulose determination by NIR spectroscopy but we didn't find many articles on poplar buds. This is a rapid qualitative analytic method for the identification of chemical compounds (especially phenolic compounds). Firstly, we weighed approximately 5 g of sample from 48 genotypes and placed in plastic bags with proper labelling.

A Luminar 5030 miniature portable NIR analyzer (Brimrose Corporation, Baltimore, 92 MD), based on the AOTF-NIR principle, was used for spectral detection.This is a portable device that can be used directly on the tree field, although in this specific case spectral surveys were conducted under laboratory conditions. Set the wavelength (1100 to 25000 nm) and arrange the external gun. Place the poplar buds (5g) in a sampling tray and measure 10 acquisitions from different positions by moving the sampling tray. while the raw spectra were detected and recorded in the transmittance. The survey was conducted in the range between 1100 and 2300 nm, with wavelength increments of 2 nm and 10 spectra per media, representing a single measurement.The spectral variations of the data sets were analysed through the Principle component analysis (PCA) directly on the raw spectra then, transmittance (T) spectra were statistically pre-treated for absorbance transformation (log 1 / T) using the SNAP 2.03 software (Brimrose).

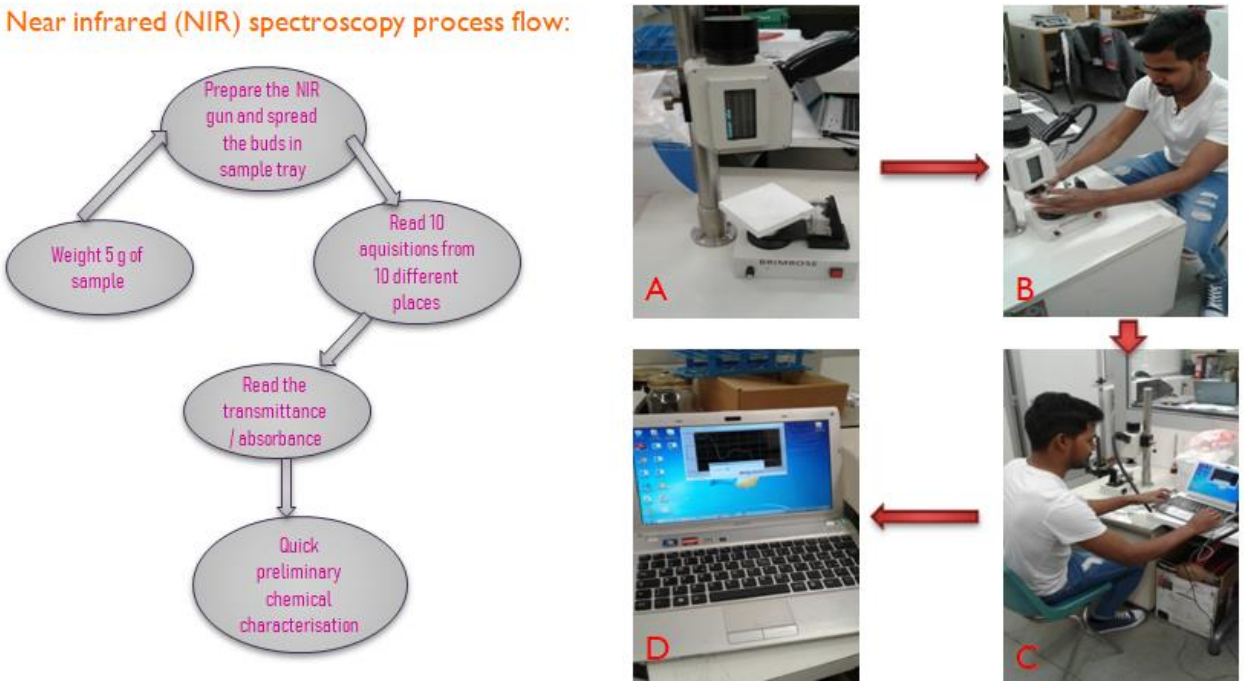



Figure 4.6 - Step by step Procedure for chemical characterization (especially phenolic compounds) of poplar buds. A. External gun set up. B. Record 10 acquisition points. C and D. Recording transmittance

Absorbance spectra, obtained as the spectral mean of each poplar sample (5g) subset, were used as X variables for the building up of the regressive model which was performed by the calculation of a PLS-DA (Partial Least Square-Discriminant Analysis). In the modelling, Y variables were represented by the genotype classes of the three poplars analysed. The mean normalization, the Multiplicative Scattering Correction (MSC) and the Standard normal variate (SNV), the first order of the Savitzky-Golay filter (6 smoothing points) or the second-order of the Savitzky-Golay filter (6 sanding points) tested, even if they were not used for final modelling. All the spectrum treatments, filtering, and chemometric procedures were performed using Matlab R2013a (MathWorks®, Natick, MA, USA) and PLS Toolbox (Eigenvector Research, Inc., Manson, WA, USA).

## 4.7 Bud extraction of phenolic compounds and chemical characterization

### 4.7.1 Chemicals and Instruments

All chemicals are purchased by 1. Sigma-Aldrich (methanol, gallic acid, sodium carbonate, and Folin-Ciocalteu reagent) 2. VWR chemical agencies (ethanol, acetone, deuterated methanol, and dichloromethane).

The following instruments have been utilized: Uv/Visible Spectrophotometer (UV-2600-SHIMADZU), Centrifuge (Centrifuge 5804 K-Eppendorf), Rotary evaporator (LABOROTA

4000-Heidolph), Analytical balance (SHIMADZU ATX124), Vacuum pump (Vaccubrand 2.5), and NMR (Bruker 400 MHZ). TLC plates purchased from Sigma-Aldrich.

### 4.7.2 Extraction procedure

**Grinding:** collected 48 genotype buds were ground by using mortar and pestle. Liquid nitrogen (collected from the University of Tuscia, Viterbo) was used to obtain a dry powder from the vegetative buds.

**Extraction:** 0.5 gr of the powdered sample into 3 replications were transferred into the round bottom flask. Prepare the extraction solutions by using ethanol:water with 70:30 concentration. Set the parameter, temperature (60°C), Time (30 min). After 30 min allows the solution to room temperature and then transfer into a falcon tube. Set the centrifugation parameters, RPM(8000) and time (10min) at room temperature. After centrifugation, collect the supernatant liquid and allow for the filtration by using Whatman filter paper to remove traces of solid particles. The solvent evaporated by using a Rotary evaporator at temperature (38-40°C). Finally obtained solid extract which was transferred into a vial and noted the finalweight with proper label.


Figure 4.7 - Step by step procedure for the extraction of Phenolic compounds from Poplar buds. The left Grinding process, in the middle distillation process, and the right final extract after evaporation by Rotary evaporator.

### 4.7.3 Chemical characterization

#### 4.7.3.1 Qualitative analysis: $H^1$NMR (Nuclear magnetic resonance) Spectra

In the late 1940s, physical chemists originally developed NMR spectroscopy to study different properties of atomic nuclei, but later found it to be useful in determining the molecular structure of organic compounds. The theory behind NMR comes from the spin of a nucleus. Just as electrons have a +1/2, -1/2 spin, certain nuclei also experience charged spins that create a magnetic field (called the magnetic moment). Nuclei with even numbers of both neutrons and protons experience NO spin and nuclei with odd numbers of both neutrons and protons have integer spins. Nuclei that have the sum of protons and neutrons equal to an odd number (like $^{1}H$ and $^{13}C$) have half-integer spins. When there is no external or applied magnetic field, the nuclear spins orient randomly. however, when there is an applied magnetic field, the nuclei orient themselves with or against the larger applied field. The α-spin state is parallel to the applied force and has lower energy than the β- spin state that is antiparallel to the applied force.

The energy difference (ΔE) between the α and β-spin states depends on the strength of the applied magnetic field. The greater the strength of the applied magnetic field, the greater is the ΔE between the α and β-spin states. The ΔE between the αand β-spin state is ~0.02 cal mol-1, which lies in the radio frequency region. The emitted energy in this region produces an NMR signal.

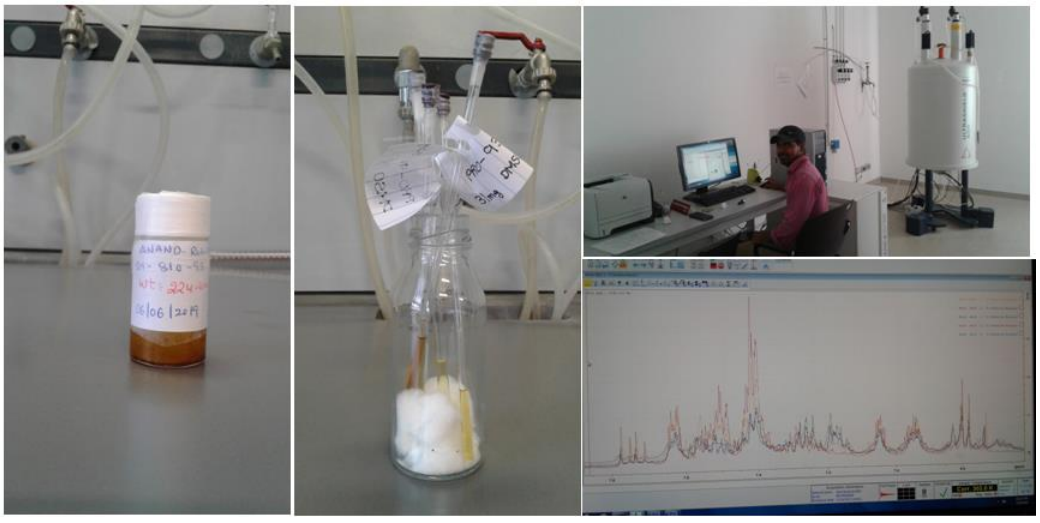


Figure 4.8 - Process flow of $H^1$ NMR spectra. The left is a sample, middle NMR sample tubes, and on the right NMR instrument with spectra.

An equivalent weight of 30-35 mg of sample, then transfers to the NMR sampling tube. Add approximately 500 µl of deuterated methanol. Place the sample tube in the NMR chamber to record the signals of the nuclear magnetic resonance of the sample.we analyzed five genotypes

from *Populus nigra* bud extract, four *P. nigra* sprout exudates(exceptgenotype‘JP’), and four *Populus deltoides* genotype bud extracts.

**Thin layer chromatography (TLC)**

TLC has two phases, one is the stationary (solid) phase and the other one is a mobile phase (liquid phase). Different molecules have different solubilities. The elution rate depends on the polarity and the molecular weight of the compound/sample. More polar and less molecular weight molecules elute fast. The selection of the mobile phase depends on the solubility of the compound.

Prepared different mobile phases with different concentrations of solvents. We used methanol and dichloromethane solvents as the mobile phase (Dichloromethane: Methanol (8:2), (8.5:1.5), (9:1), (9.5:0.5)). The TLC plates were labeled with sample names. Point a drop of sample on the baseline, dry, and run the chromatogram in the mobile phase. Continue the process until the mobile phase reaches 80-90% of the TLC plate and allow for drying. Check the spots under the UV chamber and calculate the Rf values for each spot:

Rf= Distance traveled by the compound/Distance traveled by the solvent

Figure 4.9 -Qualitative analysis by Thin Layer Chromatography, On the left sample spotting and on the right Elution of compounds under the UV lamp.

**4.7.3.2 Quantitative analysis: Total phenolic content (TPC)**

Determination of the quantity of phenolic content in various individual genotypes by using gallic acid is a standard. The main principle based on the oxidation/reduction reaction between sample phenolic compounds and Folin-ciocalteu reagent. Folin-Ciocalteu reagent is a yellow acidic solution having complex polymeric ions formed by phosphomolybdic and phosphotungstic heteropoly acids. This reagent solution contains an integrated polymeric series, contain the

general form of a central tetrahedral phosphate unit surrounded by several octahedral molybdenum oxyacid units. In this structure, tungsten can freely substitute by molybdenum. This reagent oxidizes phenolates, and the heteropoly acid becomes partially reduced from the +6 to a mixture of +6 and +5 valence state, resulting in the production of the complex molybdenum-tungsten complex.

This solution having light green-blue color and having maximum absorbance at 765 nm. The reaction temperature was used to reduce the time required to reach the maximum color (T = 37 ° C) but we allowed the vials at room temperature for 1 hr in dark to achieve maximum reaction color. the carbonate buffer is used for pH adjustment and the endpoint of the reaction was reached after 60-70 minutes at room temperature (Prior, R et, al., 2005). Generally, gallic acid is used as a reference standard compound and the results are expressed in equivalents of gallic acid (Magalhães, L. M, et, al., 2010).

**Construction of gallic acid (GA) calibration curve**

Initially, we constructed the calibration curve by using "gallic acid" (964 mg/L) as a reference with gradually increasing concentrations (250,300,350, 400, and 450 µl). Take 100 µl of gallic acid solution from each different concentrations and add 2 µl of distilled water and 200 µl of

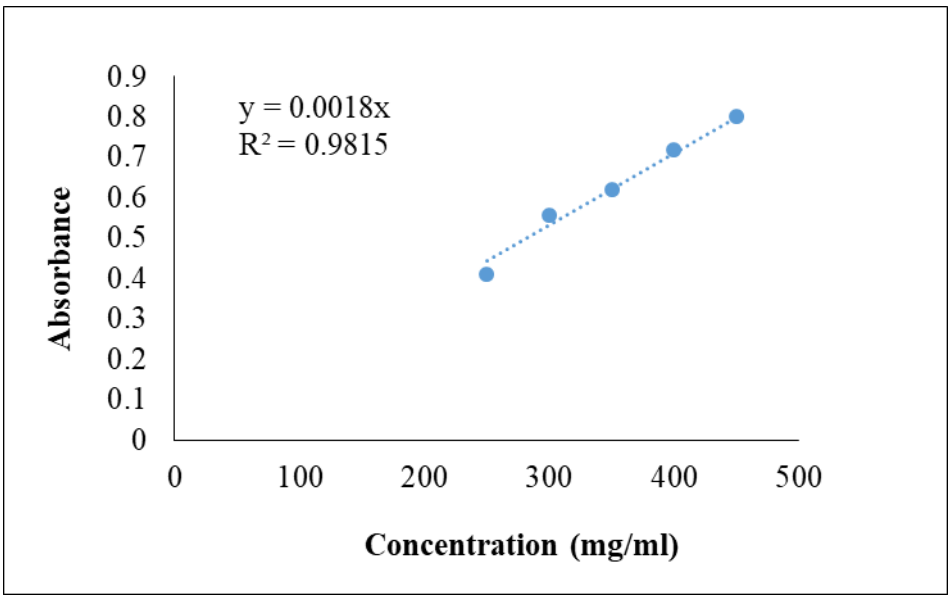



Figure 4.10 - Gallic acid calibration curve plotted between absorbance and concentration

Folinciocalteu reagent. Kept aside for 15 min and finally add 100 µl of sodium bicarbonate solution (20/80 w/v) and kept in dark place for 1 hr. Finally, check the absorbance by using a UV spectrophotometer at 765 nm.

Figure 4.11 -Quantitative analysis, Total Phenolic Content (TPC). On the right, UV spectrophotometer and on the left sample preparation.

**Total Phenolic Content of *P. nigra* and *P. deltoides* samples**

In this study, we analyzed nine samples from *P. nigra*(5) and *P. deltoides*(4). Approximately weighed 10-15 mg of *P. nigra* and *P.deltodes* samples and dissolve in 3 ml of methanol. Take 100 µl of the sample solution and add 200 µl of Folinciocalteu reagent, 2 ml of distilled water and kept aside for 3 min. Add 1 ml solution of sodium bicarbonate (20/80 w/v) and kept in dark place for an hour. The absorbance was read by using UV spectrophotometer at 765 nm.

$$\text{Total Phenolic Content (TPC)} = \frac{CV}{m}$$

Where C represents concentration (mg/L) of the sample. V represents the volume of methanol(L) and m represents dry extract mass (g). concentration C can be calculated by using the following formula. Folinciocalteu reagent. Kept aside for 15 min and finally add 100 µl of sodium bicarbonate solution (20/80 w/v) and kept in dark place for 1 hr. Finally, check the absorbance by using a UV spectrophotometer at 765 nm.

$$\text{Concentration(C)} = \frac{\text{Absorbance}}{\text{Slope}} \text{ (mg/L)}$$

SAS has several procedures for analysis of variance models, including proc ANOVA, procglm, proc varcomp and proc mixed. We will mainly use proc glm and mixed proc, that the SAS manual defines "flagship" for the analysis of variance. The same ANOVA statistical analysis model applied to biomass and bud production was used for total phenolic content (See §4.5).

## 5. Results and discussions

In the present work, 48 poplar genotypes were used for the NIR spectroscopy analysis to evaluate the qualitative chemical characterization of various poplar genotypes. Four *P. nigra* genotypes ("Poli", "58-861", "810-93", and"74-00") were characterized for their crown architecture and biomass and bud production. The results were scaled up to estimate biomass and bud production per hectare. The study has also involved the chemical characterization of the bud extracts from various *P. nigra* and *P. deltoides* genotypes. Further chemical analyses were done to compare various poplar bud extracts with sprout exudates.

### 5.1 Near-infrared (NIR) spectroscopy

Chemometrics might be used for both qualitative and quantitative analysis (Granato et, al., 2018). In this experiment, we did a qualitative analysis of different poplar genotypes, by using supervision and pattern recognition techniques based on the comparison among NIR spectra detected in the sample sets grouped and arranged as described in the M&M section. 48 genotypes from different poplar sections and some hybrids were evaluated. Obtained raw spectra are intended as a signal of transmittance detected on the poplar buds. Transmittance and, as a consequence, absorbance is due to chemical compounds present in individual poplar species and to the interaction between electromagnetic spectrum and molecular bonds related to organic compounds. As obvious, total phenolic compounds which are the most interesting metabolites investigated in the present study, contribute to the spectral response and to the potential differences findable among poplar samples. Transmittance values were also converted into absorbance by using log 1/T. The absorbance of each genotype is represented by the mean spectra relative to all acquisition points (10) of each individual genotype. Principle component analysis (PCA) was carried out on the absorbance of the NIR-AOTF spectra of grouped samples of 48 genotypes. In this Fig. 5.1, the PC1 axis consists of aresidual variance explained for 89.29% and PC2 consists of 8.55%. This classification is based on the total phenolic content present in the poplar buds. In fig. 5.1 there is a clear bounded out layer. Only one genotype out of the boundary layer and got 97.84% variance (PC1+PC2), which defined an error in explaining residual variance under 3%.Clusterization and trend to a group of the samples are graphically described by the score plot and the visual analysis.

HCA is a clustering method that explores the organization of samples in groups and between groups that represent a hierarchy (Lee & Yang, 2009). The HCA result is usually presented in a dendrogram, a diagram shows the organization of the samples and its relationships in tree form.

There are two main approaches to solving the problem of clustering in HCA, agglomerative or divisive. In the first, each sample is initially considered a cluster and then pairs of clusters are merged. In the divisive approach algorithm, starts with a cluster comprising all the samples,

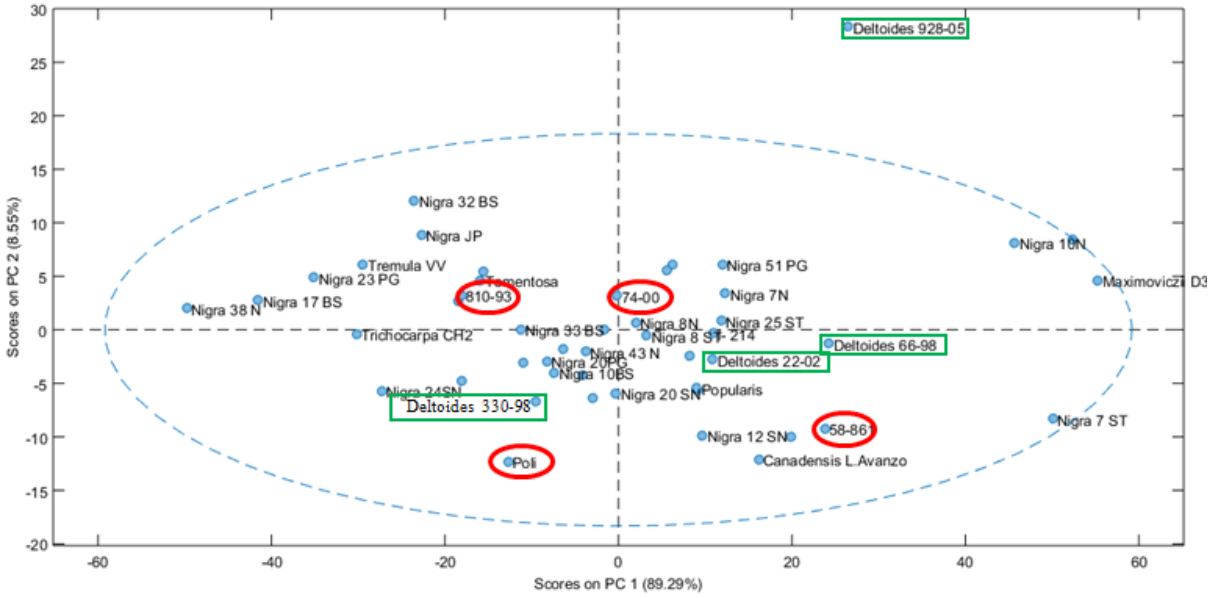



Figure 5.1 - Score plot for the Principle Component Analysis (PCA), Plotted between PC 1 and PC 2 axes. The percentage of the variance showed in the parentheses on the axes

recursive divisions are performed. This study shows that PCA/HCA methods do not always provide sufficient means to group samples according to the spectra profile which indirectly could

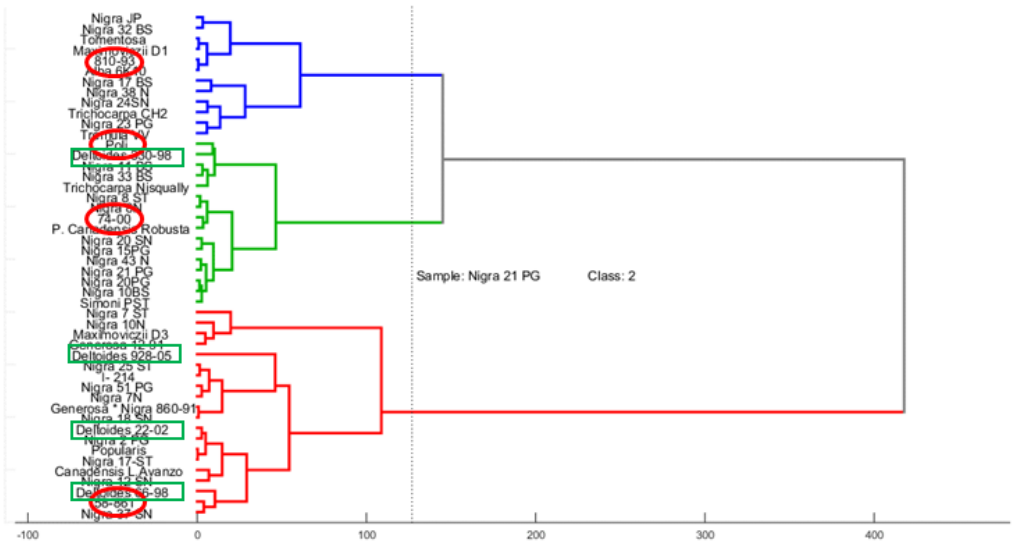



Figure 5.2 -Dendrogramof hierarchical cluster analysis for the various poplar genotypes and hybrids.

be affected by the concentrations of bioactive compounds. HCA and PCA were used with the aim to tentatively “classify” the commercial samples comparing this clusterization to the sample

separation based on the HPLC fingerprint, but no significant differentiation between samples was achieved.

In PLS-DA analysis, the visual response of the regressive method is appreciated by a score plot where PC was replaced by a latent variable of the regressive modeling which minimizes the error in classifying assigned groups the explained variable (Y). Results and errors were also reported in a confusion matrix and a confusion table (data not showed)including five classes of grouping (class1 to class 5 with different genotypes from different sections). Scorerplot result is divided into LV1 and LV2 with 89.52 and 7.90 variances, respectively.

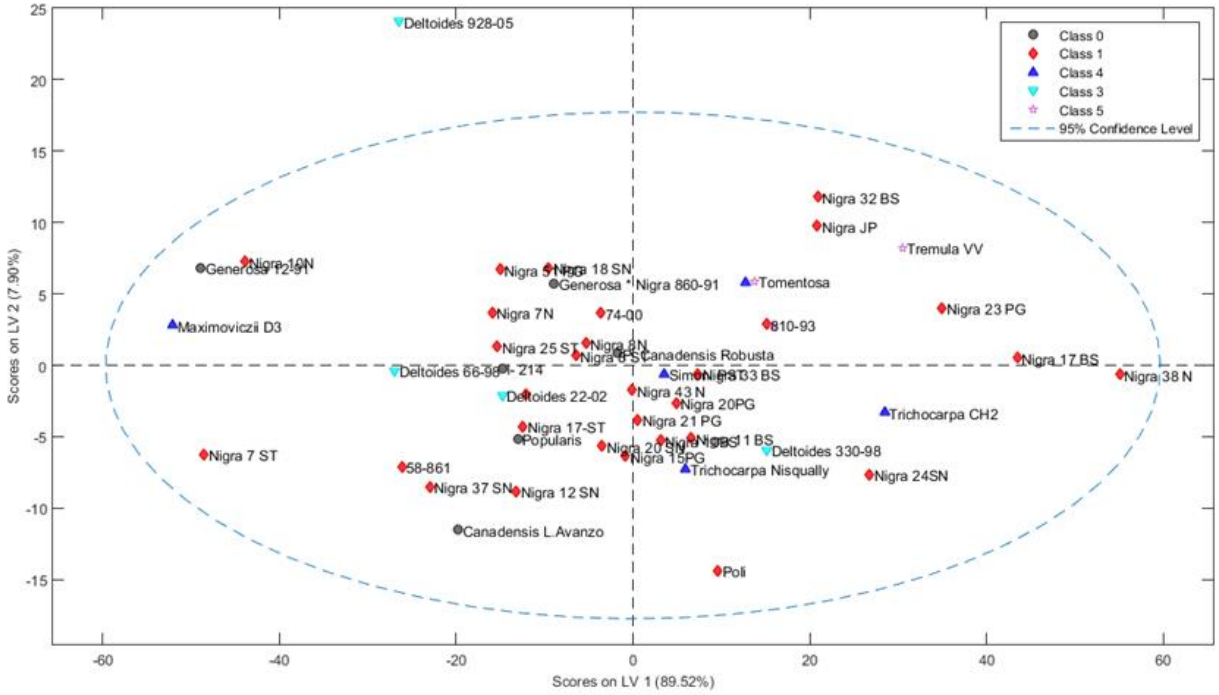



Figure 5.3 - Classification of 48 poplar genotypes by Partial least square –DA (PLS-DA) analysis

The distribution of different genotypes by PCA, HCA, and PLS were based on total phenolic content as well as other chemical compounds (which are detectable at 1100 to 2500 nm wavelength). In this experiment, we analyzed 48 genotypes from three major poplar sections (*Aigeiros, Tacamahaca,* and *Populus*) and some poplar hybrids. In the principle component analysis (PCA) and the hierarchical cluster analysis (HCA), the genotypes were scattered randomly. In fig 5.1, there is no clear separation or distinguish between the genotypes according to sections. It is a clear indication that the classification of the genotypes is not only based on the chemical composition and also based on other genetical, morphological, and physiological characteristics of the buds. In PCA analysis we absorbed maximum absorbance with 89.29% variance from PC 1 and 8.55% from PC 2 in which *P. nigra, P. deltoides* and the hybrids were scattered all over the matrix.

Both PCA and HCA analysis wereshowing similar results. In fig 5.2, genotype “810-93” and “58-861” were separated in different branches, whereas genotype “Poli”and “74-00” were grouped under the same branch. The total phenolic content results have given a strong evidence that genotype “Poli”and“74-00”havethe higher phenolic content among the fourP. nigra genotypes. Among the four *P. deltoides* genotypes, 3 genotypes (“928-05”, “22-02”, and “66-98”) were grouped under the same branch and the 4$^{th}$genotype (*330-98*)was grouped under another brach. The total phenolic content results for *P. deltoides* were not significant between the genotypes. In PLS analysis (fig 5.3), 48 genotypes were classified into 5 classes (class 0, 1, 3, 4, and 5). There is no clear separation between the genotypes due totheir great diverse chemical characteristics. All most, many genotypes were separated randomly. This means the concentration of phenolic content varies between the different sections as well as within the same section of poplar species. This technique is a rapid preliminary analysis for a large number of samples to estimate the qualitative analysis.

## 5.2 *P. nigra* crown architecture, bud, and biomass production

### 5.2.1 *P. nigra* crown architecture

Four *P. nigra* genotypes (“Poli”, “58-861”, “810-93”, and“74-00”) were selected for the study about crown architecture and the estimation of the bud and biomass production. As described in MM the four *P. nigra* genotypes were randomly replicated in the plantation and as showed in Fig. 5.4 they are significantly different for all the analysed traits of crown architecture. We measured the circumference at 1m height from the hardwood stump. There is a slight significant variation ($P \leq 0.05$) between the genotypes. Among four genotypes, “58-861” has reached the maximum growth with 95 mm while“Poli”has showed the minimum circumference values, with 53 mm. Total height for the four genotypes showed a high significant variance ($P \leq 0.001$) between the genotypes.

Genotype“810-93”was the highest with 455 cm and “Poli”reached the lower height (287 cm) compared to the other genotypes. The height of the last branch (HBr) for the four *P. nigra* genotypes has a low significant variance ($P \leq 0.05$), “810-93” showed the maximum value (324 cm) than the other genotypes. $L_{arrow}$ for the four genotypes had medium significant variance ($P \leq 0.01$), with genotype“74-00”that dominated $L_{arrow}$ length with a value of 173 cm, more than 1m if compared with “Poli”. The number of sylleptic branches had high significant variance ($P \leq 0.001$).“Poli”had the highestnumber of sylleptic branches (69), while the selected genotype “74.00” showed about one third of the branches of “Poli” with a mean value of 20 branches.

Number of branches and number of buds at 60 cm (30 cm above and below from mid-height) showed higher and medium significant variance (P≤0.001& P≤0.01), respectively. “Poli”has showed that during the growing season it produces continuously sylleptic branches with values of 24 NBr60 and only 3 Nbud60 whereas “74-00” had the minimum NBr60 (5)and maximum number of buds at 60 cm (10) among all genotypes. The number of nodes and internode length showed higher significant variance (P≤0.001).“Poli”confirmed the maximum mean number of nodes with a value of 27 and the shortest mean internode length (2.3 cm nodes$^{-1}$), whereas “810-93” had the higher internode length with a value of 14 and the shortest mean internode length (4.3 cm nodes$^{-1}$).


| Trait | Genotype | | | | ANOVA significance |
|---|---|---|---|---|---|
| | *"58-861"* | *"Poli"* | *"810-93"* | *"74-00"* | |
| **Circum100 (mm)** | 95.7 ±14.0 | 53.3 ±7.6 | 86.8 ±2.0 | 85.8 ±7.2 | * |
| **H (cm)** | 404.8 ±19.4 | 286.8 ±16.4 | 455.2 ±7.3 | 422.7 ±18.0 | *** |
| **HBr** | 305.2 ±21.2 | 244.3 ±19.7 | 324 ±13.9 | 249.5 ±20.8 | * |
| **Larrow** | 99.7 ±28.3 | 51.0 ±7.4 | 131.2 ±10.6 | 173.2 ±16.1 | ** |
| **NsyllBr Tot** | 47.2 ±3.3 | 69.2 ±7.4 | 38.5 ±1.5 | 20.8 ±2.7 | *** |
| **Ibr cm/br** | 8.7 ±0.5 | 4.4 ±0.4 | 11.9 ±0.5 | 21.9 ±2.6 | *** |
| **NBr60** | 10.3 ±2.0 | 23.7 ±2.0 | 9.3 ±0.7 | 5.0 ±1.2 | *** |
| **Nbud60** | 6.3 ±1.5 | 3.0 ±1.7 | 4.5 ±1.0 | 10.3 ±1.7 | ** |
| **Nbnodes** | 16.7 ±0.6 | 26.7 ±0.5 | 13.8 ±0.6 | 15.3 ±0.8 | *** |
| **Int L (cm nodes$^{-1}$)** | 3.6 ±0.1 | 2.3 ±0.04 | 4.3 ±0.2 | 4.0 ±0.2 | *** |

Figure 5.4 - Schematic graphical representation of *P. nigra* crown architecture with different morphological traits. ANOVA significance P≤0.001(***), P≤0.01(**), P≤0.05(*), P≤0.1(ns)

In the present study, we explored the phenotypical crown architecture variation between the four *P. nigra* genotypes. Among them, "Poli" had showed more different morphological characters compared with other genotypes."Poli"confirmed a completely different strategy of resources allocation in the crown being originated from the southern part of Italy. So it grows slowly to overcome the drought period, but also the many extreme climatical events in its native environment. *"Poli"*had showed the maximum number of sylleptic branches and nodes compared to other genotypes. Even if the other genotypes (58-861, "810-93", and"74-00") displayed some differences in the crown architecture parameters (total number of branches and related indexes), they appear more similar for many traits (Fig. 5.4).

### 5.2.2 *P. nigra* bud and biomass production

Buds and biomass were collected from the four *P. nigra* genotypes in the month of February 2019. The collected bud and biomass allowed to measure the fresh and the dry weight of the buds and of the biomass for different components of the crown architecture (branches and stem). The mean total bud dry weight of the total plant (Fig. 5.5) ranges from 1.84 g to 12.20 g, and there is a significant deifference among the four *P. nigra* genotypes.

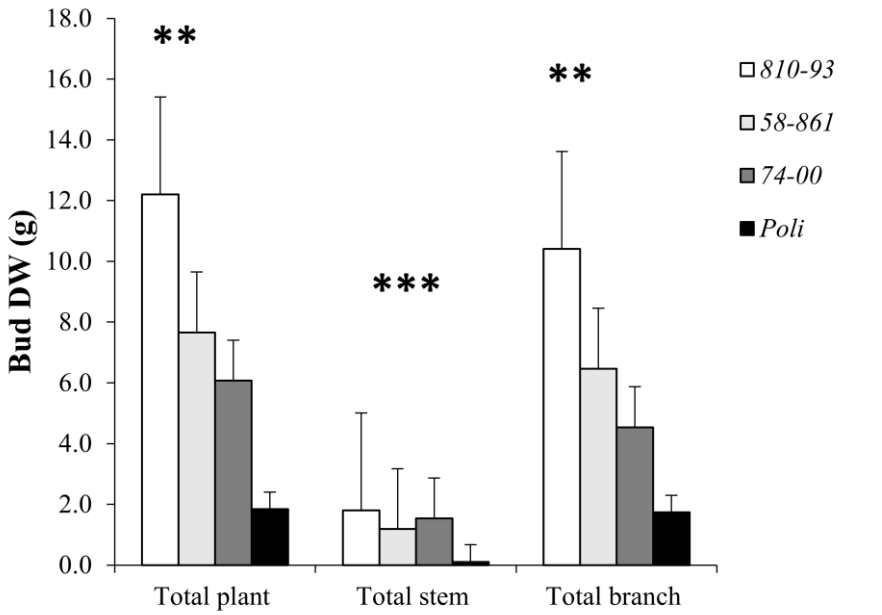



Figure 5.5 - Total bud production of four *P. nigra* genotypes ("810-93", "58-861", "74-00", and "Poli") for the stem, branch components and for total plant. ANOVA significance P≤0.001(***),P≤0.01(**),P≤0.05(*),P≤0.1(ns)

As shown in Fig 5.5, the mean dry weight of the buds from branches had a medium significant variance (P≤0.01) between the genotypes. The mean dry weight of total branch buds ranges from

1.74 g to 10.41 g. The mean dry weight of the stem bud showed the maximum significant variance (P≤0.001) among the genotypes, with a range of the total plant bud DW from 0.11 g to 1.8 g. The data shows that the most important component of the crown architecture where the buds are allocated are the branches in the plants grown under the short rotation system. The allocation of the buds on the branches makes difficult their harvesting. For this reason we also divided the plant stem in two comparetments to verify if it was present a part of the stem that can produce buds easy accessible for their collection.

The mean dry weight of the stem bud from different compartments (S1 and S2) had medium to higher significant variance. Stem bud DW from S1 compartment showed maximum significant variance (P≤0.01) among the four genotypes, whereas stem bud DW from S2 had medium significant variance (P≤0.001).

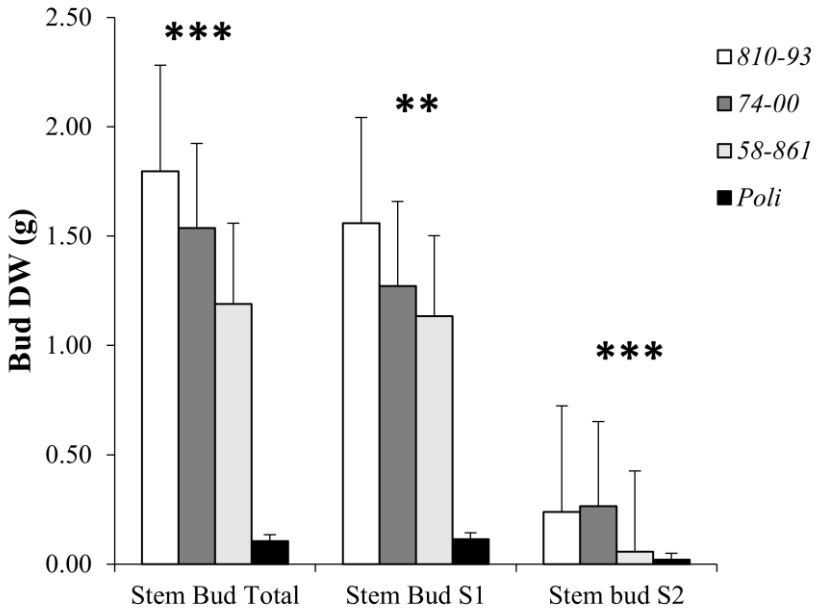



Figure 5.6 -Bud production of the four genotypes (“810-93”, “58-861”, “74-00”, and “Poli”) from different compartments of the stem. ANOVA significance P≤0.001(***), P≤0.01(**), P≤0.05(*), P≤0.1(ns)

The presented data show that the most interesting and accessible part where to concentrate the bud collection is the section S1 corresponding to the upper part of the plant. In this part of the tree, depending by the genotype its is possible to collect up to 1.5 g of *P. nigra* buds with the possibility, in perspective to mechanize this operation. Allocation of bud biomass towards the S1 compartment due to apical growth and new bud formation. Bud length also varying between the S1 (higher) and S2 (lower) compartments.

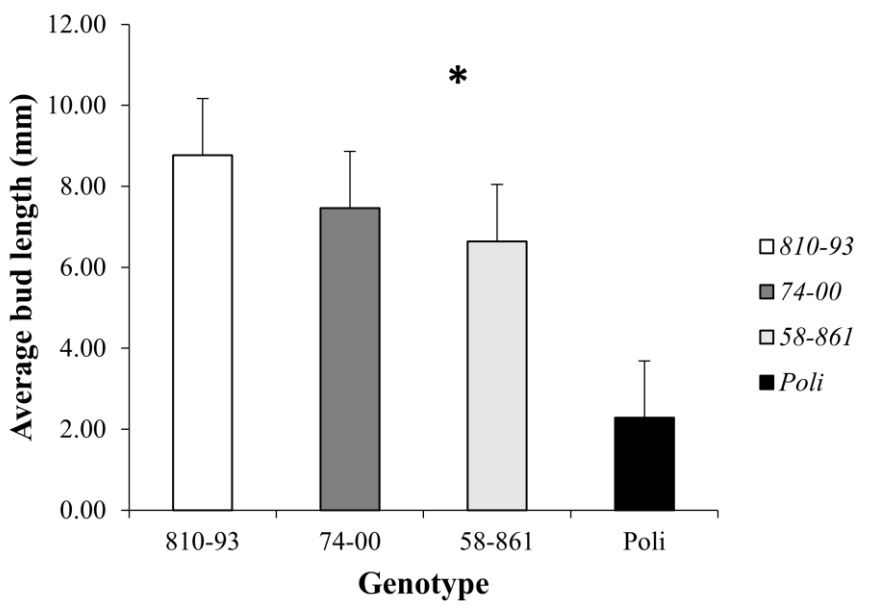



Figure 5.7 -The average bud length of four *P. nigra* ("810-93", "58-861", "74-00", and "Poli") genotypes. ANOVA significance P≤0.001(***), P≤0.01(**), P≤0.05(*), P≤0.1(ns)

The average bud length was measured by using vernier caliper. Genotype *"Poli"* had the smallest average bud length (2.28mm) and "*810.93"* had higher average bud length (8.77 mm). ANOVA results showed a minimum significance variance (P≤0.05) between the genotypes (Fig. 5.7).

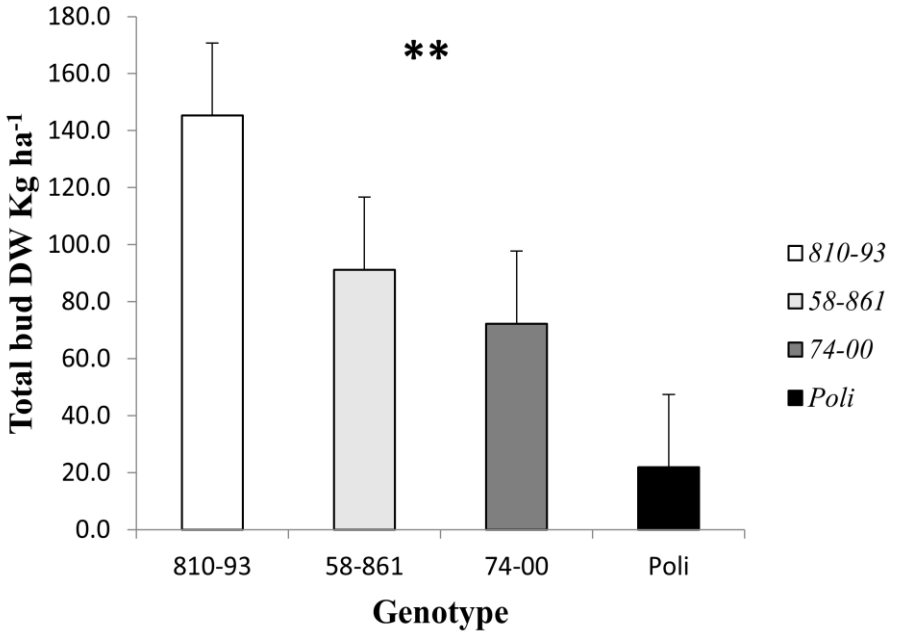



Figure 5.8 -The average total bud production of four *P. nigra* ("810-93", "58-861", "74-00", and "Poli") genotypes per Kg $ha^{-1}$. ANOVA significance P≤0.001(***), P≤0.01(**), P≤0.05(*), P≤0.1(ns)

We also calculated the average bud production per single plant and then estimated the bud production per hectare (calculated number of plants per $ha^{-1}$11905 plants). There is a medium significance variance (P≤0.01) among the genotypesfor this trait . Genotype “810-93” showed the maximum average bud production (145.29 Kg $ha^{-1}$), whereas “Poli” showed a minimum average bud production (21.96 Kg $ha^{-1}$). Genotype “58-861” and “74-00” had a medium bud production, estimated in 91.17 Kg$ha^{-1}$and 72.32 Kg $ha^{-1}$, respectively. The clear indication that the branches have a higher quantity of bud mass was not taken in account in this calculation.In this case the amount of bud production only consideringthe apical partshould reduce the productivity of the buds to about one tenth of the total bud productivity.

Interesting also to know the percentage of the bud productivityof the four *P. nigra* genotypes 58-861, “Poli”, “810-93”, and “74-00” that account to 1.07%, 0.69%, 1.60%, and 1.00% of the total biomass, respectively. The bud length is another interesting information as it determines the facility to detach the buds from their support (stem or branch). Buds of major sizes can be detached by hand or mechanically with more facility, so if we imagine to brush away the buds formthe stem our choice of plant materials will be oriented towards genotypes with big buds. Bud % for 4 *P. nigra* genotypes “58-861”, “Poli”, “810-93”, and “74-00” were 1.07%, 0.69%, 1.60%, and 1.00% respectively.

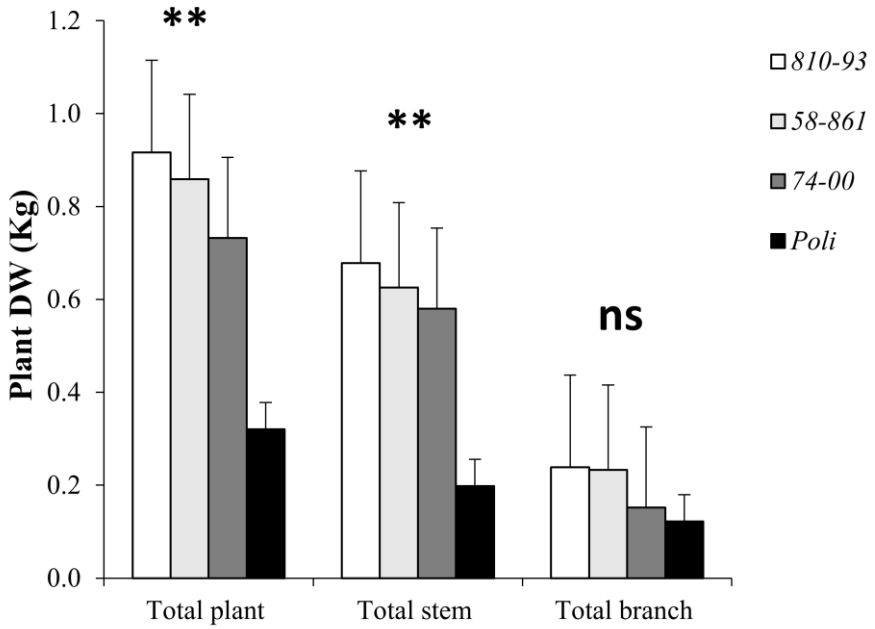



Figure 5.9 -Biomass production at plant level, stem and branchesof the four genotypes (“810-93”, “58-861”, “74-00”, “Poli”) . ANOVA significance P≤0.001(***), P≤0.01(**), P≤0.05(*), P≤0.1(ns)

Mean dry biomass per genotype was calculated and it ranges from 0.32 to 0.92 Kg per plant. The plant stem and branches DW were measured separately and finally calculated to obtain total plant DW. Total average plant DW showed medium significance variance (P≤0.01) among the genotypes.The average branch mass ranges from 0.12 Kg to 0.24 Kg per genotype. There is no significant variance P≤0.1(ns) between the genotypes for this trait. The stem average DW ranges from 0.2 to 0.68 Kg per genotype. These results showed a medium significance variance (P≤0.01) among the genotypes.

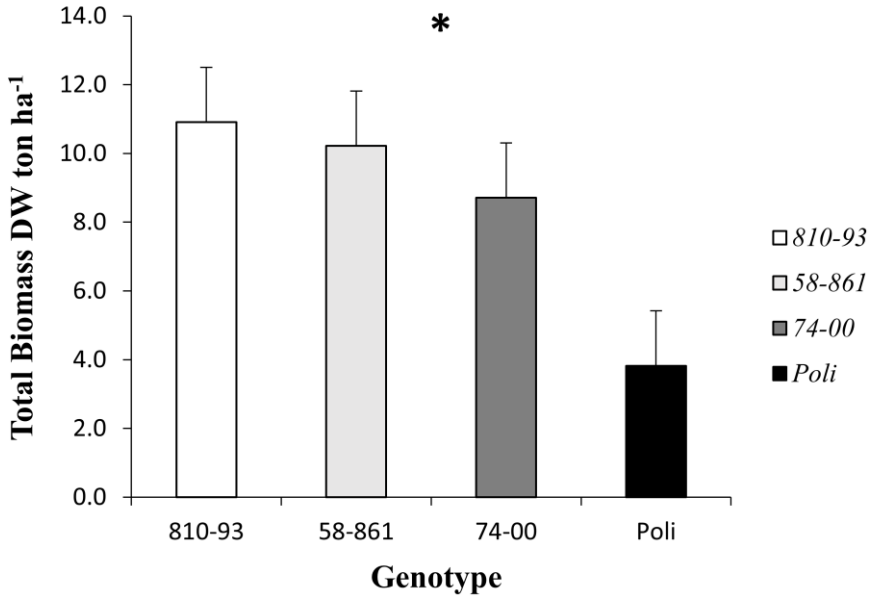



Figure 5.10 -Average total biomass production of four *P. nigra* ("810-93", "58-861", "74-00", and "Poli") genotypes. ANOVA significance P≤0.001(***), P≤0.01(**), P≤0.05(*), P≤0.1(ns)

Measured plant DW per genotype and estimated total biomass in ton $ha^{-1}$ ranges from 3.82 to 10.91 ton $ha^{-1}$. Genotype "810-93" showed the maximum biomass production and the lower biomass production was obtained from the genotype "Poli". 58-861 and "74-00" had similar biomass production when compared to the genotype"810-93". There is a low significant variance (P≤0.05) between the genotypes for this trait.

The study was conducted in the University experimental farm with plants very dense as a short rotation coppice (SRC) system. The study was done with trees atthe fourth rotation (root 4 year old, stem 1 year old, $Root_4$– $Stem_1$). The average total biomass production was higher in genotype "810-93" (10.91 ton $ha^{-1}$), whereas "Poli"has minimum average biomass production (3.82 ton $ha^{-1}$). Genotype "74-00" and 58-861 produced slightly low biomass production (8.71 ton $ha^{-1}$ and 10.22 ton $h^{-1}$) than genotype "810-93"(Fig. 5.9). The average total biomass didn't depend upon the number of branches because genotype "Poli" had the maximum number of

branches but finally, the average biomass production was lower if compared to other genotypes. The average total biomass of branches for all genotypes was lower if compared with the average biomass of the stem (Fig. 5.9). In particular, the selection of parent genetic material is a fundamental step in the development of biomass production in SRC, that can reflect also bud productivity. However, the new genotypes used in this study were selected based on their rapid juvenile growth and tolerance to biotic constraints. The assessment of coppicing ability is amongthe most important determinant of sustainable biomass production (Pontailler et al., 1999). The level of biomass production did not depend on the number of shoots per hectare, but rather on the knowledge on the self-thinning mechanisms and on the associated shoot physiology that has been shown to be dependent on the genotype (Laureysens et al., 2003). Furthermore, it has been established that suppressed shoots support the growth of the dominant shoot by supplying carbon to the stem and lower roots (Tschaplinski and Blake, 1995). Therefore, this should be taken into consideration when focusing on the physiology of shooting to reduce factors such as neighboring stool competition for vital space.

### 5.2.3 Simple linear regression analysis

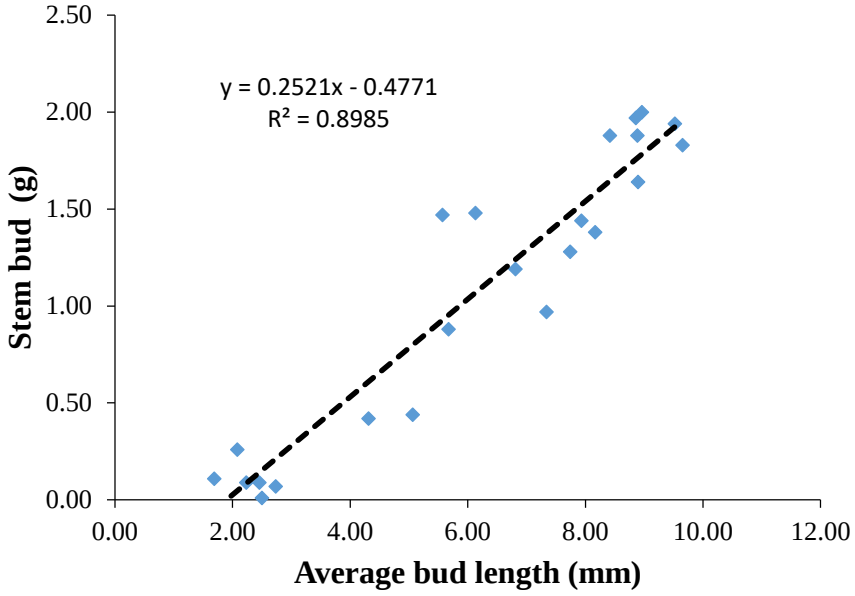



Figure 5.11- Linear regression between average bud length and total stem bud DW. The R2 or coefficient of determination value 0.89.

In the present study, the explanatory variable is considered the average bud length on the X-axis and the dependent variable is the total plant bud weight on Y-axis. The coefficient of determination $R^2$ is quite high 0.89 (Fig 5.11) showing an interesting correlation between the two

traits. In another case, the correlation between the average bud length and the internode length per node has produced a coefficient of determination $R^2$with a value of0.84(Fig 5.11).

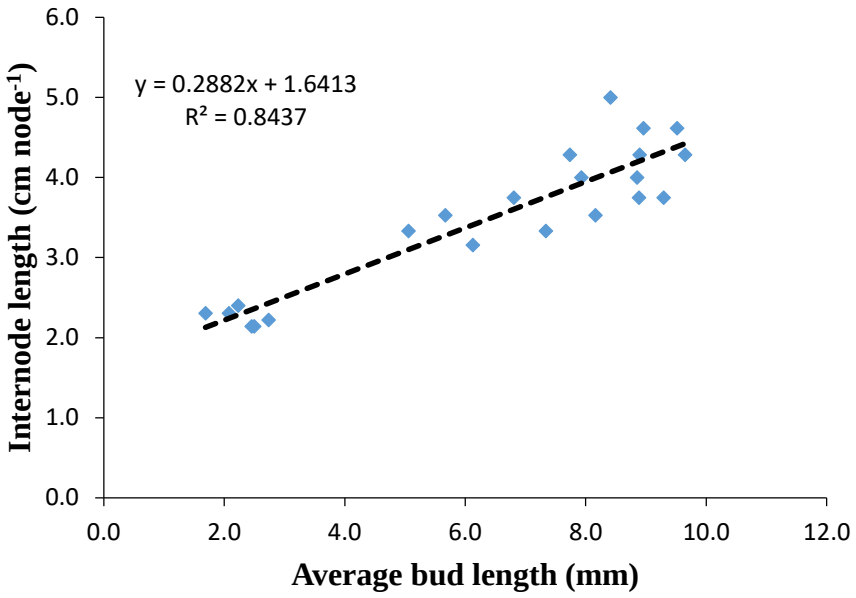



Figure 5.12 -Linear regression between average bud length and internode length per node. The $R^2$ or coefficient of determination value 0.84.

Figure 5.12 clearly states that the two variablesare positivelycorrelated as when average bud length increases the internode length also increases. Coefficient of determination $R^2$ with a value of 0.84, indicates that this correlation is well established. The positive correlation between

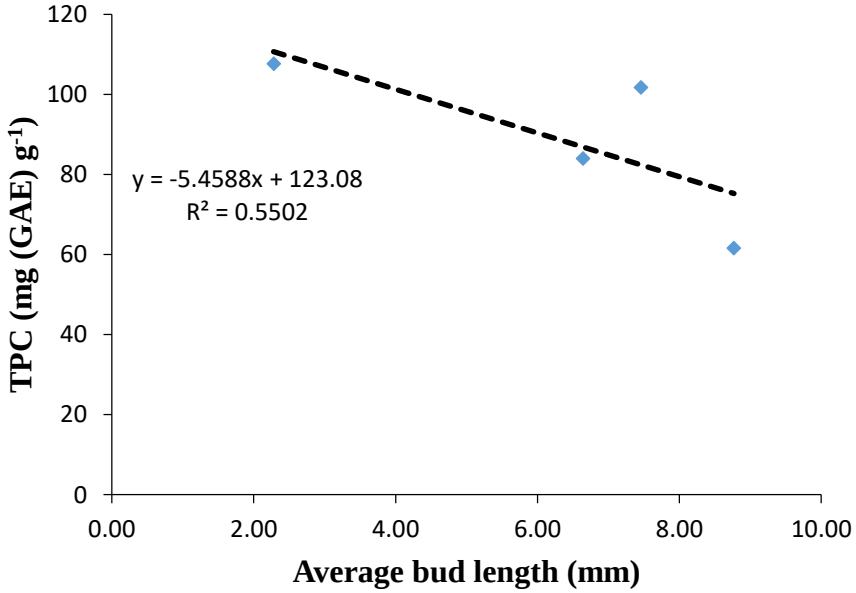



Figure 5.13 - Total Phenolic Content (TPC), mg (GAE)/g DWE and average bud length (mm). mg (GAE): mg Gallic acid equivalent; g DWE: g of the dry weight of extract

average bud length and some crown traits was not found if we correlate the X variable average bud length with the chemical characteristic of the bud among the *P. nigra* studied genotypes. In this experiment, genotype "Poli"has showed the minimum average bud length (2.28mm) and genotype "810-93"themaximum average bud length (8.77mm). The other two genotypes ( "74-00" and "58-861") havea bit lower average bud length (7.46mm and 6.64mm) than "810-93". In the case of TPC analysis, "Poli"hasthe maximum amount of TPC (107.64 mg (GAE)$g^{-1}$ of the dry weight of extract(d.w.e) compared to other genotypes. And genotype "810-93" has the minimum TPC (61.64 mg (GAE)$g^{-1}$d.w.e). "74-00" has the second-highest amount of TPC (101.75 mg (GAE)$g^{-1}$d.w.e). This results is clearly stating that there is a negative correlation between the average bud length and Total Phenolic Content, even if the coefficient of determination is not high (0.55). The interesting indication is that the percentage of Total Phenolic Content doesn't depend on the bud size. It might depend upon many other physiological, genetical and biochemical processes which are unknown.

### 5.3 Quantification of phenolic extracts from poplar buds

Extraction is a crucial and time-consuming procedure. According to TomislavMasek (2018), EtOH:$H_2O$ (70:30) ratio is the best method to extract the phenolic compounds. In Figure, 5.14

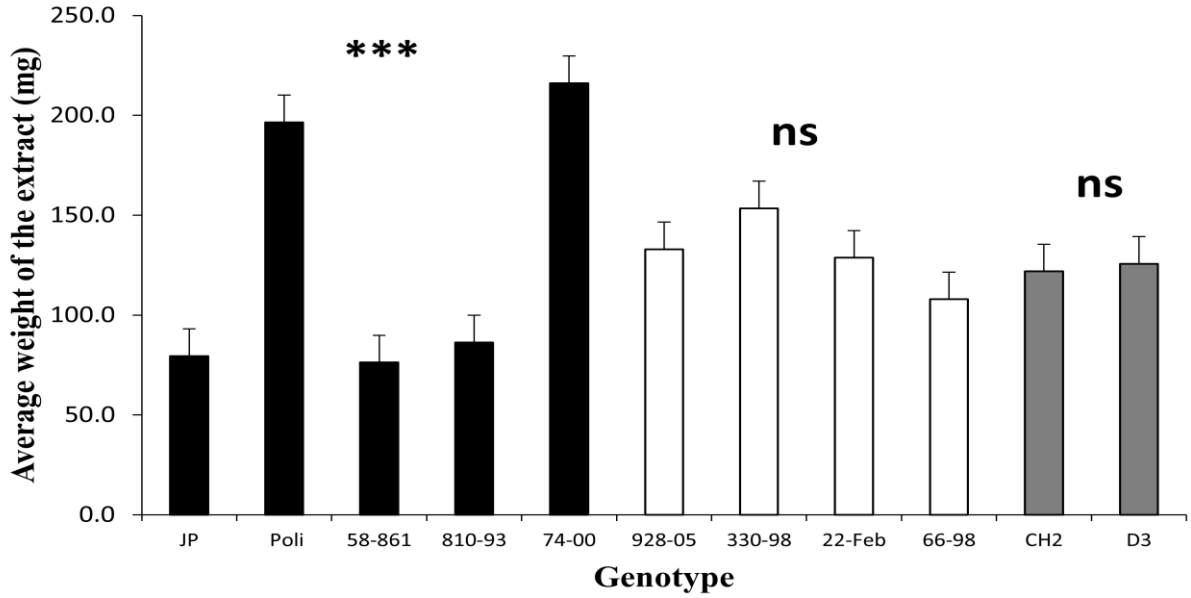



Figure 5.14 - EtOH:$H_2O$ (70:30) extraction of various poplar buds and the final average weight of the extracts. The black color representing *P. nigra*, the white color representing *P. deltoides*, and brown color representing *P. trichocarpa ("CH2")* and *P. maximoviczii ("D3").* ANOVA significance P≤0.001(***), P≤0.01(**), P≤0.05(*), P≤0.1(ns)

mentioned the average final weight (with 3 replicates) of the extract. The extraction weights vary from genotype to genotype. The total phenolic content doesn't always depend on the quantity of the extract because the extract not only contains phenols and also another group of chemical constituents. In fig 5.14, there is a significant variation in *P.nigra* extraction quantities between genotypes, whereas in *P.deltoides*, *P.trichocarpa*, and *P.maximoviczii*, no significant variation between the genotypes. In *P.nigra*, Genotype "74-00" and "Poli"showing the maximum quantity of extraction compared to other genotypes.

### 5.4 $^{1}$H NMR Spectra for chemical characterization of extracts

The profiling of chromatographic or spectroscopic fingerprints is one of the most promising tools usually used in the chemical characterization of various crude extractions and also to identify pure compounds. Among the advanced instruments, NMR and mass spectroscopy (MS are receiving considerable importance in the analysis of metabolic fingerprints in recent times (Kooyet, al., 2009).

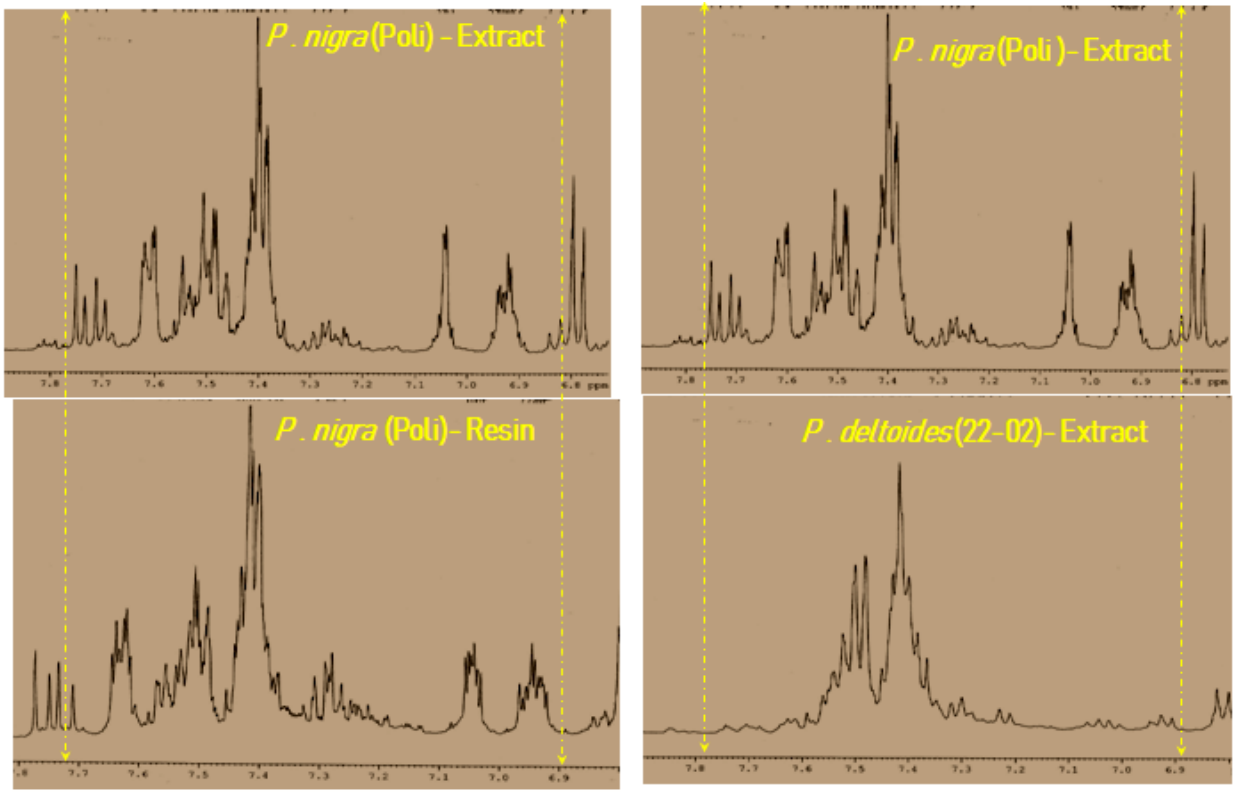



Figure 5.15 -Comparision studies of $^{1}$H NMR spectra: on left comparison between*P. nigra* budextract and *P. nigra* sprout exudates. On the right comparison between *P. nigra* budextract and *P. deltoides* bud extracts.

In the above $^{1}$H NMR (Fig. 5.15) shows the profile of the fingerprint of the $^{1}$H NMR spectra of ethanolic and aqueous extracts of Poplar bud extracts and sprout exudates according to different specific phytochemical regions (Especially Phenolic region). In this experiment, the spectrum lies between 0-12ppm with different functional group regions. The peaks occur in the region 3.5–5.0 and 5.5–8.0 ppm of the $^{1}$H NMR spectrum is respectively of sugars and phenols. Furthermore, the peaks found in the region of the 0.5–3.0 ppm spectra are terpenes and steroids (M.R Lima, 2010 and A. Porzel, 2014). In this study, considerable variations occurred only in the phenolic region (Fig 5.15), indicating the importance of phenolics in poplar bud extracts and sprout exudates (M. Kasote, et, al., 2017).

*Populus nigra* four genotypes have similar NMR spectra, that is the reason why representing only *P.nigra* ("Poli") genotype (Fig 5.15). Also did $^{1}$H NMR spectra for sprout exudates of *Populus nigra*. The NMR spectra for *P.nigra* bud extract and sprout exudates were similar. It was a very interesting result we found in these resultsby this information we can skip many processes (extraction and evaporation), can save time, and also can save huge money for the process. The $^{1}$H NMR spectra for four *Populus deltoides* showing similar results, so representing only one *P.deltoides*("22-02") genotype (Fig 5.15). In the case of *P. deltoides*, the NMR spectra were different compare to *P. nigra* (fig 5.15).

## 5.5 Total Phenolic Content (TPC) of the different poplar genotypes

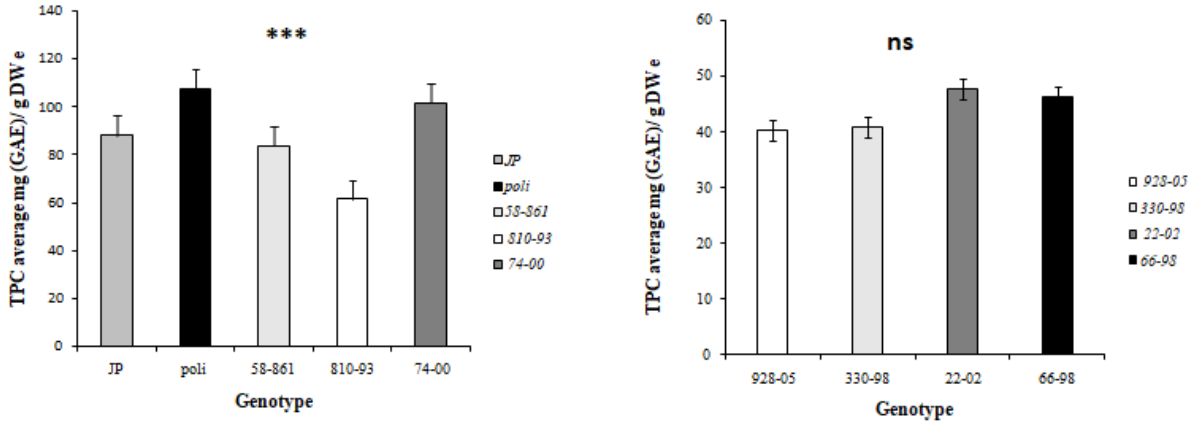



Figure 5.16 - Total Phenolic Content of five *P.nigra* and four *P.deltoides* genotypes. ANOVA significance P≤0.001(***), P≤0.01(**), P≤0.05(*), P≤0.1(ns)

The results stating that there is a significant variation between *P.nigra* genotypes, whereas *P.deltoides* doesn't show any significant variation between the genotypes. *P.nigra* "Poli" has higher concentrations of phenolic content than other genotypes. However, genotype "74-00"also

showing second- highest phenolic content. This results in giving more evidence to the NIR spectroscopy results. In NIR spectra, “Poli”and “74- 00” fit under the same class(PCA and HCA analysis). It is a clear indication that the phenolic content of a genotype doesn’t depend upon bud size, it depends upon many other physiological factors that were unknown. In *P. deltoides,* genotype “22-02” showed higher phenolic content among all four genotypesbut during extraction time, genotype *P. deltoides* “330-98*”* showed the highest extraction quantity. These results are evidence that the extraction quantity doesn’t depend upon the total phenolic content.

## 6. Conclusion

Poplars are an integral part of the landscape and of the rural economy in many European countries. In Italy, poplar natural populations of *P. nigra* and *P. alba* are found commonly along the rivers while poplar plantations are mainly concentrated in the Po valley (Northern Italy). Unfortunately, the economic benefits accruing from timber production have declined in recent years. Standing timber price from traditional poplar plantations has plummeted to the last 15-year low. According to Pra&Pettenella (2017), standing timber prices of poplars are only 80 has been % of what they were 15 years ago and prices are still declining.

Short Rotation Coppice (SRC) using poplar hybrids is a well established technique in Europe and it have been considered as an option in modern agriculture for biomass energy and fiber production in the last 30 years, with the aim of producing bioenergy feedstock in the substitution of fossil fuels(Lindegaard et al., 2016). The trend of SRC plantations in Europe peaked in the period 2005-2010. Nonetheless, despite this promising start and 30 yearsof supportive policy measures in many countries, theindustry has faltered and there are currently estimated to be 50,000 hectares of SRC plantations in the EU28 (Aebiom 2015).In many countries, Italy included, there has been a similar trend withrelatively large areas of SRC being established in a shorttime followed by a rapid decline.

The challenge for the forestry sector is to find alternative uses for timber and woody biomass and 'add value' to these resources. This is of particular importance to upland communities that rely on forestry as a source of income, where diversification into alternative enterprises is more difficult because of climatic, geological and legislative restrictions. It is estimated that only 25% of the felled tree is actually transformed into timber. The remaining material (roots, leaves, bark, small branches, off-cuts etc.), a rich composite of primary and secondary metabolites and plant fibres, is a relatively unexplored and unexploited source for potentially novel products that could complement revenue from pulp or timber outlets
(https://secure.fera.defra.gov.uk/treechemicals/review/introduction.cfm).

In this experimental study, 48 genotypes from different poplar sections(*Aigeiros, Tacahamaca*, and *Populus*) and hybrids were used to investigate vegetative bud chemical characterization. Four *Populus nigra* genotypes(“Poli”, “58-861”, “74-00” “810-93”) were also used to assess bud and biomass production. These four *Populus nigra* genotypes grown under the same environmental conditions showed different growth responses in the short-rotation coppice system.

We tried innovative chemical characterization of poplar buds and succeded by using NIR spectroscopy. The results of this study clearly indicated rapid assessment of phenolic content and

chemical characterization of poplar buds for the 48 genotypes. The calculation and the estimation of the bud and the biomass production of the four *P. nigra* genotypes on a hectare basis is the first attempt to indicate a value for bud crop from poplar cultivated under SRC system. Even if the collection of buds can be not mechanized at the moment the information can contribute to define the percentage of phenolic compounds present in chipped biomass. Extraction of phenolic compounds from poplar buds and their chemical characterization allowed also comparison studies between *P.nigra* and *P. deltoides* vegetative bud extracts and sprout exudates. NIR results given strong evidence that all genotypes don't have always similar chemical composition even though they were from the same speciesandthe same section. The NIR scoreplot scatters the genotypes based on phenolic content as well as other chemical composition. Based on this study we selected the four *P. nigra* genotypes randomly from the 48 genotypes to estimate bud and biomass production. In the four *P. nigra* genotypes, the highest allocation of bud dry mass was on the branch component. Buds on the branches are very difficult to collect separately, so the "usable buds" in the above-ground biomass are mainly in the upper part of the stem (S1compartment). Genotype "810-93" had the higher bud length compared to "Poli" while the other two genotypes "58-861", "74-00"had an intermediate bud length among the four *P. nigra* genotypes studied. Genotype "Poli" has a higher number of branches and a higher number of buds but genotype "810-93" showed maximum bud and biomass production.

Genotype "Poli" had a high amount of phenolic content followed by "74-00", "58-861" and "810-93". This context clearly states that the Total Phenolic Content doesn't depend upon bud size or productivity. We observed significant variation between the five *P. nigra* genotypes ("Poli", "58-861", "74-00", "810-93" and "JP")analyzed whereas no significant variation was found between the four *P. deltoides* genotypes ("928-05", "330-98", "22-02" and "66-98"). In terms of biomass production, genotype "810-93" is the more productive but if we consider the percentage of phenolic compounds in the bud on the basis of biomass produced on of the pharmaceutical industry, "Poli" and "74-00" are the best genotypes for the pharmaceutical industry.We found very interesting results comparing vegetative *P. nigra* bud extracts and its sprout exudates. For the same genotype, bud extracts and sprout exudates had similar chemical composition. This trend reduces the cost of the extraction process, minimizes processing time and will open a novel door for innovative and productive phytochemical production on a large scale.

The abundance and novelty of the phytochemicals present in poplar species could constitute new feedstocks for the pharmaceutical industry.

According to Devappa et al., 2015, more than 160 phytochemicals from *Populus* species have been identified. However, their natural role in plants has yet to be discovered. Most identified

phytochemicals have potential pharmaceutical applications compared to other industrial uses. Only a few have been extensively studied as pharmaceutical products (eg salicylic and phenol-based drugs) and have reached a commercial phase. However, there are over 100 phytochemicals that are still unexplored. Most of these phytochemicals exhibit diverse biological activities, for example, anticancer, antioxidant, anti-inflammatory, antidiabetic, immunostimulatory, gastroprotective, cardioprotective, among others. In 2011, the total area of the global poplar plantations in the World was estimated at 87 million hectares. Over the next decade, growing poplar species in certified forest management systems should increase for both wood and biofuels/bioenergy. This will involve the generation of huge quantities of raw materials for both traditional wood-based industries and potential pharmaceutical industries. The symbiotic existence between the forestry and biorefinery pharmaceutical industries could open new trade routes for the development of a sustainable green bioeconomy

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