



UNIVERSITY OF MOLISE

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Biosciences and Territory Department – DiBT

DOCTORAL THESIS

**“SCIENZE BIOLOGICHE, BIOMEDICHE, DELL’AMBIENTE E  
DEL TERRITORIO”**

**Cycle XXIX – 2015/2016 – S.S.D. BIO03**

***The responses of poplar plants to mechanical  
bending stress***

ELENA DE ZIO

***Supervisor: Professor Gabriella Stefania Scippa***



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*Coordinator:* **Professor Gabriella Stefania Scippa**

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PhD Student:

**Elena De Zio**

*The responses of poplar plants to mechanical  
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*University of Molise  
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DOCTORAL THESIS  
Pesche (IS) 2017



# *The responses of poplar plants to mechanical bending stress*

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## **Dedication**

To Antonio and my family.

To perseverance.

# *The responses of poplar plants to mechanical bending stress*

## **Abstract**

Mechanical stress is a common environmental perturbation that can considerably affect plant stability. In the course of evolution, plants evolved different mechanisms for coping with and mitigating the negative effects of this disadvantageous abiotic stress. Study the impact of this stress on the woody plant means to provide a scientific basis in the knowledge of the root biology and complex dynamics of the reaction wood formation, important in the context of plant productivity and utilization.

In this thesis, a controlled simulation of mechanical stress was performed in *Populus nigra* roots by using a simple experimental system. After giving an outline of the molecular mechanisms that regulate the responses to long-term bending stress between the two sides of the poplar root (the convex and the concave side), this thesis lays emphasis on the roles of main plant hormones in the stress-induced reaction wood formation.

Using the method based on liquid chromatography coupled to tandem mass spectrometry analysis (LC-MS/MS) auxins, abscisic acid and cytokinins profiling was obtained for different sectors and sides of the bent root and stem, subjected to different intensities of tension and compression forces. Data obtained were then compared in order to verify similarities and differences between root and stem response to bending.

In order to better understand the role of hormones in the induction of cambial zone of poplar root subjected to bending, a method for isolation of cambial tissue was developed and the analysis of auxins, abscisic acid and cytokinins profile was carried out.

Taking together, data of this thesis showed as poplar root responds to bending producing in the concave zone a compression wood, rich in lignin content, with features similar to the compression wood produced at stem level by gymnosperms. A high level of auxin and abscisic acid seems to accompany this development process, even in the early phases of the treatment.

# Contents

<b>List of publications .....</b>	<b>1</b>
<b>Other contributions .....</b>	<b>3</b>
<b>Abbreviations.....</b>	<b>4</b>
<b>1 Introduction .....</b>	<b>5</b>
<b>1.1 Mechanical stress.....</b>	<b>6</b>
<b>1.2 Responses of herbaceous plants to mechanical stress .....</b>	<b>8</b>
<b>1.3 Responses of woody plants to mechanical stress.....</b>	<b>11</b>
<b>1.4 Mechanical stress: a stimulus for reaction wood formation .....</b>	<b>15</b>
<b>1.5 Plant hormones and reaction wood formation.....</b>	<b>19</b>
<b>Literature cited .....</b>	<b>23</b>
<b>2 Objectives and papers.....</b>	<b>36</b>
<b>3 Conclusions and future perspectives .....</b>	<b>37</b>
<b>Acknowledgement.....</b>	<b>41</b>

# List of publications

This thesis is based on the work contained in the following papers:

**I. Poplar woody taproot under bending stress: the asymmetric response of the convex and concave sides**

Elena De Zio, Dalila Trupiano, Antonio Montagnoli, Mattia Terzaghi, Donato Chiatante, Alessandro Grosso, Mauro Marra, Andrea Scaloni, Gabriella S. Scippa.  
*(2016) Annals of botany, 118: 865-883.*

**II. Mechanical bending stress in *Populus nigra* plants: hormones profiling in root and stem**

Elena De Zio, Michal Karady, Ioanna Antoniadi, Dalila Trupiano, Antonio Montagnoli, Mattia Terzaghi, Donato Chiatante, Karin Ljung, Gabriella S. Scippa.  
*Manuscript in progress.*

**III. Hormonal profile of *Populus nigra* cambium cells in root under bending stress condition**

Elena De Zio, Michal Karady Ioanna Antoniadi, Dalila Trupiano, Antonio Montagnoli, Mattia Terzaghi, Donato Chiatante, Karin Ljung, Gabriella S. Scippa.  
*Manuscript in progress.*



The contribution of Elena De Zio to the papers was:

- I.** Performance of the molecular and biochemical analysis, analysis of results, writing the manuscript.
- II.** Planning, performance of hormonal extractions, analysis of results, writing the manuscript.
- III.** Planning, method development, analysis of results, writing the manuscript.

## Other contributions

This list includes author's contributions not related to the thesis work.

- I. **Effect of short-term cadmium stress on *Populus nigra* detached leaves**  
Tonia Lomaglio, Mariapina Rocco, Dalila Trupiano, Elena De Zio, Alessandro Grosso, Mauro Marra, Sebastiano Delfino, Donato Chiatante, Domenico Morabito, Gabriella S. Scippa.  
*(2015) Journal of Plant Physiology, 182: 40-48.*
  
- II. **Biochar and compost for growing poplar trees: negative and positive aspects**  
Elena De Zio, Dalila Trupiano, Carla Amendola, Rosaria Santamaria, Mattia Terzaghi, Antonio Montagnoli, Donato Chiatante, Gabriella S. Scippa.  
*Manuscript in progress.*
  
- III. **The impact of biochar application on tomato (*Solanum lycopersicum* L.) grown on different soils**  
Antonella Polzella, Dalila Trupiano, Elena De Zio, Carla Amendola, Andrea Scaloni, Antonio Montagnoli, Donato Chiatante, Gabriella S. Scippa.  
*Manuscript in progress.*
  
- IV. **Integrated analysis of drought and salt stressed olive (*Olea europaea* L. cv. Chétoui) plant: understanding the physiological, biochemical and proteomic response**  
Mariem Ben Abdallah, Dalila Trupiano, Antonella Polzella, Elena De Zio, Mauro Sassi, Andrea Scaloni, Mokhtar Zarrouk, Nabil Ben Youssef, Gabriella S. Scippa.  
*Manuscript submitted.*

# Abbreviations

ABA, abscisic acid  
ABS, above bending sector  
ACC, 1-aminocyclopropane-1-carboxylic acid  
BBS, below bending sector  
BS, bending sector  
CE, concave side  
CK, cytokinins  
CW, compression wood  
CX, convex side  
CZ, cambial zone  
ESTs, expressed sequence tags  
ERF, ethylene responsive protein  
GAs, gibberellins  
IAA, indole-3-acetic acid  
IAAsp, IAA-aspartate  
IAGlu, IAA-glutamate  
LRs, lateral roots  
OW, opposite wood  
oxIAA, 2-oxindole-3-acetic acid  
Ph dev, developing phloem  
PIN, PIN-FORMED auxin efflux proteins  
ROS, reactive oxygen species  
SPE, solid phase extraction  
TW, tension wood  
Xy dev, developing xylem

# 1 Introduction

Plants, as sessile organisms, need a constantly adaptation to changing of surrounding environment, and often suffering the consequences of different adverse factors (Boyer, 1982). Any extreme environmental condition that tends to inhibit the correct developmental process of a biological system could be a potential stress factor (Nilsen and Orcutt *et al.*, 1996; Borics *et al.*, 2013). In nature, plants encounter numerous stresses, which can be distinguished in two general categories: biotic stress, imposed by other organisms, and abiotic stress, imposed by physical/chemical environmental alterations (Boyer, 1982). Biotic (i.e. pathogenic infection) and abiotic (i.e. flooding/drought, salinity, light excess/deficiency, extreme temperatures, ion toxicity/deficiency, pollutants, brushing, bending) stresses can cause aberrant changes in plant growth, productivity and survival (Boyer, 1982; Dhlamini *et al.*, 2005). These different types of stress can act separately or in combination; therefore, severity, together with duration and number of exposures can significantly affect perception and response of the plant to the stress.

Plants exhibit extraordinary plasticity, responding to the stress with transient or chronic solutions, but their susceptibility depends also on the species, organ or tissue in question, stage of development and genotype (Rai and Varma, 2010; Martínez-Crego *et al.*, 2010; Reddy *et al.*, 2011).

In the course of evolution, plants developed a great number of sophisticated avoidance and adaptive mechanisms, to prevent/escape the stress exposure or withstand/tolerate stressful conditions (Levitt, 1972; Hasanuzzaman *et al.*, 2013). The resistance mechanisms that allow plants to survive in adverse conditions may be constitutive or stress-induced; in the last case, an acclimatization period (a gradual stress exposure) is necessary for the acquisition of the maximum degree of tolerance (Tuteja and Gill, 2013; Anjum, 2015). Elucidation of avoidance and tolerance mechanisms by which plants respond to various stresses is of great interest to plant biologists to know fundamental principles in cellular signaling but also to apply that knowledge in the generation of transgenic plants with increased resistance to grow under adverse environmental conditions (Reddy *et al.*, 2011; Wang *et al.*, 2003; Zhang *et al.*, 2004).

## 1.1 Mechanical stress

The mechanical stress is a common abiotic stress that can considerably affect plant stability and survival. Plants in their environment are subject to many different kinds of mechanical stress, including shaking, rubbing, brushing, wounding, bending, impedance. These disadvantageous conditions are caused by several factors, such as wind, rain, slope of soil, presence of natural/artificial physical barriers and gravity (Jaffe and Forbes, 1993).

The responses of plants to these mechanical factors involving alterations on plant growth and development are defined “thigmomorphogenesis” (Boyer, 1967; Jaffe, 1973) and can be considered as a series of physiological, morphological, biomechanical and molecular adaptations of the plant in response to mechanical stimuli (Jaffe and Forbes, 1993). The thigmomorphogenesis has been studied for a long time, for different mechanical stresses, in various plant species: in the herbaceous plant *Arabidopsis thaliana* (Braam and Davis, 1990; Paul-Victor and Rowe, 2011), in gymnosperms, as *Pinus taeda* (Telewski and Jaffe, 1986a), in woody angiosperms, as *Nicotiana tabacum* (Anten *et al.*, 2005) and *Populus* spp. (Pruyn *et al.*, 2000; DeBell *et al.*, 2002; Kern *et al.*, 2005).

Plants experience mechanical stress at any level; leaves, branches, stems, roots but also individual cells and subcellular organelles can sense and respond to changes in turgor and cell wall integrity (Braam, 2005).

Different plant species react to mechanical stimuli in different ways; in some plants the responses occur rapidly, in other slowly over time (Zhang *et al.*, 2013). In some cases, plant morphogenesis can be influenced by mechanical perturbations, resulting in visible phenotypic changes. For example, it is known that in *A. thaliana* mechanical perturbation inhibits elongation of the inflorescence stem (Paul-Victor and Rowe, 2011), touching retards flowering (Chehab *et al.*, 2009) and bending induces postembryonic lateral roots formation (Richter *et al.*, 2009). In woody plants, flexing increases stem diameter in *Ulmus* (Telewski and Pruyn, 1998) and in *Pinus* (Telewski and Jaffe, 1986a) often at the expense of elongation growth. Also in woody herbaceous plants, such *Solanum lycopersicum*, bending determines a decrease in extension growth (Coutand *et al.*, 2000).

Compression wood was detected in wind-treated *Pinus pinaster*, although no difference in the righting response was observed between leaning trees growing toward or away from the source of wind, suggesting that also other factors can determine the reorientation rate of leaning trees (Berthier and Stokes, 2005). Maritime pine (*Pinus pinaster*) exposed to wind can induce phototropic responses in the early stage of pine seedlings development (Berthier and Stokes, 2005). Herrera *et al.*, (2010) demonstrated a rapid gravitropic response in apices of inclined pine seedlings, little or no response at the stem bases of the plants.

In *Acer saccharum*, leaf petioles exposed to wind are short and narrow with less lignified tissues (Niklas, 1996). In *Spartium junceum* root system morphology, pulling strength and chemical lignin content change in slope conditions (Scippa *et al.*, 2006). An asymmetric allocation of root biomass in two preferential directions, named up-slope and down-slope, characterizes *Fraxinus ornus* growth on slope (Chiatante *et al.*, 2003).

More generally, the responses to mechanical perturbations at biomechanical level include an increase of flexural rigidity (strengthening tissue production) resulting, often, in an increase of diameter needed to counteract the reduction in stiffness and improve resistance to mechanical damage (Telewski and Jaffe, 1986b; Kern *et al.*, 2005).

In recent years, considerable progress has been made in understanding the effects of mechanical stresses at the molecular level. Signaling pathways involving various messengers that participate in stress adaptation, such as plant hormones, auxin, abscisic acid (ABA), ethylene, gibberellins (GAs), but also others signal molecules, such as  $Ca^{2+}$ , reactive oxygen species (ROS) and *miRNA* (Ditengou *et al.*, 2008; Trupiano *et al.*, 2012b; Reddy *et al.*, 2011; Braam, 2005; Lu *et al.*, 2005).

Progress in this field have been done thanks to many experimental studies conducted in controlled conditions using mechanical bending (Osler *et al.*, 1996; Countand *et al.*, 2000, 2010), primary object of this thesis.

In the following sections, I will focus on the effects of bending at root level.

## 1.2 Responses of herbaceous plants to mechanical stress

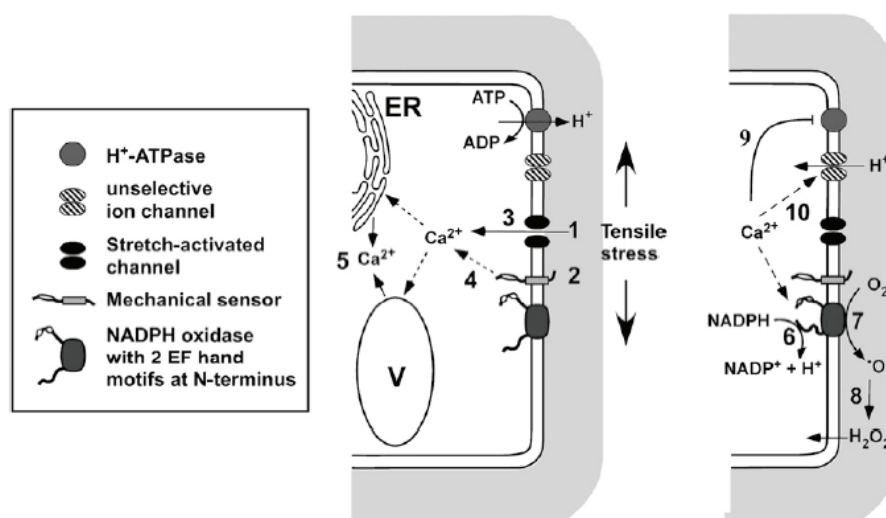
Effects of mechanical perturbations on root system have been widely studied on plant model of herbaceous species *Arabidopsis thaliana*. Richter *et al.*, (2009) have found that the application of mechanical forces on the primary root can act as one of the triggers that entrain lateral roots (LRs) production on the convex side of an imposed bend. In this study it was demonstrated that a transient bending of 20s can be sufficient to elicit this developmental program. Curve-related LRs may form also in decapped root, showing as mechanically induced bends lead to LRs formation also without a gravitropic stimulation. While, the surgical removal of the hypocotyl, to deplete *Arabidopsis* root of an acropetal source of auxin, prevented bend-induced LR emergence but still supported bend-induced primordium formation.

As regard LRs formation is known that accumulation of auxin in the pericycle is likely sufficient to convert a pericycle cell to a founder pericycle cell, responsible of LRs formation, as detailed in Péret *et al.*, (2009) and Dubrovsky *et al.*, (2008). Furthermore, *Arabidopsis* mutants defective in auxin transport or signaling show reduced LRs formation (De Smet *et al.*, 2007).

Richter *et al.*, (2009), demonstrated that the establishment of the LR primordium induced by bending was not disrupted in mutants related with auxin transport and auxin receptor/response elements. For example, mutants in the AUX1 auxin permease, responsible for auxin influx into the cell, normally exhibit a reduction in LRs density, but if these mutants are subjected to bending, show a wild-type frequency of LRs induction. These results suggest that the bending and, consequently, the stretching of root cells in the convex side, is sufficient by itself to start the development of LRs in *A. thaliana*. Cytoplasmic  $Ca^{2+}$  may act as a key messenger in regulating many stress-related developmental processes in roots.

Bending, as many other abiotic stresses, is known to elicit  $Ca^{2+}$  changes in root cells (Monshausen *et al.*, 2009), and  $Ca^{2+}$  seems play a crucial role for the bend-related signals linked to LRs formation. Monshausen *et al.*, (2009) showed that a touch stimulus in *Arabidopsis* roots elicits a cytoplasmic acidification and an apoplastic alkalization, correlated with apoplastic ROS production.

These responses regulated by intracellular calcium, show the same kinetics of  $\text{Ca}^{2+}$  increase stress-induced, and can be: a) elicited in the absence of a mechanical stimulus by artificially increasing  $\text{Ca}^{2+}$  concentrations; b) inhibited blocking  $\text{Ca}^{2+}$  variation, by using  $\text{Ca}^{2+}$ -channel blocker (e.g.  $\text{La}^{3+}$ ). Blocking these  $\text{Ca}^{2+}$  changes, can be selectively blocked LR formation in bent roots (Richter *et al.*, 2009; Monshausen *et al.*, 2009). ROS production and pH changes are independent of each other. In fact, in *Arabidopsis* root hair defective2 mutant (*rhd2*), lacking of a functional NADPH oxidase RBOH C involved in ROS production (Mori and Schroeder, 2004; Foreman *et al.*, 2003), touch stimulation still triggered pH changes but not the local increase in ROS production seen in wild-type plants (Monshausen *et al.*, 2009). A model of early signaling events related with bending was proposed by Monshausen *et al.*, (2009, Fig. 1).



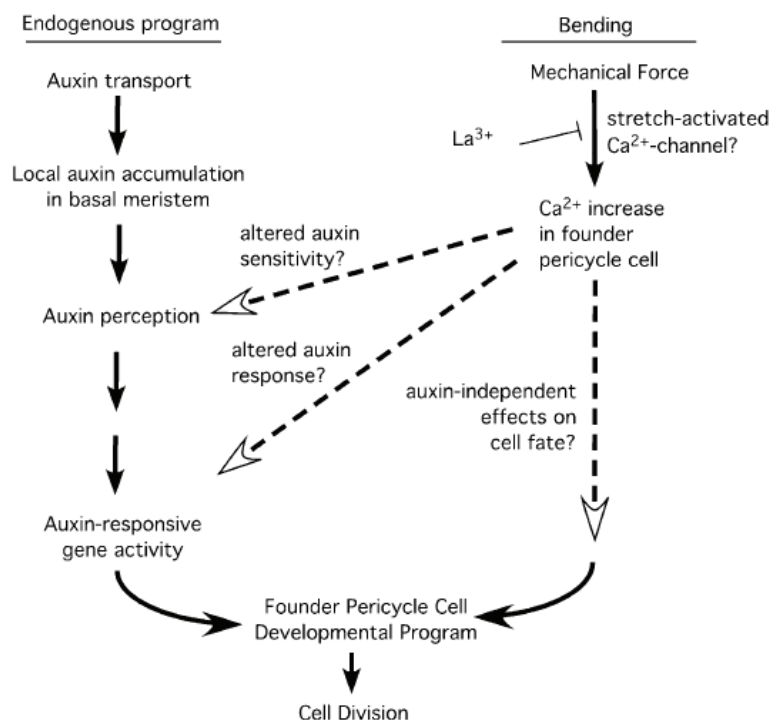
**Fig. 1. Model of signaling events upon mechanical stimulation.** The deformation of the cell wall and plasma membrane caused by mechanical stress is perceived through the activation of mechanosensors, such as  $\text{Ca}^{2+}$  permeable channels (1) and/or cell wall integrity sensors (2). The activation of these channels leads to an influx of  $\text{Ca}^{2+}$  into the cytoplasm directly (3) or indirectly (4). A signal amplification is determined by the mobilization of  $\text{Ca}^{2+}$  from internal stores (5).  $\text{Ca}^{2+}$ -dependent activation of plasma membrane NADPH oxidases results in NADPH oxidation (6) and extracellular superoxide (ROS) production (7). In the cell wall, superoxide is dismutated to  $\text{H}_2\text{O}_2$  (8), which can diffuse back into the cytoplasm. The inhibition of plasma membrane  $\text{H}^+$  ATPases (9) contribute, together with the involvement of other transporters, such as unselective cation or anion channels (10) to pH changes. ER, endoplasmic reticulum; V, vacuole (Monshausen *et al.*, 2009).



This model is applicable both to the mechanical exogenous perturbations (e.g. wind, obstacles in the soil) and internal mechanical stresses (e.g. tissue tension). In this model, mechanical stimulus determine an increase of  $\text{Ca}^{2+}$  levels, which leads to: a) an alteration in ROS and cytosolic acidification, known to elicit signaling events; b) a cell wall alkalization, known to rigidify the cell wall matrix.

Similar signaling cascades have also been described during pathogen defense responses (Garcia-Brugger *et al.*, 2006), suggesting that this changes are used by plants to protect themselves against a range of environmental insults but also to regulate cellular expansion and tissue growth.

Knowing the importance of  $\text{Ca}^{2+}$  in signaling events arising from bending of *Arabidopsis* root, Richter *et al.*, (2009) proposed a possible  $\text{Ca}^{2+}$  role in stretch-induced founder pericycle cell recruitment for LR formation (Fig. 2). Tension perception on the convex side of the bent root could activate  $\text{Ca}^{2+}$  channels, leading to an increase of  $\text{Ca}^{2+}$  in founder pericycle cell and elicit a cascade of events that operate in parallel or interact with auxin-dependent pathway.



**Fig. 2. Model of  $\text{Ca}^{2+}$  role in stretch-induced LR formation** (Richter *et al.*, 2009).

Laskowski *et al.*, (2008) observed that in *Arabidopsis* root the concentration of auxin increase along the outside of the bend, in the pericycle cells on the outside of the curve. The side of the root where LRs emerge is invariant, even in mutants closely related with auxin transport, such as PIN-FORMED (PIN) mutants, suggesting that a robust developmental pathway focuses LRs on the convex side of curves (Laskowski, 2013).

### **1.3 Responses of woody plants to mechanical stress**

The studies on woody plants, especially on root system, are quite difficult. The lack of information in the literature about woody root response to mechanical stresses may be attributed to intrinsic difficulties of analysis of these species, compared to herbaceous species, given the large sizes, longer growth cycles and the limitations related with planting and sampling procedures. In fact, excavation and cleaning procedures can considerably damage the root system, determining alterations in the analysis at any level (Scippa *et al.*, 2008).

As regards the woody plants, therefore, many studies dealing with mechanical stimuli were conducted at stem level (Pruyn *et al.*, 2000; DeBell *et al.*, 2002; Kern *et al.*, 2005; Telewski, 2006; Azri *et al.*, 2009).

Inhibition of stem elongation is often cited as a response to mechanical perturbation in many woody species (Jaffe, 1973; Paul-Victor and Rowe, 2011), together with a high radial growth rates (Osler *et al.*, 1996; Jourez *et al.*, 2001).

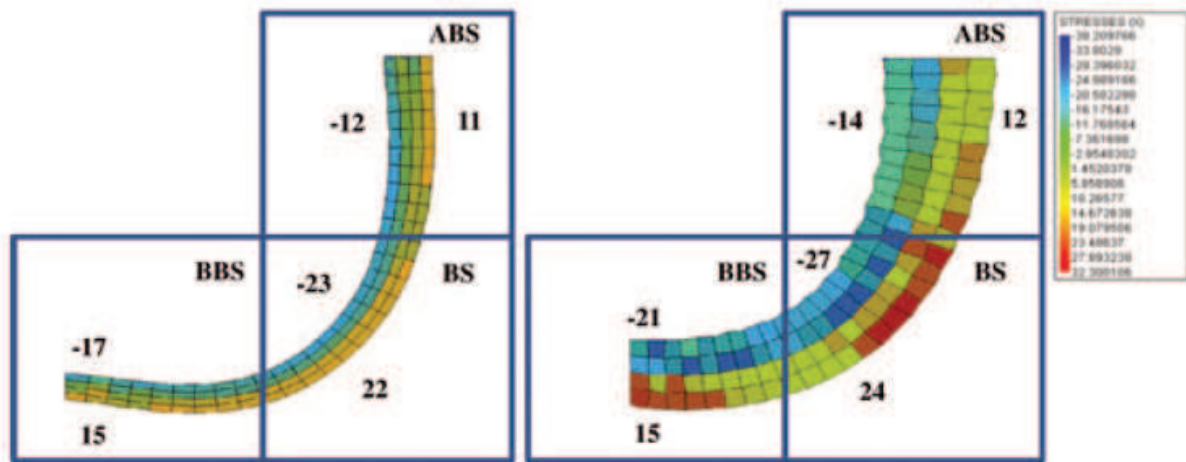
Roots, important for anchoring and mechanical support, show a great plasticity to adapt to the mechanical stress and optimize the whole plant stability. On the basis of such plasticity, root morphology and anatomy are considerably influenced by mechanical perturbations. Several studies reported that mechanical stress determines an increase of root diameter (Materchera *et al.*, 1991), and root biomass (Di Iorio *et al.*, 2005; Scippa *et al.*, 2008), an increase of LRs numbers (Potters, 2007; 2009) and, similarly to the stem, a reduction of main root elongation rate (Bengough and Mullins, 1990; Bengough *et al.*, 2011; Popova *et al.*, 2016).

In the last years, several progress has been made in the understanding of the physiological, molecular and biomechanical basis of *Populus* root responses to mechanical bending stress (Scippa *et al.*, 2008; Trupiano *et al.*, 2012a, b; Trupiano *et al.*, 2013a, b; Trupiano *et al.*, 2014; Rossi *et al.*, 2015; Saviano *et al.*, 2016).

Poplar, as model plant of woody species, is widely studied for the understanding of mechanisms that control the adaptations of woody root to a wide variety of changing environments (Kieffer *et al.*, 2008, 2009; Bohler *et al.*, 2010; Plomion *et al.*, 2006). In fact, its genome was the first forest tree genome to be decoded (Tuskan *et al.*, 2006), with more than 45,000 putative protein-coding genes identified.

The works of Scippa *et al.*, (2008) and Trupiano *et al.*, (2012a, b; 2013a, b), conducted on *Populus nigra* root subjected to bending, highlighted how, the integration of different signals (hormones, transporters, ROS, etc.) determines specific morphological alterations, such as variations in LR density, root biomass, root radial growth and lignin content.

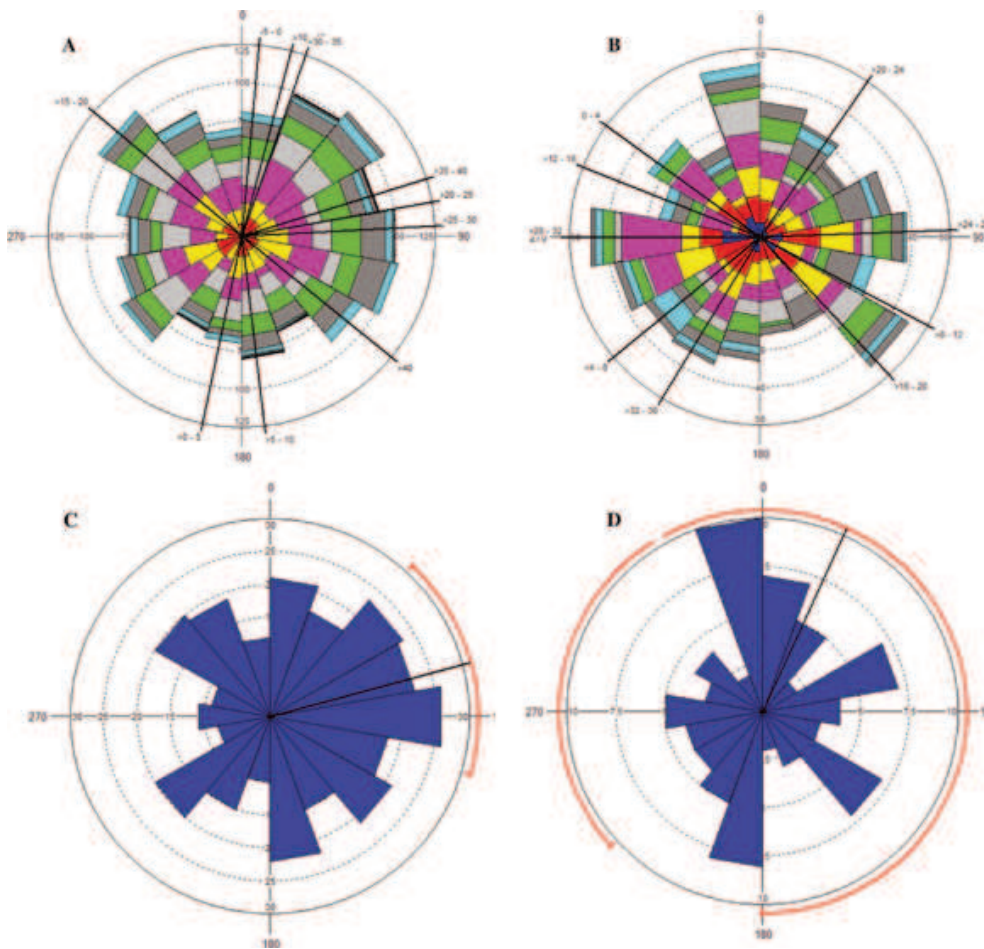
In detail, in these studies a bending stress condition was simulated using a simple experimental system, in which poplar roots were linked to right angle curved steel nets (Scippa *et al.*, 2008; Trupiano *et al.* 2012a), carrying out the analysis on three specific bent sectors (ABS, above bending sector; BS, bending sector, the point of maximum bending; BBS, below bending sector). In Trupiano *et al.*, (2012b) a model has been proposed where along the bent taproot tensile and compression forces differently distributed identifying the three different regions, ABS, BS and BBS. According to this model, both types of forces (tensile and compression) resulted particularly intense in BS and BBS regions (Fig. 3).



**Fig. 3. Model of the mechanical forces distribution along *Populus* bent taproot.** Forces distribution was analyzed in three specific bent sectors (ABS, BS and BBS) of poplar root at two time points ( $T_1$  on the left and  $T_2$  on the right). Values are indicates under tension (positive values) and compression (negatives values) conditions. ABS: above bending sector; BS: bending sector (point of maximum bending); BBS: below bending sectors (Trupiano *et al.*, 2012b).

Furthermore, in this study, it was observed that the three regions undergo diverse alterations. ABS and BS react to bending stress increasing LR emission in their convex side (Fig. 4), while BS and BBS regions respond with a predominant lignin accumulation. For this reason, a relation between these specific morphogenetic responses and forces distribution along the bent root was hypothesized.

These studies provide also first insights on molecular factors regulating the response to bending of poplar plants, through the acquisition of the first poplar proteome map (Trupiano *et al.*, 2012a). Indeed the comparative analysis of root proteome maps revealed that proteins involved in plant defense, metabolism, reaction wood formation and LR development resulted differentially expressed between the different bent sectors and control roots, seemingly in relation to the mechanical forces distribution along the stressed woody taproots.



**Fig. 4. Frequency of first-order lateral emission.** The measurements were done in stressed (A) and control (B) poplar taproot at 5 cm length intervals, and within BS sector of bent root (C) and control (D). The black lines originating from the center indicate the average emission direction. The arc red line highlights the 95% confidence interval, while 0° coincides with the root convex side (Trupiano *et al.*, 2012b).

The modulated expression in ABS and BS of the *ethylene responsive protein* (ERF), the transcription factor BTF3 or pectin acetyl esterase and  $\beta$ -1,3 glucanase enzymes resulted particularly interesting in relation with the morphogenetic responses. In fact, these factors are directly associated with the LRs formation and the wall weakening required during primordium growth and LRs emergence (van der Graaff *et al.*, 2000; Banno *et al.*, 2001; Chuck *et al.*, 2002; Xie *et al.*, 2000; Yoshida *et al.*, 2006).

Factors involved in cell differentiation and gravitropic response were identified mainly in BS and BBS regions. The overexpression of annexin protein or enzymes, such fructosebisphosphate aldolase and fructokinase, was associated with conditions of altered gravity (Clark *et al.*, 2005; Martzivanou and Hampp, 2003; Kimbrough *et al.*, 2004).

Furthermore, investigating the effects of bending during plant transition from winter dormancy to full vegetative activity, Trupiano *et al.*, (2012a) suggest that long-term bending treatment reinforces the plant's defense machinery, enabling the bent taproot to overcome winter better and resume growth earlier than unbent controls.

Gene expression data on factors associated with cell cycle regulation, as Kip-related protein (KipRP) and scarecrow (SCR), or associated with auxin transport, as PIN3, seem to support the earlier growth resumption induced by mechanical stress and the differential modulation of process involved in growth-dormancy cycle within ABS, BS and BBS regions, in relation to their dissimilar intensity of tension and compression forces (Trupiano *et al.*, 2013b).

Overall, these works provide new information on how the intensity of tension and compression forces and the direction of gravity in the bent woody root can elicit specific responses. Poplar plants subject to bending, therefore, change their root morphology emitting new LRs, and their biomechanical properties increasing the root biomass and lignin content, showing a high degree of flexibility that allow them to acclimate and resist stress condition.

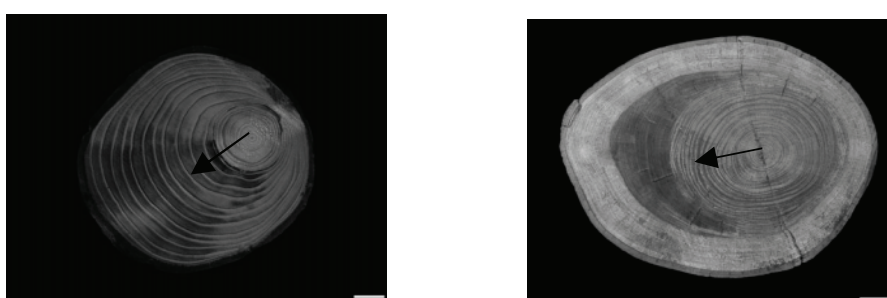
## **1.4 Mechanical stress: a stimulus for reaction wood formation**

Morphological and anatomical changes caused by mechanical perturbations in woody plant often need time to be appreciated, making the study of thigmomorphogenesis very difficult (Braam, 2005). In general, as previously mentioned, woody plants respond to a gravistimulus increasing their tissue rigidity or flexibility (Biddington, 1986; Telewski, 1995) or reducing vertical growth in favor of the radial growth, this is made possible by the production



of a specialized tissue, defined "reaction wood" (Telewski and Jaffe, 1986a; Braam and David, 1990; Braam, 2005). Reaction wood, represent a serious defect in wood utility because possesses altered anatomical and chemical proprieties, but for the plant is necessary to perform the support function when needed (Du and Yamamoto, 2007). Reaction wood is formed in gymnosperms and in woody dicotyledonous angiosperms, generally in response to a non-optimal orientation of the stem or branches and root. In the case of woody plants, in fact, a longitudinal growth promotion on the lower side, as happens in the inclined herbaceous plants, is not sufficient to overcome the bending (Du and Yamamoto, 2007).

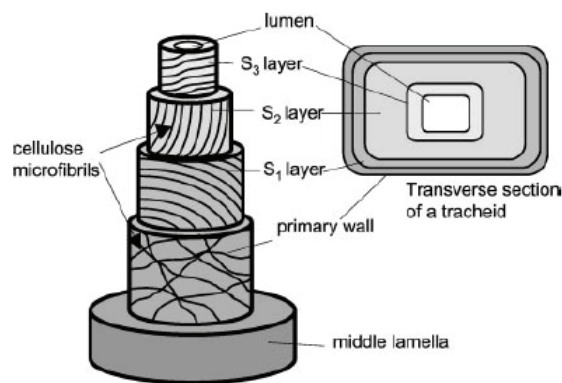
Reaction wood in gymnosperms (softwood species) is generally called compression wood (CW) and is localized in a compression zone, developing on the lower side of leaning stems or branches, while in woody dicotyledonous angiosperms (hardwood species), it is called tension wood (TW) and is formed on the upper side of the leaning, in the tension zone. The wood produced on the side opposite to the reaction wood is named opposite wood (OW) and is characterized by properties intermediate between normal and reaction wood (Timell, 1986). The formation of both CW and TW causes an eccentric radial growth (Fig. 5).



**Fig. 5. Compression wood in a stem of *Picea abies* (to the left), tension wood in a stem of *Eperua falcate* (to the right).** The force vectors are indicated with a black arrows. Scale bar = 5 cm (Ruelle, 2014).

CW and TW differ for cellulose and lignin content. CW is highly lignified and presents less cellulose than normal wood, while TW is characterized by a lower lignin content and more cellulose.

Anatomically, CW presents short and rounder tracheid (xylem cell) with thick cell walls, resulting in intercellular spaces larger than in normal wood. In the CW, tracheid secondary wall lacks of the innermost S<sub>3</sub> layer (for more details see Fig. 6), while the primary wall is the same as in normal wood. The microfibril angle in CW is much higher than in normal wood. These changes allow a longitudinally expansion of CW, necessary in order to push up the organ in the opposite direction of the bending (Timell, 1986; Du and Yamamoto, 2007).

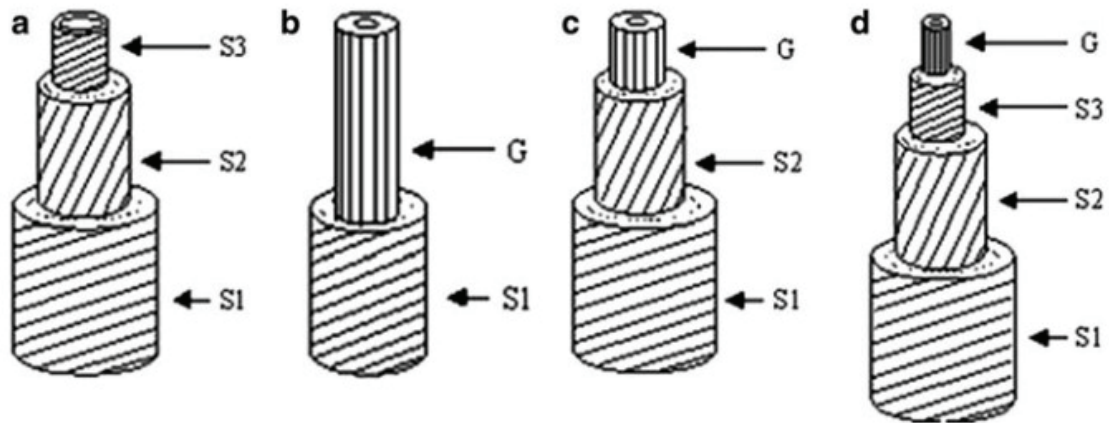


**Fig. 6. Tracheid secondary cell wall in three-dimensional structure.** The cell wall presents different layers, each characterized by a particular arrangement of cellulose microfibrils (Plomion *et al.*, 2001).

In angiosperms, as beech (*Fagus* spp.) or poplar (*Populus* spp.), TW is characterized by fibres with a particular morphology and chemical composition that form the so-called gelatinous layer (G-layer), that is the inner cell wall layer with a high crystalline cellulose content. In the G-layer the microfibril orientation is always longitudinal and parallel to the fiber axis, but fibres can show some variations: they can replace both S<sub>2</sub> and S<sub>3</sub> layers, only S<sub>3</sub> layer or they can represent the innermost layer next to the S<sub>3</sub> layer (Ruelle, 2014; Fig. 7).



Longitudinal tension wood fibres create a strong tensile tissue capable of pulling the stem upright (Timell, 1969; Felten and Sundberg, 2013; Ruelle, 2014).



**Fig. 7. Fibres in normal wood (a) and tension wood (b–d).** Lines indicate cellulose microfibril orientation. The variants in G-layer are showed: (a) Normal fibres do not develop a G-layer. (b) S2 and S3 layers replaced with G-layer. (c) S3 layer replaced with G-layer. (d) G-layer forms as the innermost layer next to the S3 layer (Ruelle, 2014).

The formation of reaction wood (CW and TW) is essentially regulated by environmental factors, such as gravity, bending, wind, compressive or tensile stimuli, but it can take place every time the tree needs to adjust the proper state of balance. Consequently, this specialized tissue, extremely different between gymnosperms and angiosperms, always forms on the side of the organ where it serves to reestablish a correct position (Du and Yamamoto, 2007).

In nature, there are also some exceptions: plants that not produce reaction wood or produce a type different from the group they belong. For instance, the angiosperm *Bruxus* produce a reaction wood with features similar to CW, including increased lignification and absence of S<sub>3</sub> layer (Yoshizawa *et al.*, 1993).

## 1.5 Plant hormones and reaction wood formation

Several studies indicate that phytohormones play a central role during reaction wood formation. Phytohormones are organic compounds usually synthesized in one tissue of the plant and then transported to another location, where they induce a specific physiological response (Antoniadi *et al.*, 2015). Auxins, cytokinins (CKs), abscisic acid (ABA), gibberellins (GAs), and ethylene are the five “classical” groups of plant hormones (Kende and Zeewart, 1997).

Auxins affect numerous aspects of plant growth and development, such as photo- and gravitropism, cell division and differentiation, apical dominance, flowering and senescence, are involved in many different biotic and abiotic stress responses and seem to regulate reaction wood formation as well (Simm *et al.*, 2016; Du and Yamamoto 2007). The major auxin found in plants is indole-3-acetic acid (IAA); its biosynthesis, mostly tryptophan-dependent, is associated with rapidly dividing and rapidly growing tissue (meristems, young leaves, seeds and root tips), although all plant tissues seem able to produce low levels of IAA (Cohen *et al.*, 2003; Ljung *et al.*, 2001; Aloni *et al.*, 2006).

Regarding the involvement of IAA in CW formation, data reported in the literature are scarce and sometimes contradictory (Table 1), while negative or no relations were observed between IAA and TW formation (Table 2, Du and Yamamoto, 2007).

Until recently, the role of auxin in the development of CW was mainly demonstrated by indirect investigations, applying exogenous auxins, auxin regulators (i.e. naphthaleneacetic acid, NAA) or inhibitors (i.e. dinitrophenol, DNP) at stem level (Hejnowicz and Tomaszewski, 1969; Kurt *et al.*, 2014; Fagerstedt *et al.*, 2016).

Results showed that high concentrations of IAA can induce the formation of CW in upright softwood stems at the site of application (Little and Savidge, 1987). In decapitated stems of *Scots pine*, the apically application of IAA induced cambial cells division and xylem production in a dose-dependent manner (Sundberg and Little, 1990). While the application of the IAA transport inhibitor NPA (N-1-naphthylphthalamic acid) to upright stems can induce CW above the site of application (Sundberg *et al.*, 1994).

**Table 1.** Reports on the relationship between endogenous IAA and compression wood formation

Source	Method	Material	Result
Onaka 1942	Bioassay	Bent stems, <i>Pinus</i>	Positive relation
Nečesaný 1958	Bioassay	Horizontal branches, <i>Pinus</i>	Positive relation
Wilson et al. 1989	HPLC	Bent branches, <i>Pseudotsuga</i>	Negative or no relation
Funada et al. 1990	GC-MS	Inclined stems, <i>Cryptomeria</i>	Positive relation
Sundberg et al. 1994	GC-MS	NPA-treated shoots, <i>Pinus</i>	Negative or no relation
Du et al. 2004a	GC-MS	Inclined stems, <i>Metasequoia</i>	Positive relation
Hellgren et al. 2004	GC-MS	Bent stems, <i>Pinus</i>	No relation

**Table 2.** Reports on the relationship between endogenous IAA and tension wood formation

Source	Method	Material	Result
Nečesaný 1958	Bioassay	Inclined shoots, <i>Populus</i>	Negative relation
Wareing et al. 1964	Bioassay	Branches, <i>Populus</i> , <i>Salix</i>	No relation
Leach and Wareing 1967	Bioassay	Horizontal stems, <i>Populus</i>	Negative relation
Andersson-Gunnerås et al. 2003		Bent stems, <i>Populus</i>	No relation (Opposite side<Tension wood side<Upright control)
Hellgren et al. 2004	GC-MS	Bent stems, <i>Populus</i>	No relation (Opposite side<Tension wood side<Upright control)

**Table. 1-2. Reports on relationship between IAA/CW or TW formation.** (Du and Yamamoto, 2007).

Du *et al.*, (2004) found a higher amount of endogenous IAA in the cambial region producing CW. Contrary, Hellgren *et al.*, (2004) suggested that IAA gradient in the cambium could not be the signal for the development of reaction wood, since it can form even without changes in auxin distribution within the cambial region of *Populus tremula* and *Pinus sylvestris* bent stem. Regarding the development of TW, reports in the literature suggest that it is induced by IAA deficiency or it formed around the stem position where IAA concentration is lowest (Timell, 1986).

The application of IAA to one side of *Populus tremula* upright stems induces TW on the opposite side (Blum, 1971). Most recently, genes expression studies in bent poplar stem showed a rapid downregulation of *PttIAA* genes during the earliest responses associated with TW formation (Moyle *et al.*, 2002). In the same study, no measurable changes in IAA content were found after days of bending in isolated stem tissues (xylem and phloem tissues) after induction of TW (Moyle *et al.*, 2002). The analysis of expressed sequence tags (ESTs) of poplar stems subjected to bending seems to confirm these

results. In fact, Jin *et al.*, (2011) identified many genes involved in cell wall biosynthesis and modification after bending treatment but also a downregulation of the majority auxin-related genes.

These experiments provided interesting and very informative results about the potential role of IAA in reaction wood formation. However, the measure of endogenous auxin concentrations *in planta* is still necessary to better validate its role in reaction wood formation.

Unlike auxins, CKs and ABA have been seldom investigated in reaction wood formation and, so far, any direct relations with reaction wood formation has been shown (Du and Yamamoto, 2007).

CKs are adenine derivatives, produced in the root cap that can move upward. Mainly based on cell division and proliferation activity, CKs are involved in many development aspects, as seed germination, shoot apical meristem activity, floral development, photomorphogenesis, leaf senescence and regulation of vascular development (Simm *et al.*, 2016; Svačinová *et al.*, 2012; Kieber and Schaller, 2014; Paul *et al.*, 2016).

Several studies suggest that CKs interact with IAA in a synergetic manner for xylem differentiation: this combination appears to be essential for induction of lignification and tracheary element differentiation (Du and Yamamoto, 2007). In growing poplars, CKs can stimulate vascular cambium cells division and promote the development of vascular cambium (Matsumoto-Kitano *et al.*, 2008). CKs can increase the auxin-responsiveness in the cambial zone, for example, regulating the level of PIN auxin efflux proteins (Paul *et al.*, 2016).

As regards the universal stress hormone, ABA, evidence demonstrated that, it plays a central role in the plasticity of plant development; it can regulate the wood formation by retarding or ending the cambial activity during winter (Carvalho *et al.*, 2013). Differently from CKs, ABA acts in an inhibitory manner with IAA in xylem differentiation (Du and Yamamoto, 2007). ABA seems to be involved in plants responses to mechanical stimuli (Trupiano *et al.*, 2012b), but its specific role in the reaction wood formation needs to be further investigated.

Among the hormones mainly involved in reaction wood formation, aside the IAA, there are gibberellins and the gaseous hormone ethylene.

GAs are a large family of tetracyclic diterpenes which promote cell and stem elongation, inducing long tracheids, in gymnosperms, and fibres, in angiosperms (Carvalho *et al.*, 2013).

GAs are known to be involved in TW formation, while their role in the development of CW is weakly supported. The application of GA to vertical stems of *Populus* plants induces the development of TW with typical G-fibres in the absence of gravistimulus (Funada *et al.*, 2008). Exogenous application of GAs on branches of cherry trees stimulated cambial growth and promoted TW formation on the upper side (Fagerstedt *et al.*, 2016).

Several GAs have been identified in the cambial region in both woody angiosperms and gymnosperms (Du and Yamamoto, 2007). However, are still needed more direct evidence on their involvement in reaction wood formation.

Ethylene, synthesized in response to various stress (wounding, flooding, wind bending) is usually measured by using application experiments or by the estimation of its precursor, the 1-aminocyclopropane-1-carboxylic acid (ACC). Applied ethylene alters polysaccharide deposition during cell wall formation and induced the activity of key enzymes in the lignin biosynthesis (Du and Yamamoto, 2007), despite this, many examples rejected its role in CW formation. In bent poplars (*P. tremula x tremuloides*), for example, an induction of *PttACO1* (ACC oxidase, the last enzyme in the ethylene biosynthesis pathway) expression was observed in the TW (Andersson-Gunnerås *et al.*, 2003). The role of ethylene in CW-forming tissue would appear mainly correlated with his positive regulation function of IAA levels and IAA sensitivity (Love *et al.*, 2009).

## Literature cited

Aloni R, Aloni E, Langhans M, Ullrich C (2006). Role of cytokinin and auxin in shaping root architecture: regulating vascular differentiation, lateral root initiation, root apical dominance and root gravitropism. *Ann Bot* 97:883-893.

Andersson-Gunnerås S, Hellgren JM, Björklund S, Regan S, Moritz T, Sundberg B (2003). Asymmetric expression of a poplar ACC oxidase controls ethylene production during gravitational induction of tension wood. *Plant J* 34:339-349.

Antoniadi I, Plačková L, Simonovik B, Doležal K, Turnbull C, Ljung K, Novák O (2015). Cell-type-specific cytokinin distribution within the *Arabidopsis* primary root apex. *The Plant Cell* 27:1955-1967.

Anjum NA (2015). Plant acclimation to environmental stress: a critical appraisal. *Front Plant Sci* 6:445.

Anten NP, Casado-Garcia R, Nagashima H (2005). Effects of mechanical stress and plant density on mechanical characteristics, growth, and lifetime reproduction of tobacco plants. *Am Nat* 166:650-660.

Azri W, Chambon C, Herbette S, Brunel N, Coutand C, Lepié JC, Ben Rejeb I, Ammar S, Julien JL, Roeckel-Drevet P (2009). Proteome analysis of apical and basal regions of poplar stems under gravitropic stimulation. *Physiol Plant* 136:193-208.

Banno H, Ikeda Y, Niu KW, Chua NH (2001). Overexpression of *Arabidopsis* ESR1 induces initiation of shoot regeneration. *Plant Cell* 13:2609-2618.

Bengough A, Mullins C (1990). Mechanical impedance to root growth: a review of experimental techniques and root growth responses. *J Soil Sci* 41:341-358.



Bengough AG, McKenzie B, Hallett P, Valentine T (2011). Root elongation, water stress, and mechanical impedance: a review of limiting stresses and beneficial root tip traits. *J Exp Bot* 62:59-68.

Berthier S, Stokes A (2005). Phototropic response induced by wind loading in Maritime pine seedlings (*Pinus pinaster* Ait.). *J Exp Bot* 56:851-856.

Berthier S, Stokes A (2005). Righting response of artificially inclined maritime pine (*Pinus pinaster*) saplings to wind loading. *Tree Physiol* 26:73-79.

Biddington NL (1986). The effects of mechanically induced stress in plants - a review. *Plant Growth Regul* 4:103-123.

Blum W (1971). Über die experimentelle Beeinflussung der Reaktionsholzbildung bei Fichten und Pappeln. *Ber Schweiz Bot Ges* 80, 225-251.

Bohler S, Sergeant K, Lefèvre I, Jolivet Y, Hoffmann L, Renaut J, Dizengremel P, Hausman JF (2010). Differential impact of chronic ozone exposure on expanding and fully expanded poplar leaves. *Tree Physiol* 30:1415-1432.

Borics G, Várбірó G, Padisák J (2013). Disturbance and stress: different meanings in ecological dynamics? *Hydrobiologia* 711:1-7.

Boyer JS (1982). Plant productivity and environment. *Science* 218:443-448.

Boyer N (1967). Modifications de la croissance de la tige de Bryone (*Bryonia dioica*) à la suite d'irritations tactiles. *CR Acad Sc Paris* 264:2114-2117.

Braam J (2005). In touch: plant responses to mechanical stimuli. *New Phytol* 165:373-389.

Braam J, Davis RW (1990). Rain-, wind-, and touch-induced expression of calmodulin and calmodulin-related genes in *Arabidopsis*. *Cell* 60:357-364.

Carvalho A, Paiva J, Louzada J, Lima-Brito J (2013). The transcriptomics of secondary growth and wood formation in conifers. *Mol Biol Int* 2013:974-324.

Chehab EW, Eich E, Braam J (2009). Thigmomorphogenesis: a complex plant response to mechano-stimulation. *J Exp Bot* 60:43-56.

Chiatante D, Sarnataro M, Fusco S, Di Iorio A, Scippa GS (2003). Modification of root morphological parameters and root architecture in seedlings of *Fraxinus ornus* L. and *Spartium junceum* L. growing on slopes. *Plant Biosyst* 137:47-56.

Chuck G, Muszynski M, Kellogg E, Hake S, Schmidt RJ (2002). The control of spikelet meristem identity by the branched silkless1 gene in maize. *Science* 298:1238-1241.

Clark G, Cantero-Garcia A, Butterfield T, Dauwalder M, Roux SJ (2005). Secretion as a key component of gravitropic growth: implications for annexin involvement in differential growth. *Gravit Space Biol Bull* 18:113-114.

Cohen JD, Slovin JP, Hendrickson AM (2003). Two genetically discrete pathways convert tryptophan to auxin: more redundancy in auxin biosynthesis. *Trends Plant Sci* 8:197-199.

Coutand C, Chevolut M, Lacoite A, Rowe NP, Scotti I (2010). Mechanosensing of stem bending and its interspecific variability in five neotropical rainforest species. *Ann of Bot* 105:341-347.

Coutand C, Julien JL, Moulia B, Mauget JC, Guitard D (2000). Biomechanical study of the effect of a controlled bending on tomato stem elongation: global mechanical analysis. *J Exp Bot* 51:1813-1824.

De Smet I, Tetsumura T, De Rybel B, Frey NF, Laplaze L, Casimiro I, Swarup R, Naudts M, Vanneste S, Audenaert D, Inzé D, Bennett MJ, Beeckman T (2007). Auxin-dependent regulation of lateral root positioning in the basal meristem of *Arabidopsis*. *Development* 134:681-690.



DeBell DS, Singleton R, Harrington CA, Gartner B (2002). Wood density and fiber length in young *Populus* stems: relation to clone, age, growth rate and pruning. *Wood Fiber Sci* 34:529-539.

Dhlamini Z, Spillane C, Moss JP, Ruane J, Urquia N, Sonnino A (2005). Status of research and applications of crop biotechnologies in developing countries: preliminary assessment. Rome: FAO, pp 1-53.

Di Iorio A, Lasserre B, Scippa GS, Chiatante D (2005). Root system architecture of *Quercus pubescens* Trees growing on different sloping conditions. *Ann of Bot* 95:351-361.

Ditengou FA, Teale WD, Kochersperger P, Flittner KA, Kneuper I, van der Graaff E, Nziengui H, Pinoso F, Li X, Nitschke R, Laux T, Palme K (2008). Mechanical induction of lateral root initiation in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* 105:18818-18823.

Du S, Sugano M, Tsushima M, Nakamura T, Yamamoto F (2004). Endogenous indole-3-acetic acid and ethylene evolution in tilted *Metasequoia glyptostroboides* stems in relation to compression wood formation. *J Plant Res* 117:171-174.

Du S, Yamamoto F (2007). An overview of the biology of reaction wood formation. *J Integr Plant Biol* 49:131-143.

Dubrovsky JG, Sauer M, Napsucially-Mendivil S, Ivanchenko MG, Friml J, Shishkova S, Celenza J, Benkova E (2008). Auxin acts as a local morphogenetic trigger to specify lateral root founder cells. *Proc Natl Acad Sci USA* 105:8790-8794.

Fagerstedt K, Tocquard K, Lopez D, Decourteix M, Thibaut B, *et al.* (2016). The molecular mechanisms of reaction wood induction. *Springer* 4:107-138.

Felten J, Sundberg B (2013). Biology, chemistry and structure of tension wood. In: Fromm J ed. Cellular aspects of wood formation, plant cell monographs 20. Springer, Heidelberg, pp 203-224.

Foreman J, Demidchik V, Bothwell JH, Mylona P, Miedema H, Torres MA, Linstead P, Costa S, Brownlee C, Jones JD, Davies JM, Dolan L (2003). Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. *Nature* 422:442-446.

Funada R, Miura T, Shimizu Y, Kinase T, Nakaba S, Kubo T, Sano Y (2008). Gibberellin-induced formation of tension wood in angiosperm trees. *Planta* 227:1409-1414.

Garcia-Brugger A, Lamotte O, Vandelle E, Bourque S, Lecourieux D, Poinssot B, Wendehenne D, Pugin A (2006). Early signaling events induced by elicitors of plant defenses. *Mol Plant Microbe Interact* 19:711-724.

van der Graaff E, Dulk-Ras AD, Hooykaas PJ, Keller B (2000). Activation tagging of the LEAFY PETIOLE gene affects leaf petiole development in *Arabidopsis thaliana*. *Development* 127:4971-4980.

Hasanuzzaman M, Nahar K, Alam MM, Roychowdhury R, Fujita M (2013). Physiological, biochemical, and molecular mechanisms of heat stress tolerance in plants. *Int J Mol Sci* 14:9643-9684.

Hejnowicz A, Tomaszewski M (1969). Growth regulators and wood formation in *Pinus silvestris*. *Physiol Plant* 22:984-992.

Hellgren JM, Olofsson K, Sundberg B (2004). Patterns of auxin distribution during gravitational induction of reaction wood in poplar and pine. *Plant Physiol* 135:212-220.

Herrera R, Krier C, Lalanne C, Ba EHM, Stokes A, Salin F, Fourcaud T, Claverol S, Plomion C (2010). (Not) keeping the stem straight: a proteomic

analysis of maritime pine seedlings undergoing phototropism and gravitropism. *BMC Plant Biol* 10:217-229.

Jaffe MJ (1973). Thigmomorphogenesis: the response of plant growth and development to mechanical stimulation. *Planta* 114:143-157.

Jaffe MJ, Forbes S (1993). Thigmomorphogenesis: the effect of mechanical perturbation on plants. *Plant Growth Regul* 12:313-324.

Jin H, Do J, Moon D, Noh EW, Kim W, Kwon M (2011). EST analysis of functional genes associated with cell wall biosynthesis and modification in the secondary xylem of the yellow poplar (*Liriodendron tulipifera*) stem during early stage of tension wood formation. *Planta* 234:959-977.

Jourez B, Riboux A, Leclercq A (2001). Anatomical characteristics of tension wood and opposite wood in young inclined stems of poplar (*Populus euramericana* cv 'Ghoy'). *IAWA J* 22:133-157.

Kende H, Zeewart JAD (1997). The five "classical" plant hormones. *Plant Cell* 9:1197-1210.

Kern KA, Ewers FW, Telewski FW, Koehler L (2005). Mechanical perturbation affects conductivity, mechanical properties and aboveground biomass of hybrid poplars. *Tree Physiol* 25:1243-1251.

Kieber JJ, Schaller GE (2014). Cytokinins. *Arabidopsis* Book 12:e0168.

Kieffer P, Dommes J, Hoffmann L, Hausman JF, Renaut J (2008). Quantitative changes in protein expression of cadmium-exposed poplar plants. *Proteomics* 8:2514-2530.

Kieffer P, Planchon S, Oufir M, Ziebel J, Dommes J, Hoffmann L, Hausman JF, Renaut J (2009). Combining proteomics and metabolite analyses to unravel cadmium stress-response in poplar leaves. *J Proteome Res* 8:400-417.

Kimbrough JM, Salinas-Mondragon R, Boss WF, Brown CS (2004). The fast and transient transcriptional network of gravity and mechanical stimulation in the *Arabidopsis* root apex. *Plant Physiol* 136:2790-2805.

Laskowski M (2013). Lateral root initiation is a probabilistic event whose frequency is set by fluctuating levels of auxin response. *J Exp Bot* 64:2609-2617.

Laskowski M, Grieneisen VA, Hofhuis H, ten Hove CA, Hogeweg P, Marée AFM, Scheres B (2008). Root system architecture from coupling cell shape to auxin transport. *PLoS Biol* 6:307.

Levitt J (1972). Responses of plants to environmental stresses. Academic Press, New York.

Little CHA, Savidge RA (1987). The role of plant growth regulators in forest tree cambial growth. *Plant Growth Regul* 6:137-169.

Ljung K, Bhalerao RP, Sandberg G (2001). Sites and homeostatic control of auxin biosynthesis in *Arabidopsis* during vegetative growth. *Plant J* 28:465-474.

Love J, Björklund S, Vahala J, Hertzberg M, Kangasjärvi J, Sundberg B (2009). Ethylene is an endogenous stimulator of cell division in the cambial meristem of *Populus*. *Proc Natl Acad Sci USA* 106:5984-5986.

Lu SF, Sun YH, Shi R, Clark C, Li LG, Chiang VL (2005). Novel and mechanical stress-responsive microRNAs in *Populus trichocarpa* that are absent from *Arabidopsis*. *Plant Cell* 17:2186-2203.

Martínez-Crego B, Alcoverro T, Romero J (2010). Biotic indices for assessing the status of coastal waters: a review of strengths and weaknesses. *J Environ Monit* 12:1013:1028.

Martizivanou M, Hampp R (2003). Hyper-gravity effects on the *Arabidopsis* transcriptome. *Physiol Plantarum* 118:221-231.

Materechera SA, Dexter AR, Alston AM (1991). Penetration of very strong soils by seedling roots of different plant species. *Plant Soil* 135:31-41.

Matsumoto-Kitano M, Kusumoto T, Tarkowski R, Kinoshita-Tsujimura K, Václavíková K, Miyawaki K, Kakimoto T (2008). Cytokinins are central regulators of cambial activity. *Proc Natl Acad Sci USA* 105:20027-20031.

Monshausen GB, Gilroy S (2009). Feeling green: mechanical signaling in plants. *Trends Cell Biol* 9:228-235.

Mori IC, Schroeder JI (2004). Reactive oxygen species activation of plant  $Ca^{2+}$  channels. A signaling mechanism in polar growth, hormone transduction, stress signaling, and hypothetically mechanotransduction. *Plant Physiol* 135:702-708.

Moyle R, Schrader J, Stenberg A, Olsson O, Saxena S, Sandberg G, Bhalerao RP (2002). Environmental and auxin regulation of wood formation involves members of the Aux/IAA gene family in hybrid Aspen. *Plant J* 31:675-685.

Niklas KJ (1996). Differences between *Acer saccharum* leaves from open and wind-protected sites. *Ann Bot* 78:61-66.

Nilsen ET, DM Orcutt (1996). The physiology of plants under stress: abiotic factors. Wiley, NY, pp 689.

Osler GHR, West PW, Downes GM (1996). Effects of bending stress on taper and growth of stems of young *Eucalyptus regnans* trees. *Trees* 10:239-246.

Paul S, Wildhagen H, Janz D, Teichmann T, Hänsch R, Polle A (2016). Tissue- and cell-specific cytokinin activity in *Populus × canescens* monitored by *ARR5::GUS* reporter lines in summer and winter. *Front Plant Sci* 7:652.

Paul-Victor C, Rowe N (2011). Effect of mechanical perturbation on the biomechanics, primary growth and secondary tissue development of inflorescence stems of *Arabidopsis thaliana*. *Ann Bot* 107:209-218.

Péret B, Larrieu A, Bennett MJ (2009). Lateral root emergence: a difficult birth. *J Exp Bot* 60:3637-3643.

Plomion C, Lalanne C, Claverol S, Meddour H, Kohler A, Bogeat-Triboulot M (2006). Mapping the proteome of poplar and application to the discovery of drought-stress responsive proteins. *Proteomics* 6:6509-6527.

Plomion C, Leprovost G, Stokes A (2001). Wood formation in trees. *Plant Physiol* 127:1513-1523.

Popova L, van Dusschoten D, Nagel KA, Fiorani F, Mazzolai B (2016). Plant root tortuosity: an indicator of root path formation in soil with different composition and density. *Ann Bot* 118:685-698.

Potters G, Pasternak TP, Guisez Y, Jansen MAK (2007). Stress-induced morphogenic responses: growing out of trouble? *Trends Plant Sci* 12:98-105.

Potters G, Pasternak TP, Guisez Y, Jansen MAK (2009). Different stresses, similar morphogenic responses: integrating a plethora of pathways. *Plant Cell Environ* 32:158-169.

Pruyn ML, Ewers BJ, Telewski FW (2000). Thigmomorphogenesis: changes in the morphology and mechanical properties of two *Populus* hybrids in response to mechanical perturbation. *Tree Physiol* 20:535-540.

Rai M, Varma A (2010). Diversity and biotechnology of ectomycorrhizae. Springer, pp 308.

Reddy ASN, Ali GS, Celesnik H, Day IS (2011). Coping with stresses: roles of calcium- and calcium/calmodulin-regulated gene expression. *Plant Cell* 23:2010-2032.

Richter GL, Monshausen GB, Krol A, Gilroy S (2009). Mechanical stimuli modulate lateral root organogenesis. *Plant Physiol* 151:1855-1866.

Rossi M, Trupiano D, Tamburro M, Ripabelli G, Montagnoli A, Chiatante D, Scippa GS (2015). MicroRNAs expression patterns in the response of poplar woody root to bending stress. *Planta* 242:339-351.

Ruelle J (2014). Morphology, anatomy, and ultrastructure of reaction wood. In: Gardiner B, Barnett J, Saranpää P, Gril J, eds. The biology of reaction wood. Berlin: Springer-Verlag 13-35.

Saviano G, Paris D, Melck D, Falasca A, Trupiano D, Iorizzi M, Scippa GS, Motta A (2016). Monitoring spatial and temporal metabolic dynamics of woody poplar root under mechanical stress conditions by NMR-based metabolomics. *Metabolomics* 12:65.

Scippa GS, Di Michele M, Di Iorio A, Costa A, Lasserre B, Chiatante D (2006). The response of *Spartium junceum* roots to slope: anchorage and gene factors. *Ann Bot* 97:857-66.

Scippa GS, Trupiano D, Rocco M, Di Iorio A, Chiatante D (2008). Unravelling the response of poplar (*Populus nigra*) roots to mechanical stress imposed by bending. *Plant Biosyst* 142:401-413.

Simm S, Scharf KD, Jegadeesan S, Chiusano ML, Firon N, Schleiff E (2016). Survey of genes involved in biosynthesis, transport, and signaling of phytohormones with focus on *Solanum lycopersicum*. *Bioinform Biol Insights* 10:185-207.

Sundberg B, Little CHA, Cui K (1990). Distribution of indole-3-acetic acid and the occurrence of its alkali-labile conjugates in the extraxylary region of *Pinus sylvestris* stems. *Plant Physiol* 93:1295-1302.

Sundberg B, Tuominen H, Little CHA (1994). Effects of the indole-3-acetic acid (IAA) transport inhibitors N-1-naphthylphthalamic acid and morphactin on endogenous IAA dynamics in relation to compression wood formation in 1-year old *Pinus sylvestris* L. shoots. *Plant Physiol* 106:469-476.

Svačinová J, Novák O, Plačková L, Lenobel R, Holík J, Strnad M, Doležal K (2012). A new approach for cytokinin isolation from Arabidopsis tissues using miniaturized purification: pipette tip solid-phase extraction. *Plant Methods* 8:17.



Telewski FW (1995). Wind-induced physiological and developmental responses in trees. In: Coutts MP, Grace J eds. Wind and trees. Cambridge University Press, Cambridge, UK, pp 237-263.

Telewski FW (2006). A unified hypothesis of mechanoperception in plants. *Am J Bot* 93:1466-1476.

Telewski FW, Jaffe MJ (1986a). Thigmomorphogenesis: anatomical, morphological and mechanical analysis of genetically different sibs of *Pinus taeda* in response to mechanical perturbation. *Physiol Plant* 66:219-226.

Telewski FW, Jaffe MJ (1986b). Thigmomorphogenesis: field and laboratory studies of *Abies fraseri* in response to wind and mechanical perturbation. *Physiol Plant* 66:211-218.

Telewski FW, Pruyn ML (1998). Thigmomorphogenesis: a dose response to flexing in *Ulmus americana* seedlings. *Tree Physiol* 18:65-68.

Timell TE (1969). The chemical composition of tension wood. *Svensk Papperstid* 72:173-181.

Timell TE (1986). Compression wood in gymnosperms. Springer-Verlag, Heidelberg, Vol 2, pp 983-1262.

Trupiano D, Di Iorio A, Montagnoli A, Lasserre B, Rocco M, Grosso A, Scaloni A, Marra M, Chiatante D, Scippa GS (2012b). Involvement of lignin and hormones in the response of woody poplar taproots to mechanical stress. *Physiol Plant* 146:39-52.

Trupiano D, Rocco M, Renzone G, Scaloni A, Viscosi V, Chiatante D, Scippa GS (2012a). The proteome of *Populus nigra* woody root: response to bending. *Ann Bot* 110:415-432.

Trupiano D, Yordanov Y, Regan S, Meilan R, Tschaplinski T, Scippa GS, Busov V (2013a). Identification, characterization of an AP2/ERF transcription factor that promotes adventitious, lateral root formation in *Populus*. *Planta* 238:271-282.



Trupiano D, Rocco M, Renzone G, Scaloni A, Montagnoli A, Terzaghi M, Di Iorio A, Chiatante D, Scippa GS (2013b). Poplar woody root proteome during the transition dormancy–active growth. *Plant Biosyst* 147:1-6.

Trupiano D, Rocco M, Renzone G, Scaloni A, Rossi M, Viscosi V, Chiatante D, Scippa GS (2014). Temporal analysis of poplar woody root response to bending stress. *Physiol Plant* 150:174-193.

Tuskan G, Di Fazio A, Jansson S, *et al.* (2006). The genome of black cottonwood, *Populus trichocarpa*. *Science* 313:1596-1604.

Tuteja N, Gill SS (2013). Plant acclimation to environmental stress. Springer Science, NY.

Wang W, Vinocur B, Altman A (2003). Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta* 218:1-14.

Xie Q, Frugis G, Colgan D, Chua NH (2000). *Arabidopsis* NAC1 transduces auxin signal downstream of TIR1 to promote lateral root development. *Genes Dev* 14:3024-3036.

Yoshida K, Imaizumi N, Kaneko S, Kawagoe Y, Tagiri A, Tanaka H, Nishitani K, Komae K (2006). Carbohydrate-binding module of a rice endo- $\beta$ -1,4-glycanase, *OsCel9A*, expressed in auxin-induced lateral root primordia, is post-translationally truncated. *Plant Cell Physiol* 47:1555-1571.

Yoshizawa N, Satoh M, Yokota S, Idei T (1993). Formation and structure of reaction wood in *Buxus microphylla* var. *insularis* Nakai. *Wood Sci Technol* 27:1-10.

Zhang JZ, Creelman RA, Zhu JK (2004). From laboratory to field. Using information from *Arabidopsis* to engineer salt, cold, and drought tolerance in crops. *Plant Physiol* 135:615-621.

Zhang Z, Zhang X, Wang S, Xin W, Tang J, Wang Q (2013). Effect of mechanical stress on cotton growth and development. PLoS ONE 8(12):e82256. doi:10.1371/journal.pone.0082256.

## 2 Objectives and papers

The aim of the research reported in this doctoral thesis was to deepen the knowledge on woody root biology with a particular focus on the mechanisms regulating the responses to mechanical stress.

Previous studies showed that a bent *Populus nigra* woody root is subjected to tension and compression mechanical forces that distributed differently along the taproot and between the convex and concave side. This different mechanical forces distribution resulted to be associated with specific responses such as lateral roots emission and reaction wood formation in an asymmetric manner.

Based on these evidence, in this thesis, it has been hypothesized that the type (tension and compression) and the intensity of mechanical forces play a pivotal role in activating specific cambium cells signal transduction pathways which in turn, lead their different activity and differentiation.

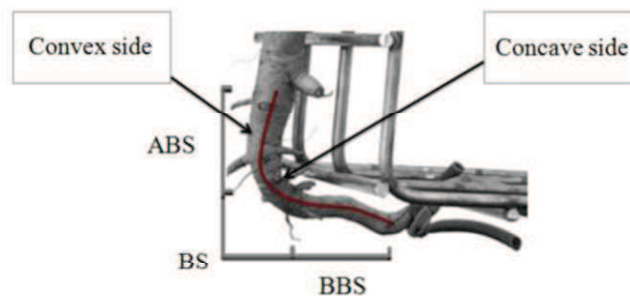
To verify this hypothesis, detailed goals of the project were:

- To analyze along the bent poplar root morphological and anatomical changes induced by different intensities of tension and compression forces, investigating the role of hormones and molecular factors (paper I).
- To investigate, through the analysis of the metabolome profile, the role of the main hormones (IAA, ABA, CKs) in the bent woody root, correlating data to those of bent poplar stem and focusing on asymmetric responses at the base of the reaction wood production (paper II).
- To develop a method for cambium cells isolation from the convex and concave sides of the bent woody root, with a consequent determination of tissue-specific hormones profiling in order to investigate their role in meristematic cells initiation and differentiation in response to mechanical stress (paper III).

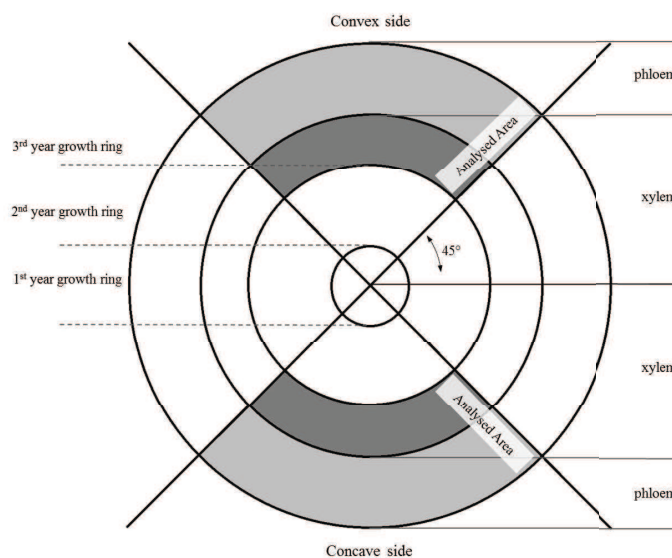
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## Supplementary information

**Figure 1S. Simulation of mechanical bending stress in *Populus nigra* root.** The roots were linked to steel nets curved at a right angle (90°). Three different regions of the bent root were defined during sampling, which occurred six months after treatment: the “Above Bending Sector” (ABS); the “Bending Sector” (BS) and the “Below Bending Sector” (BBS). Each region (5 cm long) was further divided into two sections, corresponding to the convex and the concave side of the main root. The red line shows the main root axis that separated convex and concave side of the root.



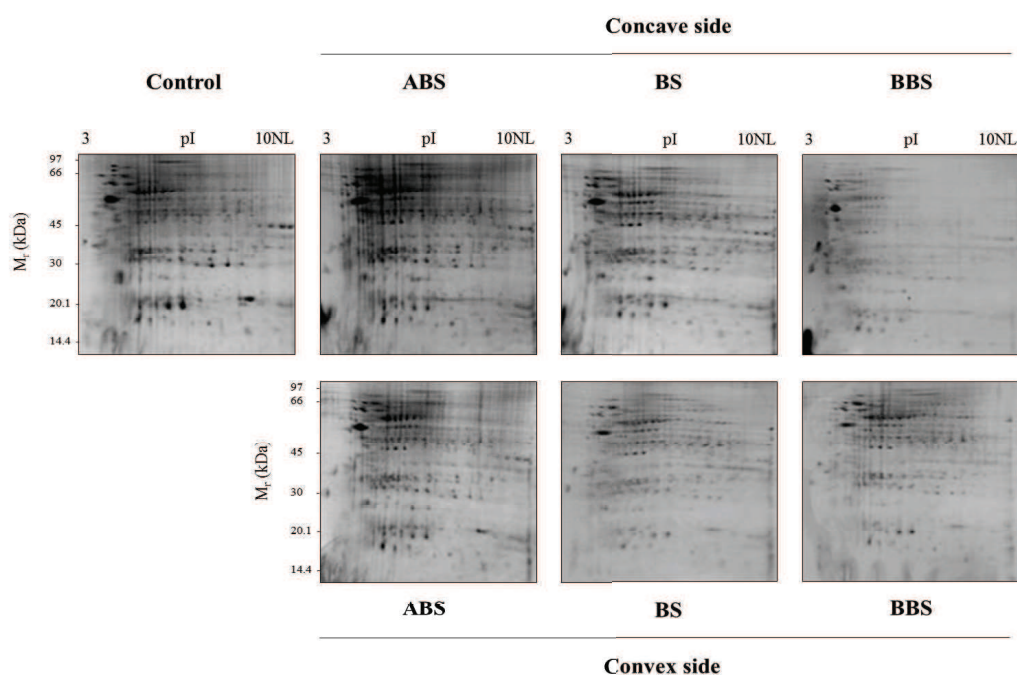
**Figure 2 S. Anatomical measurements of *Populus nigra* bent root.** Cross-sections of control and three bent root sectors (ABS, BS and BBS) were divided in four slices by a cross originating in the middle of the primary xylem stele. The reference cross was rotated of 45° in respect of the line (dashed line) dividing the root section in convex and concave sides. Measurements were done in the first ring of growth after bending application (dark grey areas, XAA: xylem analyzed area; light grey area, PAA: phloem analyzed area).



**Figure 3S. ACO sequences.** Sequence (Potri.006G151600) was obtained cloning RT-qPCR product in the pGem-T Easy Vector system I (Promega, Milan, Italy). Plasmids were isolated from individual colonies and sequenced by BMR genomics (<http://www.bmr-genomics.it/>). The obtained sequence was matched and identified in Phytozome V.9 database (<http://www.phytozome.org>).

<p><b>Potri.006G151600</b></p> <pre> GGGATCTTTATCCATCCTCCATCGTTTGAGAATTCAAGACCTGGGACATGTTTCATCTTGGAGCAAGAGTATGATTC CGCCGGCATCGGTGTGCTCTCGGAGTCCTCTCACAAGCTCTGGCCGAGGACATTCAGGGTATTTTGCCACCTTTGT TCCCACAGAAGGACCTTTAGCTCCTGAAAACGATTCCCTTTATGTAATCCTTGTCCAGTCCAAGATTCTCAGACATG AGTTGAGAAAAGCTTCTCTGCTAACTGAATCAGTTGGGCGATGTACTCATCCATTACTTTGCGAAGATTCTCCGAGA GGTTGTGGATTTCAATTGATGTTGGATGTTGGGCGATGCCAAATAAAGAAACTGCTTCCAGTCTACGTCTCCTTTA TTCATCAAGCCTTTAGCTATCTCCGACTCGTAAAAGCTTTCTTTCAAGTTTTCCCTCGTAGTGTGCATTACCAATTG CTTACCTTGCCATCAGCTTCTTCTCGAGTCCATGGTTTCTCAACCTGAA </pre>
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**Figure 4S. Two-dimensional proteome maps of *Populus nigra* woody taproots in control and mechanical stress conditions.** IEF was performed with non-linear pH 3-10 IPG strips, followed by SDS-PAGE on 12% polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue G-250 and images were analyzed using the PDQuest software. The maps present an average of about 197-383 proteins, ranging in  $M_r$  values from 97 to 14.4 (kDa). Panels show maps of control and the convex and concave sides of each bent sectors (ABS, BS, BBS). ABS: Above Bending Sector; BS: Bending Sector; BBS: Below Bending Sector.





*(Paper in preparation)*

## **MECHANICAL BENDING STRESS IN *POPULUS NIGRA* PLANTS: HORMONES PROFILING IN ROOT AND STEM**

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### **ABSTRACT**

This research is focused on understanding the role of hormones in *Populus nigra* responses to long-term bending stress. We analyzed and compared the response of different sectors of poplar root and stem to mechanical treatment, being interested in the roles played by main plant hormones. In detail, we investigated the auxins metabolite profiling, the cytokinins and abscisic acid content, using a method based on liquid chromatography coupled to tandem mass spectrometry analysis (LC-MS/MS).

We provide the hormonal profile, explaining the differences between the convex and concave side of taproot and stem. An asymmetry of auxin concentration may trigger the reaction wood formation in poplar root after bending stress, while any asymmetrical distribution of auxin seems to be at the basis of tension wood formation in the stem. Changes in IAA conjugation and degradation pathways seems to be involved in reaction wood formation as well.

Key words: bending stress, auxins, cytokinins, metabolite profiling, LC-MS/MS.



Abbreviations: ABA, abscisic acid; ABS, above bending sector; BS, bending sector; BBS, below bending sector; CE, concave side; CKs, cytokinins; CW, compression wood; CX, convex side; IAA, indole-3-acetic acid; IAAsp, IAA-aspartate; IAGlu, IAA-glutamate; oxIAA, 2-oxindole-3-acetic acid; TW, tension wood.

## INTRODUCTION

Mechanical stimuli (e.g. rain, wind, gravity, impedance, slope of soil, wounding, bending) can considerably influence plant growth and development. To optimize its anchorage and survival, a plant responds with physiological, morphological, biomechanical and molecular adaptations that, in some cases, can be quite noticeable (Jaffe and Forbes, 1993). Bending stresses experienced by a tree may involve both root system and aerial parts of the plant. Branches and trunks subjected to bending tend to reduce their elongation (Coutand and Moulia, 2000) and to increase their radial growth and their elastic resilience (Coutand *et al.*, 2009). Bending at root level leads to alterations of tissue architecture (Nicoll and Ray, 1996; Chiatante *et al.*, 2003) and mechanical properties (Goodman and Ennos, 1998).

Progress has been made in the understanding of the physiological, molecular and biochemical basis of woody root response to mechanical stress (Scippa *et al.*, 2008; Trupiano *et al.*, 2012a, b; Trupiano *et al.*, 2013a), which have highlighted some similarities with herbaceous species. Indeed, similar to *Arabidopsis thaliana*, in poplar trees bending of roots causes the initiation of lateral root primordia toward the convex side (Ditegou *et al.*, 2008; Monshausen *et al.*, 2009; Richter *et al.*, 2009; Trupiano *et al.*, 2012b). However, differently from herbaceous species, compression forces can also elicit reaction wood formation in the concave side of the bent roots (De Zio *et al.*, 2016).

Reaction wood possesses altered properties and forms as part of a developmental process important to re-orient a plant in response to a gravistimulus. In gymnosperms it is referred to as compression wood (CW) and develops on the lower side of leaning stems or branches, whereas, in dicotyledonous angiosperms, it forms on the upper side and is called tension wood (TW; Du and Yamamoto 2007). Interestingly, in *Populus* roots a CW

similar to that observed in gymnosperm stems forms after bending (De Zio *et al.*, 2016).

The anatomical and biochemical features of reaction wood in woody stems and roots have been the subject of several studies, and although the physiological and molecular signals inducing the formation of reaction wood remain poorly described, evidence on the involvement of plant hormones have been provided (for review, see Elo *et al.*, 2009; Nieminen *et al.*, 2012; Ursache *et al.*, 2013; Trupiano *et al.*, 2012b; De Zio *et al.*, 2016).

The functional role of auxin in plant response to mechanical stress has been an active area of research on woody stems. However, findings on the relationship between endogenous auxin levels and the formation of CW or TW are still scarce and sometimes contradictory and remain to be elucidated (Du and Yamamoto, 2007). Indeed, Hellgren *et al.* (2004) found that the formation of tension and compression wood in poplar and pine bent stems is not mediated by changes in the indole-3-acetic acid (IAA) level in the cambial tissues, whereas Funada *et al.* (1990) and Du *et al.* (2004) detected higher amount of endogenous IAA at the side of the cambial region forming CW. In the case of poplar bent roots, in a recent work we found that wood formation is induced by compression forces and mediated by high levels of IAA (De Zio *et al.*, 2016).

Since the role of auxin in reaction wood formation has been demonstrate essentially by indirect investigations, applying exogenous auxins, auxin regulators or inhibitors (Hejnowicz and Tomaszewski, 1969; Fagerstedt *et al.*, 2016), and few studies analyzed the IAA endogenous level within the reaction wood (Du and Yamamoto, 2007) a documentation of auxin concentration and auxin metabolism *in planta* in the reaction wood-forming tissue resulted necessary, especially at root level.

In this study, a recent developed plant hormone profiling method (Novák *et al.*, 2012) was used, to unravel the crosstalk between hormones and broaden the knowledge of the hormonal regulation at the basis of woody plant bending stress and reaction wood formation. This method offers the possibility to know endogenous levels of IAA, abscisic acid (ABA) and cytokinins (CKs) and, simultaneously, to know the degree of regulation of IAA, through the quantification of auxin amino acid conjugates, IAAsp (IAA-aspartate) and

IAGlu (IAA-glutamate), and principal auxin degradation product, the 2-oxindole-3-acetic acid (oxIAA, Ljung *et al.*, 2002; Novák *et al.*, 2008, 2012). In many species and plant tissues, mechanisms such as conjugation with amino acids or degradation are essential to convert the auxin active form to the inactive form, maintaining its appropriate homeostasis at cellular level (Tran and Pal, 2014). IAA metabolism (biosynthesis, conjugation/deconjugation and degradation) and IAA transport between tissues and cell compartments define the net concentration of auxin within a plant cell. Environmental mechanical stimuli can considerably influence the rate of removal or release of free auxin in the cells, as well as the content of other hormones. There are evidences about cytokinins (CKs) mechanically induced stress responsiveness (Sanyal and Bangerth, 1998), and about the role of abscisic acid (ABA) in responses to mechanical stimuli (Jaffe, 1980; Scippa *et al.*, 2008; Trupiano *et al.*, 2012b; De Zio *et al.*, 2016), although their role in reaction wood formation remains to be elucidated (Du and Yamamoto, 2007). In view of this, here we provide a detailed hormones profiling considering different sectors and sides (convex and concave) of poplar bent roots and stems in relation of the different intensity of tension and compression forces perception during the bending stress.

## **MATERIALS AND METHODS**

### ***Plant material and bending conditions***

Long-term bending stress was applied to four years-old *P. nigra* plants. The root bending simulation was performed tying taproots around right angle curved steel nets, as previously described in Scippa *et al.* (2008), Trupiano *et al.* (2012a) and De Zio *et al.* (2016). The same bending angle ( $\sim 90^\circ$ ) and similar supporters were used to impose the stress at stem level of other poplar plants. Differently, control plants were linked to vertical steel nets localized at root or stem level. All plants were grown in a growth chamber for five months under temperature and water controlled conditions, using LED lights ( $\lambda_{420}$  -  $\lambda_{740}$ ) to emulate a natural photoperiod.

After the treatment, control samples collection was made cutting randomly: (i) a zone between 12-27 cm from the root collar zone (equivalent of stem

base) downward (root control); (ii) a zone between 12-27 cm from the root collar zone upward (stem control).

A detailed spatial analysis was performed in the case of bent samples. Firstly, three transversal sectors, each 5 cm long, were taken in the root as like as in the stem: the Above Bending Sector (ABS), corresponding to the region just above the bending zone; the Bending Sector (BS), representing the point of maximum bending and the Below Bending Sector (BBS), corresponding to the region just below the bending zone. In the case of bent roots, ABS was localized at 12-17 cm distant from the root collar, BS at 17-22 cm and BBS at 22-27 cm. In the case of bent stems, ABS was localized at 22-27 cm distant from the root collar (stem base), BS at 17-22 cm and BBS at 12-17 cm.

Secondly, each of these regions (ABS, BS and BBS) was further divided lengthwise into two parts to collect both the convex (CX) and concave side (CE) [Fig. 1 Supplementary information]. Samples were frozen in liquid nitrogen and stored at -80°C for successive analysis.

### ***Auxins, abscisic acid and cytokinins purification***

For the simultaneous purification of auxins, ABA and CKs, we used a modified Dobrev and Kamínek (2002) method. Briefly, 20 mg of fresh weight of plant material was grounded in liquid nitrogen and mixed with 1 mL of cold extraction mixture of methanol/water/formic acid (modified Bielecki buffer - 15/4/1, v/v/v). Labeled internal standards (Olchemim Ltd, Czech Republic) were supplemented in this first extraction step. After adding 3 ceramic beads, the samples were homogenized using a MixerMill MM 301 bead mill (Retsch) at a frequency of 25 Hz for 8 min, incubated for 10 min at 4°C with continuous shaking, and centrifuged for 15 min, 14000 rpm at 4°C. Supernatants were reconstituted in 7 mL of 1 M formic acid and purified by solid-phase extraction (SPE) on MCX 1cc/30mg columns (Oasis Extraction Cartridges - Waters). Columns were conditioned with methanol and water, equilibrated with 1 mL 50% (v/v) nitric acid, 2 mL of water, and 1 mL of 1 M formic acid. After samples application, the columns were washed with 1 mL of 1 M formic acid and eluted with following order of solutions: 1 mL of methanol (to collect the auxin and ABA fraction); 1 mL of 0.35 M ammonium hydroxide and 2 mL of 0.35 M ammonium hydroxide in 60% (v/v) methanol solution (to collect the cytokinin fraction). The eluates were vacuum-dried

using a SpeedVac concentrator, dissolved in 40  $\mu$ L of 10% methanol and stored at -20°C until mass analysis.

### ***UHPLC-MS/MS method***

The ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) was used to analyze the auxins metabolites and detect ABA and CKs. Separation and determination of compounds was performed using a 1290 Infinity LC system and 6490 Triple Quadrupole LC/MS system (Agilent Technologies). Auxins mass analysis was done according to Novák *et al.*, (2012), with subsequent ABA detection, while CKs mass analysis was carried out in accordance with Novák *et al.*, (2008). IAA metabolites and ABA were expressed as  $\text{pg mg}^{-1}$  of fresh weight, while CKs as  $\text{pmol g}^{-1}$  of fresh weight. MassHunter software (version B.05.02; Agilent Technologies) was used to determine the concentrations of all examined compounds using stable isotope dilution.

### ***Statistical analysis***

When needed, variables were square root or log transformed to ensure normal distributions and equal variances for the use of parametric statistics. For each variable, a one-way ANOVA was used to compare different plant compartments (root and stem), regions (ABS, BS, BBS) and sides (CX and CE). Post-hoc LSD-tests were conducted to detect overall differences between convex and concave sides for each region of each plant compartment. Analyses were applied on a 95% significance level. Statistical analysis was carried out using statistical software package SPSS 17.0 (SPSS Inc, Chicago IL, USA).

## **RESULTS AND DISCUSSION**

### ***IAA metabolites response to bending***

Based on isotope feeding experiments and a sensitive mass spectrometry-based method we identified and quantified the major primary IAA catabolite, oxIAA and the conjugates IAGlu and IAAsp, in *Populus* woody root and stem subjected to long-term bending stress.

In order to ascertain the root/stem zones involved in the major contribution to the degradation and conjugation of IAA, we analyzed the concentration of the hormone and its metabolites along and across the main root and stem, focusing our attention on two aspects: i) differences and similarities between the two tissues analyzed; ii) characterization of the asymmetric response between the concave and convex side from each tissue.

As regards root tissue, in a previous study we showed that bending stress in *P. nigra* induces drastic changes in cells shape and tissues organization (De Zio *et al.*, 2016). Stretched and compressed cells, distributed in the convex and concave side, respectively, perceive differently tensile and compression forces along the taproot and this results in an asymmetric distribution of lateral roots and reaction wood formation in the main root (De Zio *et al.*, 2016).

In accordance with previous observations, here we showed that in poplar root the concentration of IAA was higher in the concave side of BS and BBS regions, compared to the opposite side, confirming, once again, the presence of an asymmetry in these root zones. This asymmetry was greater in BBS, with the highest IAA value in BBS concave side (Fig. 1D).

In De Zio *et al.*, (2016) has been hypothesized, that the stress-related anatomical changes, expressed through reaction wood development in the concave side of the main root [**Fig. 2 Supplementary information**], could be due to an increase of cambial activity, associated with an increase of auxin level in that side. The role of IAA in the regulation of cambial growth (Little and Pharis, 1995; Sundberg *et al.*, 2000) has been well established; furthermore, there are evidence of the IAA role in the regulation of reaction wood formation (Funada *et al.*, 1990; Sundberg *et al.*, 1994; Du *et al.*, 2004). However, most of the information regarding the reaction wood development comes from experiments involving applications of exogenous IAA or IAA-transport inhibitors at stem level.

In general, it is known that the reaction wood is induced by differential endogenous IAA levels in both gymnosperm and angiosperm tree species (Hellgren *et al.*, 2004). A widely accepted model suggests that the TW requires a difference in auxin concentration around the stem and forms in the region deficient in IAA, whereas CW is induced by an increase of auxin concentration (for review, see Timell, 1986; Little and Savidge, 1987;



Srivastava, 2002). Accordingly with this model, we found a reduction of IAA in the upper portion of the stem (ABS region), where TW formed (for more details see later and Fig. 1D).

Compared to the control, IAA catabolism, measured through the accumulation of oxIAA, in the root is induced in the point of maximum bending (BS region, Fig. 1A), but without any differences between the two sides of this region. This result seems to indicate that the asymmetrical IAA reduction in BS convex side may not be provided by the increase of IAA catabolism, but rather caused by asymmetrical decrease of IAA biosynthesis, considering that conjugation processes were also affected in that root zone (BS convex side).

The strong reduction of IAA observed in BBS convex side of the root was not accompanied by a high level of oxIAA catabolite, although, also in this sector the conjugations with glutamate and aspartate were affected (Fig. 1).

Although the IAA biosynthesis pathways in *Populus* have so far not been well elucidated (Vayssières *et al.*, 2015), it has been reported that oxIAA can be induced by IAA biosynthesis (Band *et al.*, 2012), and that the accumulation of oxIAA, is a typical response to high IAA levels, as observed in IAA-overproducing mutants (Novák *et al.*, 2012; Pencik *et al.*, 2013).

It has been demonstrated that localized auxin accumulation increases both ROS and oxIAA. Furthermore, oxIAA seems not to be transported from cell to cell, although it appears to be a substrate for the ATP-binding cassette subfamily G (ABCG) transporters, positioned primarily on the outer lateral surface of the root epidermis (Peer *et al.*, 2013). Several lines of evidence suggest a relationship between IAA oxidation and cellular redox status. For instance, Peer *et al.*, (2013) demonstrated that flavonoid mutants with altered ROS scavenging present high oxIAA levels.

Richter *et al.*, (2009) and Monshausen *et al.*, (2009) demonstrated the importance of Ca<sup>2+</sup> and ROS in bent *Arabidopsis* root for the lateral roots emission in the convex side. Therefore, knowing that ROS represented an important signal for lateral roots emission and that auxin maximum correlates with a change in redox status, as oxidation increases where auxin level is high, we might hypothesize that the asymmetrical increase of oxIAA in ABS convex side, could be strongly associated with lateral roots emission. In this

particular region of the bent root, in fact, was previously found a greater lateral roots emission (Trupiano *et al.*, 2012b).

Interesting, the higher content of IAA in BS root concave side was accompanied by an increase of IAGlu conjugate in respect to the control and the opposite site (Fig. 1C). An asymmetry about IAGlu content was also found in ABS, whit the highest value in the convex side, also characterized by a high level of oxIAA.

Contrary to IAGlu conjugate, IAAsp content was reduced in all stressed sectors of the root compared to the control, except for ABS convex side and BBS concave side, which showed similar values to the control and higher values compared to the opposite side.

In our measurements, IAAsp and IAGlu conjugates were detected in the stressed samples as like as in the root control, suggesting that IAA conjugation occurs during *Populus* root development and is not inhibited, although reduced in some root zones, by the mechanical stress. Normally, IAA amino acid conjugates are present in much lower quantities compared to oxIAA (Pencík *et al.*, 2013; Vayssières *et al.*, 2015). In the root, we noticed, in fact, a concentration range equal to  $\sim 100\text{-}300\text{ pg mg}^{-1}$  of FW for oxIAA,  $\sim 9\text{-}25\text{ pg mg}^{-1}$  of FW for IAGlu and  $\sim 7\text{-}15\text{ pg mg}^{-1}$  of FW for IAAsp.

The auxin conjugation pathway is differentially regulated in various tissues and during plant development, upon abiotic and biotic stresses, and can be activated in response to exogenous auxin (Ljung *et al.*, 2013). As IAA degradation pathway, conjugation can be induced by high levels of auxin (Pencík *et al.*, 2013).

Concerning stress-tolerance roles, it has been demonstrated the involvement of IAA metabolites in the adaptation to high salinity in poplar plant (Junghans *et al.*, 2006), in pathogen infection responses (Ludwig-Müller, 2011) and in the interaction plant-rhizosphere (Vayssières *et al.*, 2015). The formation of IAA conjugates may serve other functions as well, including storage and protection against oxidative degradation (Tran and Pal, 2014).

In rice, has been demonstrated that overexpression of *GH3-8* recombinant auxin-responsive gene, encoding IAA-amino acid synthetase, promotes IAAsp conjugates formation and enhance resistances to pathogens, reducing the auxin-induced cell wall loosening, through the suppression of expansin genes (wall loosening proteins; Ding *et al.*, 2008).



Furthermore, there is evidence that IAAsp is induced after exogenous application of IAA, showing a function in adventitious root formation in cuttings of *P. tremula* (Plüss *et al.*, 1989). The percentage of adventitious root formation in *Betula pendula* seems to respond to IAAsp conjugate as well, and seems to be correlated with gravitropism of the stem (Makhnev *et al.*, 2012).

In light of the above knowledge, we can suppose that the increase of IAGlu in BS root concave side could be related with the signaling that regulate the lignin deposition and the stabilization/thickening of cell wall, in this region, where compression forces triggered CW formation, characterized by high lignin content (De Zio *et al.*, 2016). However, considering the complexity of auxin pathway and the limited amount of information about root bending-auxin metabolism this hypothesis requires further investigation.

As mentioned earlier, in the stem, our results revealed a reduction of IAA content compared to control only in ABS region, but any statistical difference was found between the concave and convex sides (Fig. 1D).

Hellgren *et al.* (2004) found that the formation of TW and CW in poplar and pine bent stems is not mediated by changes in the IAA level in the cambial tissue, but there are several evidences that TW forms in the region deficient in IAA (for review, see Timell, 1986; Little and Savidge, 1987; Srivastava, 2002).

According to these observations, we found a TW [**Fig. 2 Supplementary information**], characterized by high levels of carbohydrates and low lignin content (data not showed), in the upper region of the bent stem, ABS, and towards the convex side, where the levels of IAA measured were lower than the other regions of the stem.

Furthermore, results showed a reduction of oxIAA content compared to the control in all convex side of the stem. The decrease of oxIAA in BBS convex side of the bent stem is significant compared to the control but also to the corresponding opposite side (Fig. 1A).

Interestingly, in the stem, IAAsp conjugate was detected only in ABS region, with a lower level in the convex side (Fig. 1B). The absence of IAAsp in the control and in the other bent sectors suggests that in the stem IAA balance is regulated mainly by IAA biosynthesis and degradation. The IAAsp absence in BS and BBS regions could be associated with a positive regulation of stem

elongation process; in fact, there are evidences that members of the *GH3* auxin-responsive gene family, involved in auxin signal transduction by IAAsp production, negatively regulate shoot cell elongation (Nakazawa *et al.*, 2001). This result could be also correlated with the high level of CKs (known to be involved in stem elongation) in BS region (Makhnev *et al.*, 2012).

In this regard, however, it cannot be ruled out that the presence of IAAsp conjugates exclusively in ABS region is connected with the levels of IAGlu: below the detection limit in ABS convex side and reduced in ABS concave side compared to the stem control.

As concerns IAGlu conjugate, a reduction was also found in the concave side of BS, significant compared to the stem control and the corresponding opposite side. Interestingly, IAGlu in BS concave side showed an opposite trend between root and stem (Fig. 1C).

### ***ABA and CKs response to bending***

In order to ascertain the contributors of other important plant hormones in the response of *Populus* to bending stress, we analyzed the content of ABA and CKs as well, in all bent sectors of the root and stem (Fig. 2). Surprisingly, our results showed that in the bent root ABA levels were not higher than control, nevertheless, a decrease of this hormone was found in BS and BBS convex side in respect to the control and the opposite concave side (Fig. 2A). This result appears to be contradictory, since various stresses, including drought, salinity, cold, induce ABA synthesis (Zhu, 2002; Mahajan and Tuteja, 2005; Swamy *et al.*, 1999; Verma *et al.*, 2016). Nevertheless, in the stressed samples, the most significant differences in ABA distribution were observed between the two sides of BS and BBS regions, according with previous results (De Zio *et al.*, 2016), confirming a potential role in counteracting the deformation of conduits and changes in hydraulic conductivity, related with the high compression forces perception and the CW formation (Trupiano *et al.*, 2012b; Mayr and Cochard, 2003).

Similarly to ABA distribution, we observed a reduction of total CKs in all stressed samples compared to root control (Fig. 2B). Root represents an important source of CKs that are required for normal plant growth and development, but it was recently demonstrated that CKs can regulate

negatively some abiotic stress responses. Nishiyama *et al.*, (2011) showed as in salt and drought stress conditions, plants with reduced CKs activity exhibited a strong stress-tolerant phenotype, associated with an increase of cell membrane integrity and ABA hypersensitivity.

Based on the above, we could hypothesize that also in mechanical stress condition there is a mutual regulation between CKs and ABA, aimed at raising the adaptation to the stress through the regulation of cell elasticity and integrity, particularly important in presence of strong curvature.

Contrary to the bent root, the bending in the stem determined a significant increment of CKs in ABS convex side and BS region compared to the control, the variation in ABS convex side was significant also in respect to the opposite concave side (Fig. 2B). The high CKs content in BS region could be related with well-established function of CKs in cell division, tracheid differentiation and stem elongation (Little and Savidge, 1987; Aloni *et al.*, 2006; Simm *et al.*, 2016; Kieber and Schaller, 2014; Paul *et al.*, 2016). In the literature, there are examples of increased CKs activity in xylem exudate in plants subject to constant shaking stress (Beyl and Mitchell, 1983). So far, any direct relation was found for CKs and ABA with reaction wood formation, even if, it was reported an increase of kinetin, a particular type of cytokinin, in TW in IAA-treated stems (Blum, 1971), and a localization of kinetin in the upper side of horizontally placed willow stems (Wareing, 1970; Lepp and Peel, 1971). Taking into account the CKs asymmetrical distribution in ABS, we could hypothesize a possible role of CKs in TW formation after bending imposition in *Populus*.

As in the case of the root, the bending in the stem may induce a water stress condition. Parkhurst *et al.*, (1972) observed cavitation and conductance losses in the xylem of bent stem, resulting also in a decline of transpiration rates. In our experiment, BBS region showed lower levels of ABA, and this can be related with a lower perception of mechanical forces in that stem zone. Surprisingly, we detected a low ABA level in ABS concave side as well, significant in respect to the control and the corresponding convex side (Fig. 2A). This result is not easily explained, considering the curvature influence and the intensity of compression forces in that stem zone. Therefore the asymmetrical distribution of ABA in ABS region must be clearly considering also, the ABA interaction with IAA in xylem differentiation (Sundberg *et al.*,

2000; Mellerowicz *et al.*, 2001; Muday and DeLong, 2001), or its role in the regulation of cambial activity associated with the reaction wood formation (Aloni, 2007).

### ***Differences of woody root and stem in response to bending***

Valuating the differences in the bending response between the two different tissues analyzed, it appeared that the most important changes concerned amino acid conjugates concentrations. In fact, IAAsp in the stem was detected just in ABS region, while in the root tissue this conjugate has been measured in all bent sectors and in the control. In the stem, contrary to IAAsp, the conjugation with glutamate was not affected by the bending stress, and the IAGlu levels resulted higher than root ranges, except for the differences observed in ABS region. The levels of oxIAA in the bent stem were higher compared with the same sector of the root, excepted for the convex side of ABS and BS, where any statistical difference in oxIAA content was found between the two tissues.

Despite we did not find significant variations in IAA levels between control root and control stem, results showed that bending affected IAA concentration in an opposite manner between the two tissue in the different regions: ABS showed lower levels in the stem, while BS region and BBS convex side lower levels in the root.

In addition, we found higher ABA levels in all stem samples compared to root samples and a high value of CKs in BS convex side of the stem compared with the same sector of the root, while between the two controls, a high value of CKs was detected in the root.

## **CONCLUSIONS**

In this study we investigated the influence of mechanical forces on *Populus* root and stem, providing new data on hormonal profiles in the convex and concave side of the bent plants.

In particular, we analyzed how auxin metabolism is affected by the bending and by different tension and compression forces intensities.

Metabolic profiling showed great variation of IAA levels in stressed samples between root and stem tissues and noticeable changes in IAA conjugation and

degradation pathways during bending stress, highlighting how the two organs perceive and respond differently to the stimulus.

The confirmed asymmetry of auxin accumulation in the concave side of bent poplar root provides additional evidence for the hypothesis that root responds to the bending producing an IAA-mediated reaction wood at the compressed side. While the reduction of IAA in the upper region of the stem (ABS region) seems to be at the basis of TW formation. In both root and stem IAA signaling is attenuated by IAA catabolism, but so far, IAA catabolic/oxidation pathways in plants has not been well established, making difficult to study the specific regulation and function of IAA catabolism.

According to the current knowledge, this study represent the first approach to quantify the major known IAA metabolites in *P. nigra* root and stem subjected to mechanical bending stress. However, understanding of processes, such conjugation or degradation, and their relative importance in different root/stem zones remains rudimentary and needs to be further analyzed in depth.

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## LITERATURE CITED

Aloni R (2007). Phytohormonal mechanisms that control wood quality formation in young and mature trees. In: Entwistle K, Harris P, Walker J, eds. The compromised wood workshop. Christchurch, New Zealand: The wood technology research centre, University of Canterbury, pp 1-22.

Aloni R, Aloni E, Langhans M, Ullrich C (2006). Role of cytokinin and auxin in shaping root architecture: regulating vascular differentiation, lateral root initiation, root apical dominance and root gravitropism. *Ann Bot* 97:883-893.

Band LR, Wells DM, Larrieu A, Sun J, Middleton AM, French AP, Brunoud G, Sato EM, Wilson MH, Péret B (2012). Root gravitropism is regulated by a transient lateral auxin gradient controlled by a tipping-point mechanism. *Proc Natl Acad Sci USA* 109:46668-4673.

Beyl CA, Mitchell CA (1983). Alteration and growth, exudation rate, and endogenous hormone profiles in mechanically dwarfed sunflower. *J Amer Soc Hort Sci* 108:257-262.

Blum W (1971). Über die experimentelle Beeinflussung der Reaktionsholzbildung bei Fichten und Pappeln. *Ber Schweiz Bot Ges* 80:225-251.

Chiatante D, Scippa GS, Di Iorio A, Sarnataro M (2003). The influence of steep slope on root system development. *J Plant Growth Regul* 21:247-260.

Coutand C, Martin L, Leblanc-Fournier N, Decourteix M, Julien JL, Moulia B (2009). Strain mechanosensing quantitatively controls diameter growth and PtaZFP2 gene expression in poplar. *Plant Physiol* 151:223-232.

Coutand C, Moulia B (2000). Biomechanical study of the effect of a controlled bending on tomato stem elongation: local strain sensing and spatial integration of the signal. *J Exp Bot* 51:1825-1842.

De Zio E, Trupiano D, Montagnoli A, Terzaghi M, Chiatante D, Grosso A, Marra M, Scaloni A, Scippa GS (2016). Poplar woody taproot under bending stress: the asymmetric response of the convex and concave sides. *Ann Bot* 118:865-883.

Ditengou FA, Teale WD, Kochersperger P, Flittner KA, Kneuper I, van der Graaff E, Nziengui H, Pinosa F, Li X, Nitschke R, Laux T, Palme K (2008). Mechanical induction of lateral root initiation in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* 105:18818-18823.

Ding X, Cao Y, Huang L, Zhao J, Xu C, Li X, Wang S (2008). Activation of the indole-3-acetic acid-amido synthetase *GH3-8* suppresses expansin expression and promotes salicylate- and jasmonate-independent basal immunity in rice. *Plant Cell* 20:228-40.

Dobrev PI, Kamínek M (2002). Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed-mode solid-phase extraction. *J Chromatogr A* 950:21-9.

Du S, Uno H, Yamamoto F (2004). Roles of auxin and gibberellin in gravity induced tension wood formation in *Aesculus turbinata* seedlings. *IAWA Journal* 25:337-347.

Du S, Yamamoto F (2007). An overview of the biology of reaction wood formation. *J Integr Plant Biol* 49:131-143.

Elo A, Immanen J, Nieminen K, Helariutta Y (2009). Stem cell function during plant vascular development. *Semin Cell Dev Biol* 20:1097-1106.

Fagerstedt K, Tocquard K, Lopez D, Decourteix M, Thibaut B, *et al.* (2016). The molecular mechanisms of reaction wood induction. *Springer* 4:107-138.

Funada R, Mizukami E, Kubo T, Fushitani M, Sugiyama T (1990). Distribution of indole-3-acetic acid and compression wood formation in the stems of inclined *Cryptomeria japonica*. *Holzforschung* 44:331-334.



Goodman AM, Ennos AR (1996). A comparative study of the response of the roots and shoots of sunflower and maize to mechanical stimulation. *J Exp Bot* 47:1499-1507.

Hejnowicz A, Tomaszewski M (1969). Growth regulators and wood formation in *Pinus silvestris*. *Physiol Plant* 22:5 984-992.

Hellgren JM, Olofsson K, Sundberg B (2004). Patterns of auxin distribution during gravitational induction of reaction wood in poplar and pine. *Plant Physiol* 135:212-220.

Jaffe MJ (1980). Morphogenetic responses of plants to mechanical stimuli or stress. *BioSci* 30:239-243.

Jaffe MJ, Forbes S (1993). Thigmomorphogenesis: the effect of mechanical perturbation on plants. *Plant Growth Regul* 12:313-324.

Junghans U, Polle A, Düchting P, Weiler E, Kuhlmann B, Gruber F, Teichmann T (2006). Adaptation to high salinity in poplar involves changes in xylem anatomy and auxin physiology. *Plant Cell Environ* 29:1519-1531.

Kieber JJ, Schaller GE (2014). Cytokinins. *Arabidopsis Book* 12:e0168.

Little CHA, Pharis RP (1995). Hormonal control of radial and longitudinal growth in the tree stem. In: Gartner BL, ed, *Plant stems: physiology and functional morphology*. Academic Press, San Diego, CA, pp 281-319.

Lepp NW, Peel AJ (1971). Distribution of growth regulators and sugars by the tangential and radial transport systems of stem segments of willow. *Planta* 99:275-282.

Little CHA, Savidge RA (1987). The role of plant growth regulators in forest tree cambial growth. *Plant Growth Regul* 6:137-169.



Ljung K (2013). Auxin metabolism and homeostasis during plant development. *Development* 140:943-950.

Ljung K, Hull AK, Kowalczyk M, Marchant A, Celenza J, Cohen JD, Sandberg G (2002). Biosynthesis, conjugation, catabolism and homeostasis of indole-3-acetic acid in *Arabidopsis thaliana*. *Plant Mol Biol* 50:309-332.

Ludwig-Müller J (2011). Auxin conjugates their role for plant development and in the evolution of land plants. *J Exp Bot* 62:1757-1773.

Mahajan S, Tuteja N (2005). Cold, salinity and drought stresses: an overview. *Arch Biochem Biophys* 444:139-158.

Makhnev AK, Degtyarev ES, Migalina SV (2012). Intraspecific variability of triterpene content in the leaves of *Betula pendula* Roth. *Contemp Probl of Ecol* 5:179-184.

Mayr S, Cochard H. (2003). A new method for vulnerability analysis of small xylem areas reveals that compression wood of Norway spruce has lower hydraulic safety than opposite wood. *Plant Cell Environ* 26:1365-1371.

Mellerowicz EJ, Baucher M, Sundberg B, Boerjan W (2001). Unravelling cell wall formation in the woody dicot stem. *Plant Mol Biol* 47:239-274.

Monshausen GB, Gilroy S (2009). Feeling green: mechanical signaling in plants. *Trends Cell Biol* 9:228-235.

Muday GK, DeLong A (2001). Polar auxin transport: controlling where and how much. *Trends Plant Sci* 6:535-542.

Nakazawa M, Yabe N, Ichikawa T, Yamamoto YY, Yoshizumi T, Hasunuma K, Matsui M (2001). DFL1, an auxin-responsive GH3 gene homologue, negatively regulates shoot cell elongation and lateral root formation, and positively regulates the light response of hypocotyl length. *Plant J* 25:213-21.

Nicoll BC, Ray D (1996). Adoptive growth of tree root systems in response to wind action and site conditions. *Tree Physiol* 16:891-898.

Nieminen K, Robischon M, Immanen J, Helariutta Y (2012). Towards optimizing wood development in bioenergy trees. *New Phytol* 194:46-53.

Nishiyama R, Watanabe Y, Fujita Y, Le DT, Kojima M, Werner T, Vankova R, Yamaguchi-Shinozaki K, Shinozaki K, Kakimoto T, Sakakibara H, Schmülling T, Tran LSP (2011). Analysis of cytokinin mutants and regulation of cytokinin metabolic genes reveals important regulatory roles of cytokinins in drought, salt and abscisic acid responses, and abscisic acid biosynthesis. *Plant Cell* 23:2169-2183.

Novák O, Hauserová E, Amakorová P, Doležal K, Strnad M (2008). Cytokinin profiling in plant tissues using ultra-performance liquid chromatography-electrospray tandem mass spectrometry. *Phytochemistry* 69:2214-2224.

Novák O, Hényková E, Sairanen I, Kowalczyk M, Pospíšil T, Ljung K (2012). Tissue-specific profiling of the *Arabidopsis thaliana* auxin metabolome. *Plant J* 72:523-536.

Parkhurst DF, Pearman GI, Neel PL, Harris RW (1972). Tree seedling growth: effects of shaking. *Science* 175:918-919.

Paul S, Wildhagen H, Janz D, Teichmann T, Hänsch R, Polle A (2016). Tissue- and cell-specific cytokinin activity in *Populus × canescens* monitored by *ARR5::GUS* reporter lines in summer and winter. *Front Plant Sci* 7:652.

Peer WA, Cheng Y, Murphy AS (2013). Evidence of oxidative attenuation of auxin signaling. *J Exp Bot* 64:2629-2639.

Pencík A, Simonovik B, Petersson SV, Henyková E, Simon S, Greenham K, Zhang Y, Kowalczyk M, Estelle M, Zazimalová E, Novák O, Sandberg G, Ljung K (2013). Regulation of auxin homeostasis and gradients in

*Arabidopsis* roots through the formation of the indole-3-acetic acid catabolite 2-oxindole-3-acetic acid. *The Plant Cell* 25:3858-3870.

Plüss R, Jenny T, Meier H (1989). IAA-induced adventitious root formation in greenwood cuttings of *Populus tremula* and formation of 2-indolone-3-acetylaspartic acid, a new metabolite of exogenously applied indole-3-acetic acid. *Physiol Plant* 75:89-96.

Richter GL, Monshausen GB, Krol A, Gilroy S (2009). Mechanical stimuli modulate lateral root organogenesis. *Plant Physiol* 151:1855-1866.

Sanyal D, Bangerth F (1998). Stress induced ethylene evolution and its possible relationship to auxin-transport, cytokinin levels, and flower bud induction in shoots of apple seedlings and bearing apple trees. *Plant Growth Regul* 24:127-134.

Scippa GS, Trupiano D, Rocco M, Di Iorio A, Chiatante D (2008). Unravelling the response of poplar (*Populus nigra*) roots to mechanical stress imposed by bending. *Plant Biosystems* 142:401-413.

Simm S, Scharf KD, Jegadeesan S, Chiusano ML, Firon N, Schleiff E (2016). Survey of genes involved in biosynthesis, transport, and signaling of phytohormones with focus on *Solanum lycopersicum*. *Bioinform Biol Insights* 10:185-207.

Srivastava LM (2002). *Plant growth and development*. Academic Press, London, pp 329-339.

Sundberg B, Tuominen H, Little CHA (1994). Effects of the indole-3-acetic acid (IAA) transport inhibitors N-1-naphthylphthalamic acid and morphactin on endogenous IAA dynamics in relation to compression wood formation in 1-year old *Pinus sylvestris* L. shoots. *Plant Physiol* 106:469-476.

Sundberg B, Uggla C, Tuominen H (2000). Cambial growth and auxin gradients. In: Savidge R, Barnett J, Napier R, eds. Cell and molecular biology of wood formation. Oxford: BIOS Scientific Publishers, 169-188.

Swamy PM, Smith B (1999). Role of abscisic acid in plant stress tolerance. *Curr Plant Sci* 76:1220-1227.

Timell TE (1986). Compression wood in gymnosperms. Springer-Verlag, Heidelberg, Vol 2, pp 983-1262.

Tran LSP, Pal S (2014). Phytohormones: a window to metabolism, signaling and biotechnological application. Springer Science.

Trupiano D, Di Iorio A, Montagnoli A, Lasserre B, Rocco M, Grosso A, Scaloni A, Marra M, Chiatante D, Scippa GS (2012b). Involvement of lignin and hormones in the response of woody poplar taproots to mechanical stress. *Physiol Plantarum* 146:39-52.

Trupiano D, Rocco M, Renzone G, Scaloni A, Viscosi V, Chiatante D, Scippa GS (2012a). The proteome of *Populus nigra* woody root: response to bending. *Ann Bot* 110:415-432.

Trupiano D, Yordanov Y, Regan S, Meilan R, Tschaplinski T, Scippa GS, Busov V (2013a). Identification, characterization of an AP2/ERF transcription factor that promotes adventitious, lateral root formation in *Populus*. *Planta* 238:271-282.

Ursache R, Nieminen K, Helariutta Y (2013). Genetic and hormonal regulation of cambial development. *Physiol Plant* 147:36-45.

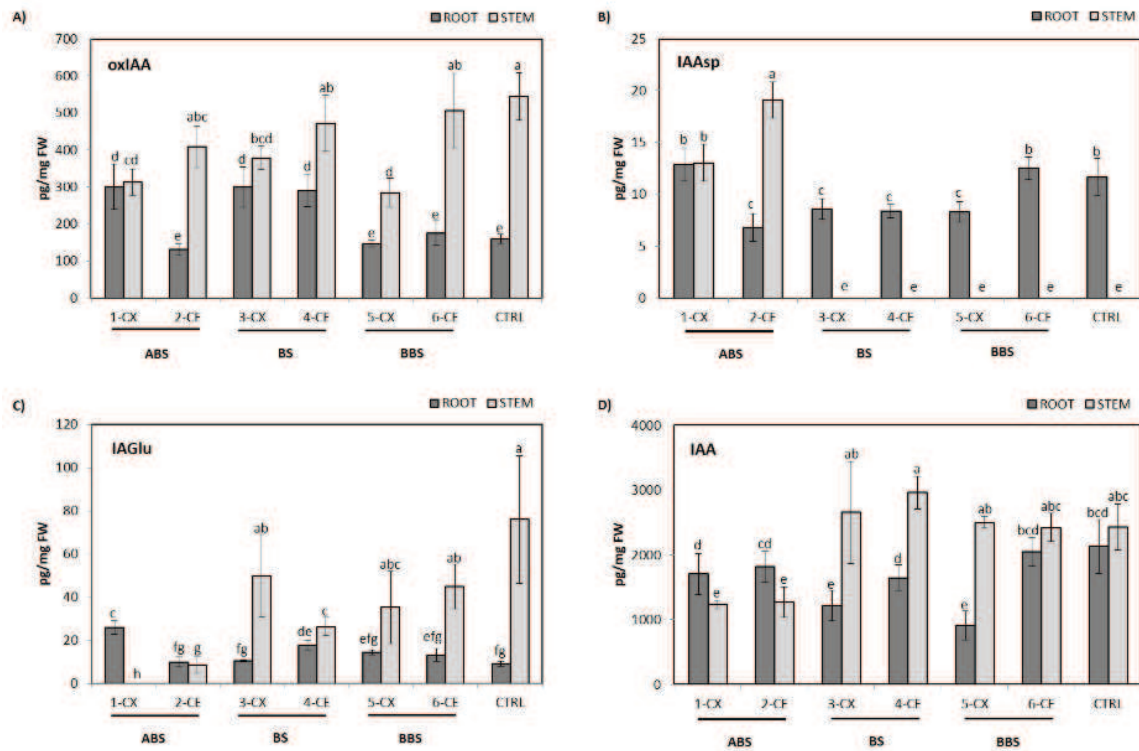
Vayssières A, Pěňčík A, Felten J, Kohler A, Ljung K, Martin F, Legué V (2015). Development of the poplar-*Laccaria bicolor* ectomycorrhiza modifies root auxin metabolism, signaling, and response. *Plant Physiol* 169:890-902.

Verma V, Ravindran P, Kumar PP (2016). Plant hormone-mediated regulation of stress responses. *BMC Plant Biol* 16:86.

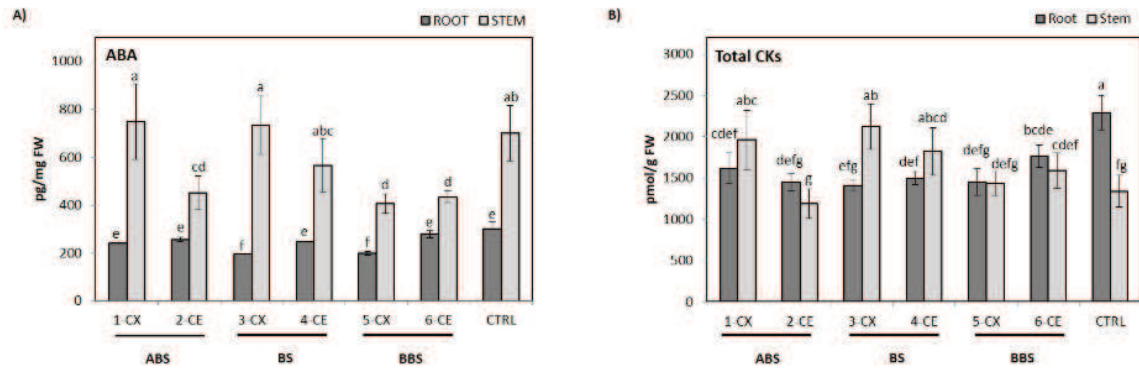
Wareing PF (1970). Growth and its coordination in trees. In: Luckwill LC, Cutting CV, eds. *The physiology of tree crops*. Academic Press, London.

Zhu JK (2002). Salt and drought stress signal transduction in plants. *Ann Rev Plant Physiol* 53:247-273.

## FIGURES

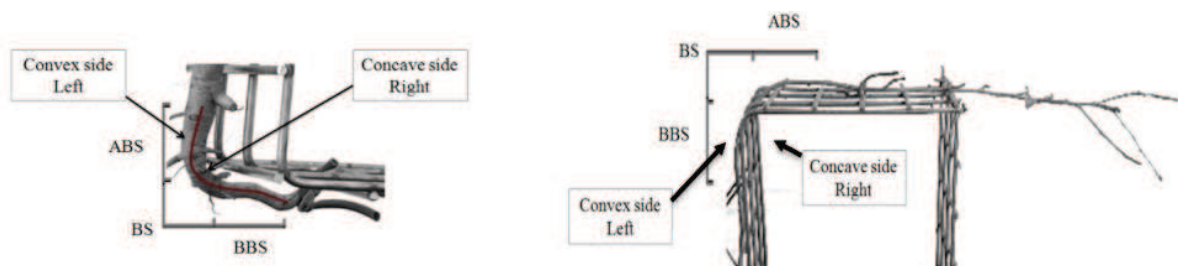


**Fig. 1. Auxin metabolites profiling in different bent sectors of *P. nigra* root and stem.** Concentrations of oxIAA, IAAsp, IAGlu and IAA were analyzed by UHPLC-MS/MS. The values are expressed in  $\text{pg mg}^{-1}$  of fresh weight. Data represent the mean of three independent extractions  $\pm$  SD. Values marked with the same letter are not statistically significant (Post-hoc LSD-tests,  $p < 0.05$ ). ABS, above bending sector; BS, bending sector; BBS, below bending sector; CX, convex side; CE, concave side.

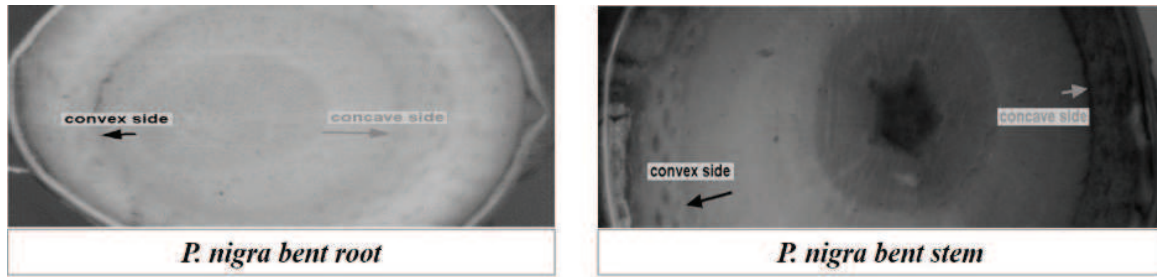


**Fig. 2. ABA and CKs content in different bent sectors of *P. nigra* root and stem.** Concentrations were analyzed by UHPLC-MS/MS. The values are expressed in  $\text{pg mg}^{-1}$  of fresh weight for ABA and in  $\text{pmol g}^{-1}$  of fresh weight for CKs. Data represent the mean of three independent extractions  $\pm$  SD. Values marked with the same letter are not statistically significant (Post-hoc LSD-tests,  $p < 0.05$ ). ABS, above bending sector; BS, bending sector; BBS, below bending sector; CX, convex side; CE, concave side.

## SUPPLEMENTARY INFORMATION



**Fig. 1S. Simulation of bending stress in *P. nigra* root and stem.** Roots and stems were linked for five months to steel nets curved at a right angle ( $90^\circ$ ). Three different regions of the taproot and stem were defined during sampling: the Above Bending Sector (ABS); the Bending Sector (BS) and the Below Bending Sector (BBS). To analyze the convex (CX) and concave side (CE), each region (ABS, BS and BBS) was further divided into two parts.



**Fig. 2S. Reaction wood in *P. nigra* root and stem after 5 months of bending treatment.** The reaction wood was produced at the compressed side (concave side) of *P. nigra* root and tension side (convex side) of the stem.





***(Paper in preparation)***

## **HORMONAL PROFILE OF *POPULUS NIGRA* CAMBIUM CELLS IN ROOT UNDER BENDING STRESS CONDITION**

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### **ABSTRACT**

Tissue-specific hormonal profile was carried out in *Populus* root subjected to short-time bending stress, with the aim of investigating the differences between the concave and convex side within the cambial zone of the bent taproot. The analysis were focused on taproot zone corresponding to the point of maximum bending. Auxins, abscisic acid and cytokinins metabolites were separated and quantified using liquid chromatography coupled to tandem mass spectrometry analysis (LC-MS/MS).

A strong modulation of auxin gradient across the cambial zone occurred in the concave side of the bent root, in the side where stress-related compression wood (CW) developed. The levels of auxin metabolites in the cambial zone also varied between the two sides analyzed, suggesting a correlation between the auxin metabolism and CW formation, even from the early phases of the stress treatment. A role of abscisic acid and cytokinins also was hypothesized in CW formation after bending stress imposition.

Key words: bending stress, vascular cambium, cryosectioning, auxins, cytokinins, metabolite profiling, LC-MS/MS.

Abbreviations: ABA, abscisic acid; BS, bending sector; CE, concave side; CKs, cytokinins; CW, compression wood; CX, convex side; CZ, cambial zone; IAA, indole-3-acetic acid; IAAsp, IAA-aspartate; IAGlu, IAA-glutamate; oxIAA, 2-oxindole-3-acetic acid; Ph dev, developing phloem; TW, tension wood; Xy dev, developing xylem.

## INTRODUCTION

The vascular cambium is a secondary meristem that ensures postembryonic secondary growth, through the production of secondary phloem and xylem in roots and shoots of dicotyledonous angiosperms and gymnosperms (Larson, 1994). Cambium *sensu stricto* is formed by one layer of juvenile cells, called initials, whose division produces phloem and xylem mother cells. Cambial initials, together with mother cells, constituted the so-called cambial zone (CZ, Plomion *et al.*, 2001).

The cambial cells division determines the formation of secondary xylem or wood on the inner side and secondary phloem on the outer side, the rate of cell division regulates the amount/quality of wood biomass (Plomion *et al.*, 2001; Sorce *et al.*, 2013). Cambial activity and xylogenesis are regulated by many environmental factors, such as temperature (Arend and Fromm, 2007) or nutrient/photoassimilates availability (Escalante-Perez *et al.*, 2009; Berta *et al.*, 2010; Krabel, 2000), and by several endogenous signal molecules (Sorce *et al.*, 2013).

One of the endogenous components that acts both as a morphogen, as well as a transmitter of environmental signals, is indole-3-acetic acid (IAA), the most abundant auxin in plants (Schrader *et al.*, 2003).

Another group of plant hormones essential in cambial growth are cytokinins (CKs), which have a well-established function in cell division (Du and Yamamoto, 2007). While auxin is considered to play a crucial role in formation of vascular strands and xylem differentiation, other hormones have been shown to be involved in these processes by interacting with IAA in a synergetic (CKs) or inhibitory (abscisic acid, ABA) manner (Davies, 1995; Sundberg *et al.*, 2000; Mellerowicz *et al.*, 2001; Muday and DeLong, 2001; Schrader *et al.*, 2003; Du and Yamamoto, 2007; Simm *et al.*, 2016).

Auxin responsiveness in the plants is essentially determined by establishment of concentration gradients across tissues and organs (Petersson *et al.*, 2009; Sorefan *et al.*, 2009; Novák *et al.*, 2012). Areas of maxima and minima of auxin concentration confer positional information to the developing cells and trigger differential development responses (Schrader *et al.*, 2003; Novák *et al.*, 2012). The control of auxin concentration in the tissues occurs through the regulation of its biosynthesis, transport, degradation, and conjugation. The auxin conjugation with amino acid, such as aspartate and glutamate, forming IAAsp and IAGlu respectively, is a process that serves to maintain proper auxin homeostasis in the cells (Tuominen *et al.*, 1994; Ljung *et al.*, 2002; Pencik *et al.*, 2013; Tran and Pal, 2014).

In response to environmental stimuli (e.g., in cases of tropic growth) the auxin gradient is rapidly established, but there are also cases where it has a more stable nature (e.g., during primary root development; Schrader *et al.*, 2003). Knowing the importance of the endogenous hormones in cambial activity regulation, xylem differentiation and wood formation and considering that hormones and their metabolites level can considerably vary between different plant tissues (Novák *et al.*, 2012), in this work we provide a detailed hormonal tissue analysis across the cambial zone of *Populus nigra* root subjected to mechanical bending stress. We used a method that permits, from small amounts of plant material, to analyze simultaneously IAA, its majority catabolite, the 2-oxindole-3-acetic acid (oxIAA), its conjugates (IAAsp and IAGlu), ABA and CKs, combining one-step solid phase extraction (SPE) purification method with liquid chromatography/tandem mass spectrometry (LC-MS/MS) analysis (Novák *et al.*, 2008; 2012).

The mechanical stress in plants can be caused by several factors, such as wind, rain, slope of soil, presence of natural/artificial physical barriers and gravity, these disadvantageous conditions can influence drastically the plant stability and survival (Jaffe and Forbes, 1993). Plants, equipped with a great plasticity, respond to these severe perturbations adapting their growth behavior. In particular, the woody species, react to the mechanical stress producing a specialized tissue, defined "reaction wood" (Telewski and Jaffe, 1986a; Braam and David, 1990; Braam, 2005) in order to adjust the non-optimal orientation of affected organ and improve the support function when needed (Du and Yamamoto, 2007).

Studies performed by Scippa *et al.*, (2008), Trupiano *et al.*, (2012a,b, 2013b) offer several progress in the understanding of biochemical, physiological and molecular processes related with bending responses in the woody root. In particular, in our recent work it was demonstrated that the diverse perception of mechanical forces (tension and compression forces) along *Populus* root influences differently the cambium cells activity between the two sides (convex and concave) of the taproot, stimulating the reaction wood formation in the concave side of the maximum point of root bending (De Zio *et al.*, 2016).

Considering the central role of cambium in wood production, the investigation of the role played by the major plant hormones in this secondary meristem is fundamental to increase knowledge on response to bending in the woody root, where it is difficult gaining access to the cambial zone, without affecting tissue proprieties and biological activity of signal molecules. In fact, most of the research in the literature concerning the study of mechanical stress/reaction wood induction in woody plants/vascular cambium isolation were conducted at stem level (Savidge *et al.*, 1983; Hallgren *et al.*, 2004; Goué *et al.*, 2008, 2012).

In this work, a method was developed to isolate cambium tissue and the surrounding areas (developing phloem and xylem) from the concave and convex side of poplar bent root, in order to obtain tissue-specific hormonal information, know hormones role in meristematic cells initiation and contribute in establishing their still unknown role in the reaction wood formation (Du and Yamamoto, 2007).

## MATERIALS AND METHODS

### ***Plant material and bending stress conditions***

Taproots of four-years-old *P. nigra* plants were subjected to short-term bending stress. To simulate the mechanical perturbation, taproots were tying around right angle curved steel nets [**Fig. 1 Supplementary information**], as previously described in Scippa *et al.* (2008), Trupiano *et al.* (2012a) and De Zio *et al.* (2016).

Plants were grown for two months in a growth chamber with normal photoperiod simulated by LED lights ( $\lambda_{420}$  -  $\lambda_{740}$ ), ensuring temperature and

water controlled conditions. During sampling, on each plant, a transversal root sector, 5 cm long, was defined, corresponding to the point of maximum root bending, the Bending Sector (BS), localized at 17-22 cm from the root collar (equivalent of stem base) downward. Each BS whole sector was immediately snap-frozen in liquid N<sub>2</sub> and then stored at -80°C.

BS regions were used in the successive cryosectioning procedures. The cambial zone (CZ) and its adjacent areas, the developing phloem (Ph dev) and xylem (Xy dev, Fig. 1A) were isolated from the left/convex (CX) and right/concave (CE) sides of each BS region.

### ***Isolation of vascular cambium***

The complete procedure for vascular cambium isolation from BS region of *P. nigra* root can be summarized in four steps: **a)** preparation of wood block; **b)** cryosectioning on wood block, performed through two different cutting directions; **c)** lyophilization of sections in «glass sandwiches»; **d)** samples storage in silica gel until hormones analysis [**Fig. 2 Supplementary information**].

Frozen plant material (BS region), with a diameter of about 10 mm, was cut in a block (2 x 13 x 10 mm). Cubic wood block was fixed on the cryotome holder with O.C.T (TissueTek, Agar Scientific, England) and used for the successive sectioning procedures carried out to the cryomicrotome (HM505E, Microm Laborgeräte, Walldorf, Germany) [**Fig. 3 Supplementary information**]. Transverse sections (50 µm) were cut from each side of the block, corresponding to the left/convex (CX) and right/concave (CE) side of BS region. Slide-mounted transverse sections were photographed using a Zeiss Axioplan 2 microscope equipped with Zeiss AxioCamMR3 digital camera (Carl Zeiss, Inc., Thornwood, NY, USA). Images were analyzed by Zeiss AxioVision Software in order to calculate the distance cortex-cambium and cortex-Ph dev/Xy dev and be able to collect tissues of interest, performing, subsequently, a serial tangential cryosectioning [**Fig. 4 Supplementary information**]. This cryosectioning method was validated staining vascular cambium tissue with safranin and alcian-blue solution [**Fig. 5 Supplementary information**].

Tangential sections (50 µm) of cambium and developing phloem and xylem from the convex and concave side of BS regions were collected on

microscope slides and protected within «glass sandwiches». Sections were lyophilized for 2 h, collected into 2.0 mL eppendorf tube and stored in silica gel until hormonal extraction.

### ***Auxins, abscisic acid and cytokinins analysis***

Auxins, ABA and CKs were extracted from 2 mg of lyophilized tissues, using a modified Dobrev and Kamínek (2002) method. Hormones and metabolites were purified by solid-phase extraction (SPE) on MCX 1cc/30mg columns (Oasis Extraction Cartridges, Waters) and analyzed by ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) as described elsewhere (Novák *et al.*, 2008, 2012). MassHunter software (version B.05.02; Agilent Technologies) was used to determine the concentrations of all examined compounds using stable isotope dilution. Concentrations of IAA metabolites and ABA were expressed as  $\text{pg mg}^{-1}$  of dry weight, while CKs as  $\text{pmol g}^{-1}$  of dry weight.

### ***Statistical analysis***

When needed, variables were square root or log transformed to ensure normal distributions and equal variances for the use of parametric statistics. For each variable, a one-way ANOVA was used to compare different root tissues (Ph dev, CZ, Xy dev) and sides (CX and CE). Post-hoc LSD-tests were conducted to detect overall differences between convex and concave sides for all root tissues. Analyses were applied on a 95% significance level. Statistical analysis was carried out using statistical software package SPSS 17.0 (SPSS Inc, Chicago IL, USA).

## **RESULTS AND DISCUSSION**

### ***IAA metabolites response to bending in the cambial zone***

A sensitive mass spectrometry-based method (LC-MS/MS) was carried out to profile IAA and auxin metabolites, oxIAA, IAAGlu and IAAsp in *P. nigra* root subjected to bending stress. In detail, in order to explore the IAA regulation during cambium development and reaction wood formation a detailed tissue analysis was performed, isolating vascular cambium together



with the developing phloem and xylem from the convex and concave side of the point of maximum root bending (BS region).

Our results showed a considerable increase of IAA in CZ sampled in the concave side of the BS region, compared to both the surrounding areas (Ph and Xy dev zones, characterized by the same IAA level) and CZ of the opposite convex side (Fig. 1B, 2D).

In our previous work we showed that bending stress in *P. nigra* root induces a strong asymmetry in the cambium cells activity between the two sides (convex and concave) of the root, with a very high number of cambium cells in the concave side of BS region, where a compression wood (CW) formed, triggered by high levels of compression forces (De Zio *et al.*, 2016).

In the literature, a clear model regarding the role of auxin in the reaction wood formation does not exist yet, although its involvement in the early steps of the process has not been ruled out (Fagerstedt *et al.*, 2016).

Differently, roles of auxin in the vascular tissue during normal development are well documented, IAA is thought to induce cambium cells division and regulate vascular cells differentiation (Sachs, 1981; Aloni, 2004; Aloni *et al.*, 2006a; Sorce *et al.*, 2013).

High levels of IAA were found in the side of cambial region producing CW in *Cryptomeria japonica* (Funada *et al.*, 1990) and *Metasequoia glyptostroboides* (Du *et al.*, 2004) and in the cambium initial mother cells in *Pinus sylvestris* (L.) trees during secondary growth. In this last case, Uggla *et al.*, (1996) suggested a link between the high IAA levels in the cambium region and its role in maintaining cambial cells meristematic state, proposing the first evidence on IAA radial concentration gradient in developing tissues. Evidence from the literature support the occurrence of this radial gradient, with highest concentration of IAA in the cambial tissue, where the hormone promotes cambium cells division, and decreasing concentrations towards the maturing xylem and phloem (Tuominen *et al.*, 1997; Sundberg *et al.*, 2000). At decreasing concentration, IAA regulates the expansion of xylem and phloem derivative cambial cells and, later the onset of the maturation stage, the secondary cell wall apposition and lignification (Sorce *et al.*, 2013).

Despite such evidence, Hallgren *et al.*, (2004) proposed that the IAA gradient across the cambial zone in *Populus tremula* and *Pinus sylvestris* stem under



gravistimuli might not be the signal to maintain the cells in a reaction wood development state.

In all cases, the strong asymmetry in IAA content between the two sides of CZ in bent *Populus* root reported in this work supports the hypothesis, proposed in De Zio *et al.*, (2016), of the role of auxin in stress-related CW formation in the concave side of the taproot.

Our results showed that in BS convex side, contrary to the concave side, does not exist an auxin gradient between CZ and the surrounding areas, although the IAA level in Xy dev convex side was lower compared to Ph dev of the same side (Fig. 1B,2D). In the convex side of the bent root lower auxin concentrations in the CZ resulted in a slower rate of cambium cells division (De Zio *et al.*, 2016) and probably in a slower xylem differentiation. Interesting, the Ph dev tissues showed comparable IAA content between the convex and concave side, whereas differences were found between the two sides of Xy dev, with lower IAA content in the convex side, confirming the asymmetry in xylem development and wood formation.

The role of auxin in the regulation of xylem development and wood formation is well established (Uggla *et al.*, 1996; Sundberg *et al.*, 2000). The radial conveying of IAA regulate the duration of developmental processes, such as xylem fiber expansion, affecting essential anatomical traits of the xylem (Tuominen *et al.*, 1997). Moyle *et al.*, (2002) found that a specific IAA signal transduction gene in mature xylem responds to lower levels of auxin. Bhalerao and Bennett (2003) suggested that different concentrations of auxin regulate the expression level of IAA-related genes: high concentrations of IAA in the cambium serve to maintain cambial cells identity, while lower concentrations of the hormone are necessary to promote xylem development. Most recently, it has been suggested that the transcription of few auxin-responsive genes responds dynamically to the changes of auxin levels regulating the xylem development (Nilsson *et al.*, 2008).

Based on the above reported evidence, we could hypothesize that the lower levels of auxin found in the Xy dev, especially in the convex side of the bent root, are related with the signaling involved in xylem differentiation and development, but is the strong asymmetry in auxin concentration to trigger the CW formation in the concave side.

The analysis of auxin metabolome profiling showed that the major auxin degradation product, oxIAA, and the auxin aspartate conjugate, IAAsp, followed the same distribution trend of IAA concentrations in the different tissues analyzed (Fig. 2A,B,D), whereas the auxin conjugation with glutamate was completely affected by short-time bending stress in *Populus* root, since concentrations of IAGlu were low detection limit in all samples analyzed (Fig. 2C).

Therefore, the levels of oxIAA and IAAsp in CZ concave side were higher compared to the CZ of the convex side, following the same trend of auxin. Generally, the accumulation of oxIAA, as well as amino acid conjugates, is a typical response to high IAA levels, and this would explain such distribution trend (Novák *et al.*, 2012; Pencík *et al.*, 2013). In detail, oxIAA level in the CZ concave side was in a 1:25 ratio compared to IAA concentration (Fig. 2A), while much lower quantities of IAAsp conjugate were found in the same tissue (Fig. 2B). In the literature is reported that IAA amino acid conjugates normally are present in much lower quantities compared to oxIAA (Pencík *et al.*, 2013; Vayssières *et al.*, 2015). Sundberg *et al.*, (1990) observed the occurrence of higher concentration of ester conjugates than amide conjugates in cambial tissue of *P. sylvestris*. Interesting, in the concave side, IAAsp, contrary to oxIAA, presented a distribution gradient within the three tissue analyzed. In fact, despite no difference in IAA levels was observed between Ph and Xy dev concave side, Xy dev showed a lower IAAsp level compared to Ph dev of the same side (Fig. 2B).

In the convex side, we have not measured differences in oxIAA content within the three isolated tissues (Fig. 2A); but we observed an increase of IAAsp in Ph dev convex side in respect to the other zones of the same side (Fig. 2B). Comparable levels in oxIAA and IAAsp were found between Ph dev convex/concave and Xy dev convex/concave side.

The minor rate of conjugation with aspartate in the Xy dev (concave and convex) could be explained by the correlation with corresponding minor auxin levels. Nevertheless, most plant organs contain significant pools of IAA conjugates, but in wood-forming tissues of softwoods and hardwoods only trace amounts have been found (Sundberg *et al.*, 1990; Tuominen *et al.*, 1995).

### ***ABA and CKs response to bending in the cambial zone***

The levels of ABA and CKs were also measured in the isolated tissues in order to understand their influence in vascular tissue of poplar root subjected to bending and broaden the knowledge of the tissue-specific hormonal crosstalk during the stress condition.

Interestingly, our results showed an asymmetric ABA and CKs concentrations between the convex and concave side of the CZ, with higher ABA and CKs levels in the concave side (Fig. 3A,B).

Nevertheless, no difference in ABA content was observed between the three tissues (Ph dev, CZ, Xy dev) in the concave side (Fig. 3A). Whereas, concerning the measurements of CKs, in the concave side, our results showed a lower content of this hormone in the Xy dev compared with the other tissues of the same side (Fig. 3B). In the convex side of BS region, instead, an increase of ABA and CKs was observed in Ph dev compared to CZ and Xy dev of the same side (Fig. 3A,B).

Unlike the auxin, CKs and ABA have seldom been investigated in the vascular cambium of trees (Mellerowicz *et al.*, 2001). Endogenous ABA and CKs are known to exist in the cambial region of trees, but usually at levels much lower than auxin (Moritz and Sundberg, 1996; Funada *et al.*, 2001). In accordance with this, we measured lower levels of ABA and CKs compared to auxin, in both cambial zones.

At lower levels compared to IAA, ABA regulates IAA biosynthesis and activity, while CKs promotes cytokinesis and the sensibility of cambial initials and derivatives to auxin, stimulating vascular differentiation into xylem cells (Baum *et al.*, 1991; Aloni, 1995; Aloni *et al.*, 2006b). Furthermore, it is known that CKs can increase the sensitivity of cambium to the free auxin also preventing the IAA conjugation (Coenen and Lomax, 1997; Fromm, 2013). In addition to auxin, CKs has also a pivotal role in the regulation of cambium development. In quadruple *ipt* mutant, in which CKs levels are severely decreased, the stem diameter is greatly reduced, together with the number and size of the vascular bundles (Matsumoto-Kitano *et al.*, 2008).

These roles could be closely related with the strong asymmetry found in this work between the convex and concave side of the cambial region, with the

high levels of ABA and CKs in the concave side and the events that in the concave side lead to CW formation.

In this regard, it should be underlined that despite CKs have a role in regulating tracheid differentiation and lignin biosynthesis (Savidge, 1988), as well as ABA has a role in avoidance of cell wall loosening, prerequisite for cell expansion (Gimeno-Gilles *et al.*, 2009), a their direct involvement in CW formation has not yet been demonstrated (Du and Yamamoto, 2007).

## **CONCLUSIONS**

Concluding, this work provides new insights in the complex dynamics of hormones regulation in poplar plant under bending stress condition. Performing a detailed tissue analysis, we evaluated the hormones profile in the cambial zone and surrounding areas (developing phloem and xylem), taking in account, for each tissue, of the differences between the convex and concave side of the bent woody root.

Data showed a noticeable increase of IAA, ABA and CKs in the vascular cambium isolated from the concave side compared to the opposite side. This asymmetrical response in the cambial region suggests an involvement of these hormones in the early phases of CW development in the concave side. Furthermore, our results revealed the occurrence of IAA gradient in the concave side, absent in the opposite side, where no difference in IAA levels was found within the three zones analyzed.

Despite these results, the consolidation of ABA and CKs role in the CW formation deserves further investigations.

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## LITERATURE CITED

Aloni R (1995). The induction of vascular tissues by auxin and cytokinin. In: Davies PJ ed. Plant hormones: physiology, biochemistry and molecular biology. Kluwer, Dordrecht, pp 531-546.

Aloni R (2004). The induction of vascular tissue by auxin. In: Davies PJ, ed. Plant hormones: biosynthesis, signal transduction, action! Dordrecht: Kluwer, 471-492.

Aloni R, Aloni E, Langhans M, Ullrich C (2006a). Role of cytokinin and auxin in shaping root architecture: regulating vascular differentiation, lateral root initiation, root apical dominance and root gravitropism. *Ann Bot* 97:883-893.

Aloni R, Aloni E, Langhans M, Ullrich CI (2006b). Role of auxin in regulating *Arabidopsis* flower development. *Planta* 223:315-328.

Arend M, Fromm J (2007). Seasonal change in the drought response of wood cell development in poplar. *Tree Physiol* 27:985-992.

Baum SF, Aloni R, Peterson CA (1991). The role of cytokinin in vessel regeneration in wounded *Coleus* internodes. *Ann Bot* 67:543-548.

Berta M, Giovannelli A, Sebastiani F, Camussi A, Racchi ML (2010). Transcriptome changes in the cambial region of poplar (*Populus alba* L.) in response to water deficit. *Plant Biol* 12:341-354.

Bhalerao RP, Bennett MJ (2003). The case for morphogens in plants. *Nat Cell Biol* 5:939-943.

Braam J (2005). In touch: plant responses to mechanical stimuli. *New Phytol* 165:373-389.

Braam J, Davis RW (1990). Rain-, wind-, and touch-induced expression of calmodulin and calmodulin-related genes in *Arabidopsis*. *Cell* 60:357-364.

Coenen C, Lomax TL (1997). Auxin–cytokinin interactions in higher plants: old problems and new tools. *Trends Plant Sci* 2:351-356.

Davies PJ (1995). The plant hormones: their nature, occurrence and functions. In: Davies PJ, ed. *Plant Hormones*, 2nd edn. Kluwer Academic Publishers, Dordrecht, pp 1-12.

De Zio E, Trupiano D, Montagnoli A, Terzaghi M, Chiatante D, Grosso A, Marra M, Scalonì A, Scippa GS (2016). Poplar woody taproot under bending stress: the asymmetric response of the convex and concave sides. *Ann Bot* 118:865-883.

Du S, Sugano M, Tsushima M, Nakamura T, Yamamoto F (2004a). Endogenous indole-3-acetic acid and ethylene evolution in tilted *Metasequoia glyptostroboides* stems in relation to compression wood formation. *J Plant Res* 117:171-174.

Du S, Yamamoto F (2007). An overview of the biology of reaction wood formation. *J Integr Plant Biol* 49:131-143.

Escalante-Perez M, Lautner S, Nehls U, Selle A, Teuber M, Schnitzler JP, Teichmann T, Fayyaz P, Hartung W, Polle A, Fromm J, Hedrich R, Ache P (2009). Salt stress affects xylem differentiation of grey poplar (*Populus x canescens*). *Planta* 229:299-309.

Fagerstedt K, Tocquard K, Lopez D, Decourteix M, Thibaut B, *et al.* (2016). The molecular mechanisms of reaction wood induction. *Springer* 4:107-138.

Fromm J (2013). Cellular aspects of wood formation. Springer-Verlag, Berlin, Heidelberg, pp 128.

Funada R, Kubo T, Tabuchi M, Sugiyama T, Fushitani M (2001). Seasonal variations in endogenous indole-3-acetic acid and abscisic acid in the cambial

region of *Pinus densiflora* Sieb. et Zucc. Stems in relation to earlywood-latewood transition and cessation of tracheid production. *Holzforschung* 55:128-134.

Funada R, Mizukami E, Kubo T, Fushitani M, Sugiyama T (1990). Distribution of indole-3-acetic acid and compression wood formation in the stems of inclined *Cryptomeria japonica*. *Holzforschung* 44:331-334.

Gimeno-Gilles C, Lelièvre E, Viau L, Malik-Ghulam M, Ricoult C, Niebel A, Leduc N, Limami AM (2009). ABA-mediated inhibition of germination is related to the inhibition of genes encoding cell-wall biosynthetic and architecture: modifying enzymes and structural proteins in *Medicago truncatula* embryo axis. *Mol Plant* 2:108-119.

Goué N, Noël-Boizot N, Vallance M, Magel E, Label P (2012). Microdissection to isolate vascular cambium cells in poplar. *Silva Fenn* 46:5-16.

Goué N, Lesage-Descauses MC, Mellerowicz EJ, Magel E, Label P, Sundberg B (2008). Microgenomic analysis reveals cell type-specific gene expression patterns between ray and fusiform initials within the cambial meristem of *Populus*. *New Phytol* 180:45-56.

Hellgren JM, Olofsson K, Sundberg B (2004). Patterns of auxin distribution during gravitational induction of reaction wood in poplar and pine. *Plant Physiol* 135:212-220.

Jaffe MJ, Forbes S (1993). Thigmomorphogenesis: the effect of mechanical perturbation on plants. *Plant Growth Regul* 12:313-324.

Krabel D (2000). Influence of sucrose on cambial activity. In cell and molecular biology of wood formation. Savidge RA, Barnett JR, Napier R, eds. BIOS Scientific Publishers, Oxford, UK, pp 113-126.

Larson PR (1994). The vascular cambium: development and structure. Springer Verlag, Berlin.



Ljung K, Hull AK, Kowalczyk M, Marchant A, Celenza J, Cohen JD, Sandberg G (2002). Biosynthesis, conjugation, catabolism and homeostasis of indole-3-acetic acid in *Arabidopsis thaliana*. *Plant Mol Biol* 50:309-332.

Matsumoto-Kitano M, Kusumoto T, Tarkowski R, Kinoshita-Tsujimura K, Václavíková K, Miyawaki K, Kakimoto T (2008). Cytokinins are central regulators of cambial activity. *Proc Natl Acad Sci USA* 105:20027-20031.

Mellerowicz EJ, Baucher M, Sundberg B, Boerjan W (2001). Unravelling cell wall formation in the woody dicot stem. *Plant Mol Biol* 47:239-274.

Moritz T, Sundberg B (1996). Endogenous cytokinins in the vascular cambial regions of *Pinus sylvestris* during activity and dormancy. *Physiol Plant* 98:693-698.

Moyle R, Schrader J, Stenberg A, Olsson O, Saxena S, Sandberg G, Bhalerao RP (2002). Environmental and auxin regulation of wood formation involves members of the Aux/IAA gene family in hybrid Aspen. *Plant J* 31:675-685.

Muday GK, DeLong A (2001). Polar auxin transport: Controlling where and how much. *Trends Plant Sci* 6:535-542.

Nilsson J, Karlberg A, Antti H, Lopez-Vernaza M, Mellerowicz E, Perrot-Rechenmann C, Sandberg G, Bhalerao RP (2008). Dissecting the molecular basis of the regulation of wood formation by auxin in hybrid aspen. *Plant Cell* 20:843-855.

Novák O, Hauserová E, Amakorová P, Doležal K, Strnad M (2008). Cytokinin profiling in plant tissues using ultra-performance liquid chromatography-electrospray tandem mass spectrometry. *Phytochemistry* 69:2214-2224.

Novák O, Hényková E, Sairanen I, Kowalczyk M, Pospíšil T, Ljung K (2012). Tissue-specific profiling of the *Arabidopsis thaliana* auxin metabolome. *Plant J* 72:523-536.



Pencík A, Simonovik B, Petersson SV, Henyková E, Simon S, Greenham K, Zhang Y, Kowalczyk M, Estelle M, Zazimalová E, Novák O, Sandberg G, Ljung K (2013). Regulation of auxin homeostasis and gradients in *Arabidopsis* roots through the formation of the indole-3-acetic acid catabolite 2-oxindole-3-acetic acid. *The Plant Cell* 25:3858-3870.

Petersson SV, Johansson AI, Kowalczyk K, Makoveychuk A, Wang JY, Moritz T, Grebe M, Benfey PN, Sandberg G, Ljung K (2009). An auxin gradient and maximum in the *Arabidopsis* root apex shown by high-resolution cell-specific analysis of IAA distribution and synthesis. *Plant Cell* 21:1659-1668.

Plomion C, Leprovost G, Stokes A (2001). Wood formation in trees. *Plant Physiol* 127:1513-1523.

Sachs T (1981). The control of patterned differentiation of vascular tissues. *Adv Bot Res* 9:151-262.

Savidge RA (1988). A biochemical indicator of commitment to tracheid differentiation in *Pinus contorta*. *Can J Bot* 66:2009-2012.

Savidge RA, Mutumba GMC, Heald JK, Wareing PF (1983). Gas chromatography-mass spectroscopy identification of 1-aminocyclopropane-1-carboxylic acid in compression wood vascular cambium of *Pinus contorta* Dougl. *Plant Physiol* 71:434-436.

Schrader J, Baba K, May ST, Palme K, Bennett M, Bhalerao RP, Sandberg G (2003). Polar auxin transport in the wood-forming tissues of hybrid aspen is under simultaneous control of developmental and environmental signals. *Proc Natl Acad Sci USA* 100:10096-10101.

Scippa GS, Trupiano D, Rocco M, Di Iorio A, Chiatante D (2008). Unravelling the response of poplar (*Populus nigra*) roots to mechanical stress imposed by bending. *Plant Biosystems* 142:401-413.

Simm S, Scharf KD, Jegadeesan S, Chiusano ML, Firon N, Schleiff E (2016). Survey of genes involved in biosynthesis, transport, and signaling of

phytohormones with focus on *Solanum lycopersicum*. *Bioinform Biol Insights* 10:185-207.

Sorce C, Giovannelli A, Sebastiani L, Anfodillo T (2013). Hormonal signals involved in the regulation of cambial activity, xylogenesis and vessel patterning in trees. *Plant Cell Rep* 32:885-898.

Sorefan K, Girin T, Liljegren SJ, Ljung K, Robles P, Galván-Ampudia CS, Offringa R, Friml J, Yanofsky MF, Østergaard L (2009). A regulated auxin minimum is required for seed dispersal in *Arabidopsis*. *Nature* 459:583-586.

Sundberg B, Little CHA, Cui K (1990). Distribution of indole-3-acetic acid and the occurrence of its alkali-labile conjugates in the extraxylary region of *Pinus sylvestris* stems. *Plant Physiol* 93:1295-1302.

Sundberg B, Uggla C, Tuominen H (2000). Cambial growth and auxin gradients. In: Savidge RA, Barnett JR, Napir R, eds. *Cell and molecular biology of wood formation*. BIOS Scientific Publishers, Oxford, pp 169-188.

Telewski FW, Jaffe MJ (1986a). Thigmomorphogenesis: anatomical, morphological and mechanical analysis of genetically different sibs of *Pinus taeda* in response to mechanical perturbation. *Physiol Plant* 66:219-226.

Tran LSP and Pal S (2014). *Phytohormones: a window to metabolism, signaling and biotechnological application*. Springer Science.

Trupiano D, Di Iorio A, Montagnoli A, Lasserre B, Rocco M, Grosso A, Scaloni A, Marra M, Chiatante D, Scippa GS (2012b). Involvement of lignin and hormones in the response of woody poplar taproots to mechanical stress. *Physiol Plantarum* 146:39-52.

Trupiano D, Rocco M, Renzone G, Scaloni A, Montagnoli A, Terzaghi M, Di Iorio A, Chiatante D, Scippa GS (2013b). Poplar woody root proteome during the transition dormancy–active growth. *Plant Biosyst* 147:1-6.

Trupiano D, Rocco M, Renzone G, Scaloni A, Viscosi V, Chiatante D, Scippa GS (2012a). The proteome of *Populus nigra* woody root: response to bending. *Ann Bot* 110:415-432.

Tuominen H, Ostin A, Sandberg G, Sundberg B (1994). A novel metabolic pathway for indole-3-acetic acid in apical shoots of *Populus tremula* (L.) x *Populus fremuloides* (Michx.). *Plant Physiol* 106:1511-1520.

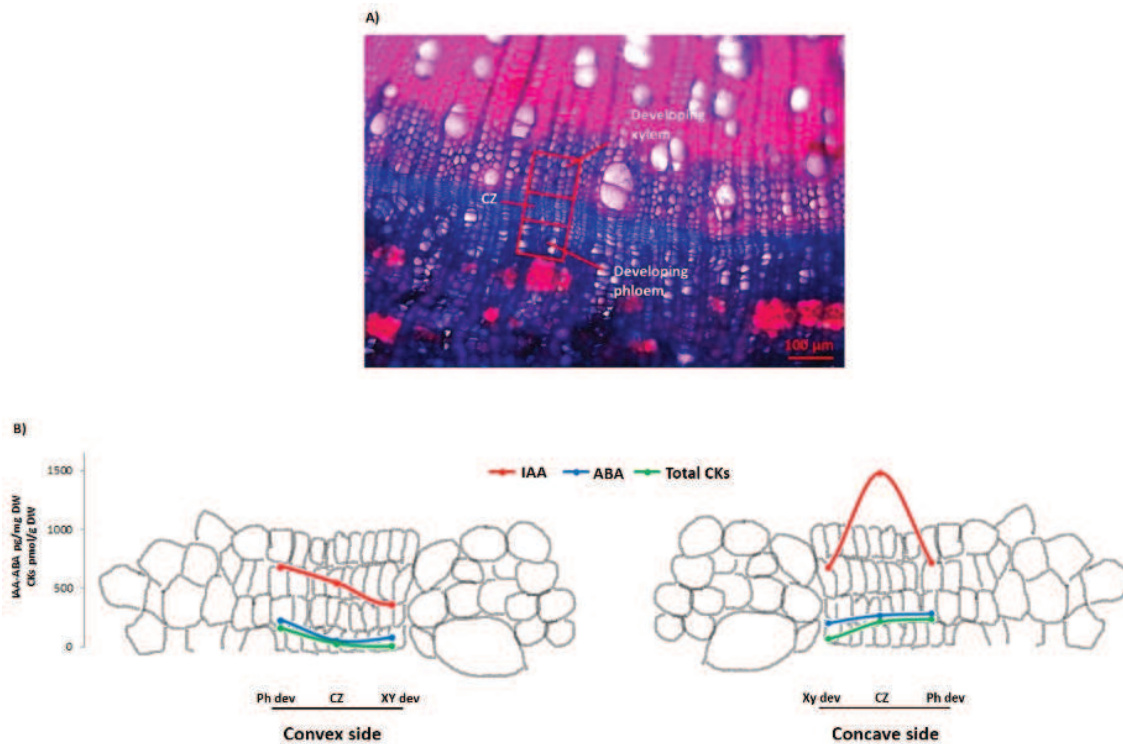
Tuominen H, Puech L, Fink S, Sundberg B (1997). A radial concentration gradient of indole-3-acetic acid is related to secondary xylem development in hybrid aspen. *Plant Physiol* 115:577-585.

Tuominen H, Sitbon F, Jacobsson C, Sandberg G, Olsson O, Sundberg B (1995). Altered growth and wood characteristics in transgenic hybrid aspen expressing *Agrobacterium tumefaciens* T-DNA indoleacetic acid-biosynthetic genes. *Plant Physiol* 109:1179-1189.

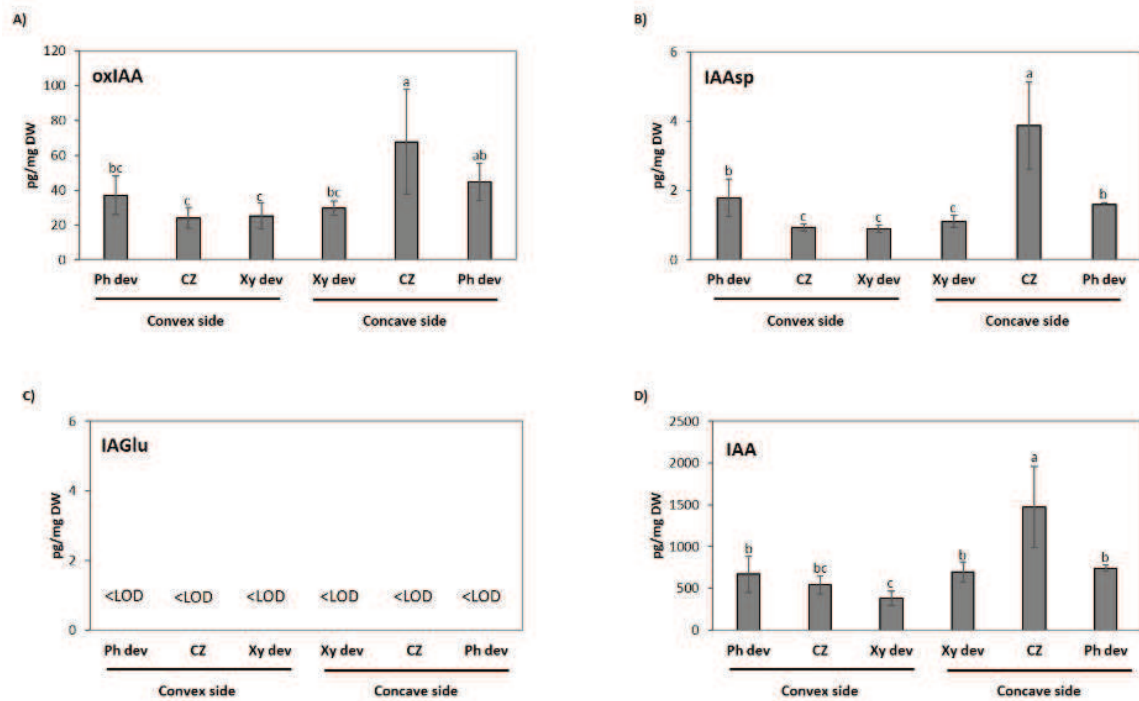
Uggla C, Moritz T, Sandberg G, Sundberg B (1996). Auxin as a positional signal in pattern formation in plants. *Proc Natl Acad Sci USA* 93:9282-9286.

Vayssières A, Pěnčík A, Felten J, Kohler A, Ljung K, Martin F, Legué V (2015). Development of the poplar-*Laccaria bicolor* ectomycorrhiza modifies root auxin metabolism, signaling, and response. *Plant Physiol* 169:890-902.

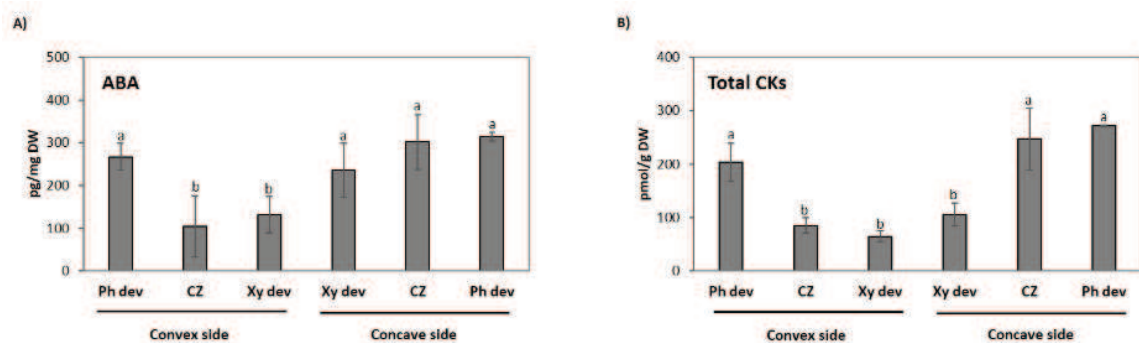
## FIGURES



**Fig. 1. (A) Transverse section of BS region in bent *P. nigra* root.** Section was stained in one part safranin (1% w/v safranin in 50% ethanol) and two parts alcian-blue (1% v/w alcian-blue, 1% v/v formalin, 36% formaldehyde). Tissues used for successive hormones extractions are indicated in the squares. Each tissue was sampled from the convex and concave side of BS region of the bent root. Scale bar=100  $\mu\text{m}$ . **(B) Comparison of IAA, ABA, CKs concentrations between the convex and concave side of BS region for each analyzed tissue.** The values are expressed in  $\text{pg mg}^{-1}$  of dry weight for IAA and ABA and in  $\text{pmol g}^{-1}$  of dry weight for CKs. Ph dev, developing phloem; CZ, cambial zone; Xy dev, developing xylem.



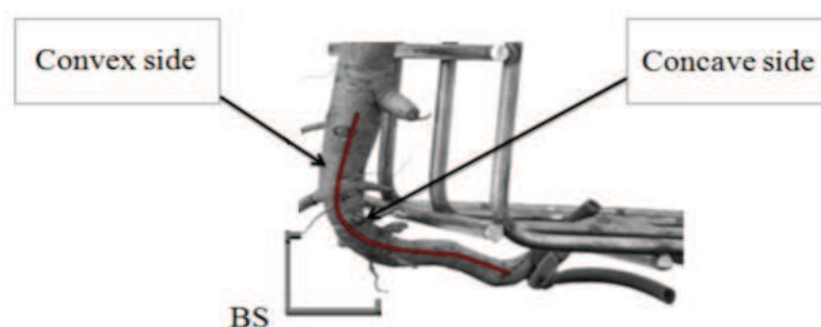
**Fig. 2. Tissue-specific auxin metabolites profiling in bent *P. nigra* root.** Concentrations of oxIAA, IAAsp, IAGlu and IAA were analyzed by UHPLC-MS/MS. Cambium tissue was isolated from the convex and concave side of BS region, the point of maximum root bending, together with the convex and concave developing phloem and xylem. The values are expressed in  $\text{pg mg}^{-1}$  of dry weight. Data represent the mean of three independent extractions  $\pm$  SD. Values marked with the same letter are not statistically significant (Post-hoc LSD-tests,  $p < 0.05$ ). Ph dev, developing phloem; CZ, cambial zone; Xy dev, developing xylem.



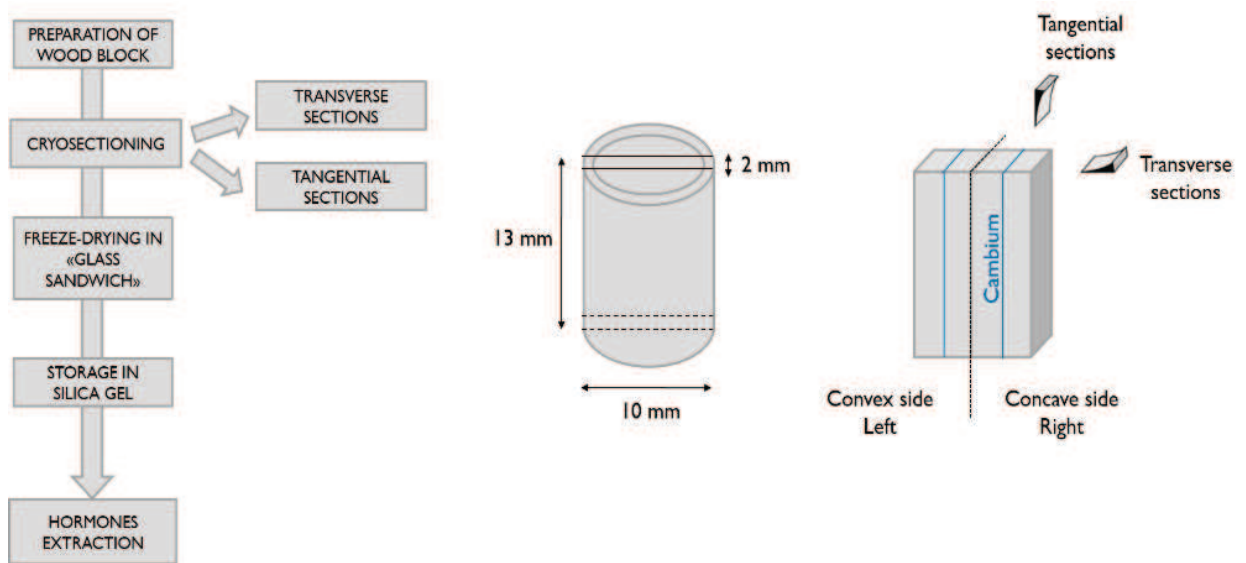
**Fig. 3. Tissue-specific ABA and CKs content in bent *P. nigra* root.** Concentrations were analyzed by UHPLC-MS/MS. Hormones were extracted from the convex and concave side of

BS region, focusing for each side, on the cambial zone and developing phloem and xylem zones. The values are expressed in  $\mu\text{g mg}^{-1}$  of dry weight for ABA and in  $\text{pmol g}^{-1}$  of dry weight for CKs. Data represent the mean of three independent extractions  $\pm$  SD. Values marked with the same letter are not statistically significant (Post-hoc LSD-tests,  $p < 0.05$ ). Ph dev, developing phloem; CZ, cambial zone; Xy dev, developing xylem.

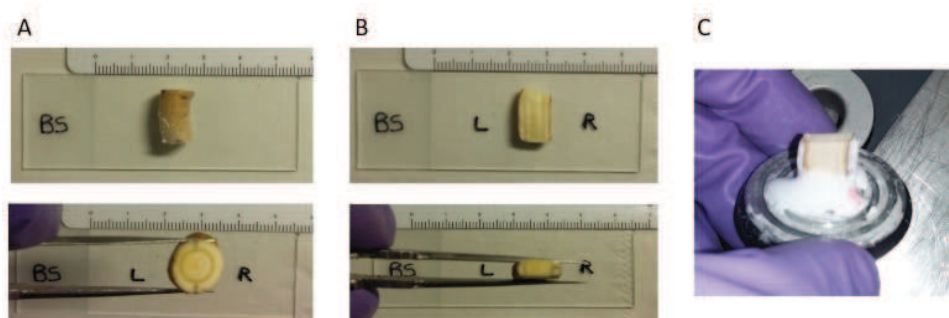
## SUPPLEMENTARY INFORMATION



**Fig. 1S. Simulation of bending stress in *P. nigra* root.** Taproots were linked to  $90^\circ$  curved steel nets and sampled after two months of treatment. The Bending Sector (BS) region, localized at 17-22 cm from the root collar, was defined during sampling on each stressed plant. The outer side (left side) of the main root is referred to as convex side (CX), the inner side (right side) is called concave side (CE).

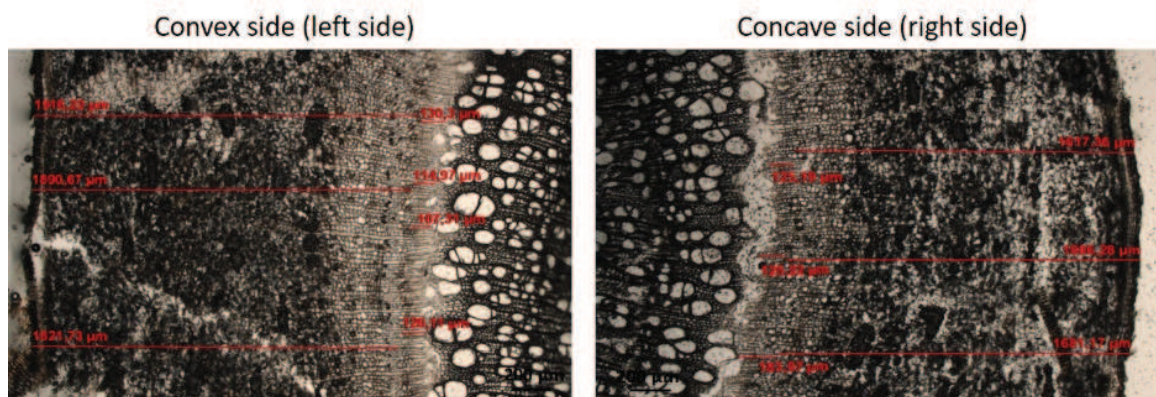


**Fig. 2S. Procedure used for cambium isolation from BS region of *P. nigra* root.** The preparation of wood block was done by cutting a cube (2 x 13 x 10 mm) from the central part of the root, where the cambium ring appeared more linear. Cryosectioning procedures were carried out on cubic wood block; firstly, transverse sections were collected, in order to calculate the distances cortex-cambium and continue, subsequently, with the serial tangential cryosectioning. Vascular cambium tissue, together with developing phloem and xylem, was collected from the convex (left) and concave (right) side of BS root sector. Different cutting directions on wood structure are showed on the right.

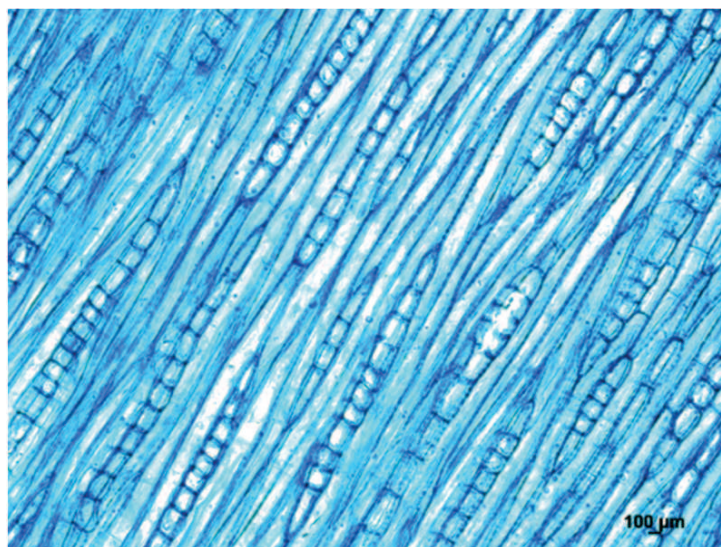


**Fig. 3S. Preparation of cubic wood block from BS region of *P. nigra* root.** (A) The entire BS region. (B) Wood block taken from the central part of BS region and characterized by following dimensions: 2 mm tangentially x 13 mm longitudinally x 10 mm radially. (C) Cryosectioning on cubic wood block.





**Fig. 4S. Transverse sections of the convex and concave side of BS region.** Slide-mounted sections were viewed using a Zeiss Axioplan 2 microscope with a Zeiss AxioCamMR3 digital camera and analyzed by Zeiss AxioVision Software (Carl Zeiss, Inc., Thornwood, NY, USA). A detailed analysis of distances cortex-tissues of interest was performed using the transversal sections. Scale bar=200 µm.



**Fig. 5S. Tangential section of vascular cambium isolated by cryosectioning procedures from BS region of *P. nigra* bent root.** Section was stained in one part safranin (1% w/v safranin in 50% ethanol) and two parts alcian-blue (1% v/w alcian-blue, 1% v/v formalin, 36% formaldehyde). Scale bar=100 µm.



### 3 Conclusions and future perspectives

In this thesis, the hypothesis that the type (tension and compression) and the intensity of mechanical forces differently distributed along a bent poplar woody root regulate specific responses was verified through a multi-disciplinary approach.

In paper I, morphological and anatomical changes induced by different intensities of tension and compression forces along the bent woody root were investigated together with proteomic analysis, hormones assay and chemical determination of lignin content.

Results of this first work clearly indicated the presence of important morphological/anatomical changes in the root area affected by the maximum intensity of mechanical compression forces (BS sector concave side), where was observed: a) an increase of cambial activity; b) an increase of lignin content and xylem thickness; c) production of reaction wood; d) an asymmetrical over-expression of key factors controlling cell wall deformation, lignification and xylem development and differentiation (such as, Ara4-interacting protein or CDC48); e) an asymmetrical under-expression of factors (such as, AlaT1 or MMSDH) related with LR formation, that were mainly produced at the tension side (convex side) of the taproot.

Hormones analysis suggested that IAA and ABA concentrations were likely to be responsible for regulating these alterations.

In paper II, a detailed analysis of main hormones (IAA, ABA, CKs) and their metabolites (oxIAA, IAAsp, IAGlu) was carried out, using an innovative and sensitive method based on liquid chromatography coupled to tandem mass spectrometry analysis (LC-MS/MS). Different sectors/sides of bent poplar root were profiled, together with the corresponding sectors/sides of bent poplar stem, in order to compare hormonal responses between two organs, especially related with the reaction wood formation process.

Considering together the results obtained in paper I and II, seems that in the root, bending, more than age, growth conditions, and stress duration influences the asymmetric responses between the concave and convex side. The increase of IAA and ABA concentrations in the concave side of the root,

found in both works, even using different techniques, suggests a role of these hormones in the reaction wood formation bending-induced. Regarding the production of this special wood, our results proved the existence of an important exception within the hardwood species, in fact in poplar root, mechanically stimulated, reaction wood was induced by high compression forces at the concave side, showing features similar to those observed in gymnosperm CW at the stem level.

Hormones analysis in the root indicated that, IAA plays an important role in the stimulation of cambial cells activity, enhancing the cell wall stiffening through lignin deposition, to trigger the CW formation in the compressed side; while the increase of ABA could serve to counters the deformation of conduits and changes in hydraulic conductivity determined by bending.

Data obtained in paper II showed also that, the root sector subjected to the highest compression forces and affected by significant anatomical, biochemical and molecular changes (BS concave side) is also one of the sectors more involved in IAA conjugation with glutamate. This result suggests a stress-tolerance role of IAGlu in that sector and the existence of a correlation between IAGlu, lignin deposition and cell wall stabilization. The increase of IAA catabolism and conjugation (increase of oxIAA, IAAsp and IAGlu) in ABS convex side, instead, has been associated with LRs formation process and the probable alteration of redox state that regulate the LRs emission.

Paper II also gives an outline on the hormones profile of poplar stem subjected to bending, in this case, TW formed in the upper side of the leaning (corresponding to the ABS sector), in a zone characterized by the lowest IAA content, in accordance with literature data.

Since no asymmetric distribution of auxin between the two sides of ABS sector seems to support TW formation, differently from what happens for CW in the root, a role of ABA and CKs in TW formation was hypothesized, considering also their high concentrations in ABS convex side.

Interestingly, metabolites analysis indicated that, ABS, is the unique sector of the stem in which the conjugation with aspartate takes place. This result suggests an involvement of IAAsp in TW formation, but represent also an example of stress-tolerance metabolites-mediated. In fact, the absence of IAAsp in the rest of bent sectors and control could be associated to a positive

regulation of stem elongation, necessary to overcome the inhibition of stem elongation caused by bending.

In this context, it should be underlined that the spatial analysis designed in the root has followed the different intensities of mechanical forces along the taproot, in accordance with an accepted model of tensile and compression forces distribution. This model has not been defined yet for the bent stem, an elaboration of this data, correlated with a detailed stem morphological/anatomical analysis, would allow to widen the knowledge of the different response of poplar stem to applied bending and better specify the links with the hormones/metabolites distribution.

Furthermore, an investigation of the role of ethylene and GAs could be carry out, in the view of know their influence in stress-related reaction wood formation in poplar root/stem and present a complete picture of hormonal crosstalk.

In a forward-looking vision of this work, could be important also: a) valuate the IAA biosynthesis (e.g., through the detention of IAA precursor by using LC-MS/MS), to test, for example, the presumed reduction in the convex side of the root; b) analyze the expression pattern of IAA degradation/conjugation-associated genes (e.g., *GH3* gene family) or auxin influx/efflux carriers-associated genes (e.g., *AUXINI/LIKE-AUX1*, *AUX/LAX* or *PIN-FORMED*, *PIN* genes families) to harmonize data obtained; c) determine the level of ROS, to know the real influence of the redox state on IAA degradation/conjugation processes and find the correlation redox state-LRs/reaction wood formation in the different poplar bent sectors/sides.

In paper III, differently from paper I and II, poplar woody taproots were subjected to short-time bending stress and a tissue-specific hormonal profiling was carried out using LC-MS/MS, investigating the differences between the convex and concave side of the cambium ring at the point of maximum root bending (BS sector).

The occurrence of a strong IAA radial gradient across the concave side of the bent root confirms the role of this hormone in the CW formation bending-induced, manifested through the stimulation of cambial activity. This work showed that the mechanisms aimed to counteract the stress condition take place from the early phases of the treatment and the region particularly

involved in the stress responses belongs, once again, to the concave side of the taproot. Results showed as IAA at high concentrations in the cambial zone of the concave side promotes cambium cells division and at decreasing concentrations, towards the maturing xylem, regulates the expansion of xylem and secondary cell wall apposition and lignification. The asymmetry in IAA content between the xylem developing zone of the concave and convex side reflects the asymmetry in xylem development and wood formation.

Across the cambial zone IAA metabolites (oxIAA and IAAsp) follow the same IAA concentration trend, while the conjugation with glutamate is affected by short-time bending stress, showing as IAGlu signal can be stronger during the time, in a more developed phase of CW (paper II).

Interestingly, while data obtained in paper I and II suggested a marginal/negative role of CKs in the root after long-term bending stress and a mutual regulation between ABA and CKs, paper III showed an increase of these hormones in the cambial zone of the concave side, suggesting a their involvement in the early phases of the treatment, probably related with IAA biosynthesis, cytokinesis and prevention of IAA conjugation.

In a prospective vision for this research may be further proposed: a) the investigation of the involvement of ABA and CKs in CW formation at tissue level using poplar mutant; b) the analysis of the expression pattern of cambium-specific key genes (e.g., *WUSCHEL-RELATED HOMEODOMAIN BOX4*, *WOX4* or *WOX14* genes) to better validate the used tissue-isolation technique or approach to tissue isolation through a more innovative and specific technique (e.g., fluorescence activated cell sorting, FACS or laser microdissection, LMD) to separate, instead of enriched tissues, single cambium cell.

At this regards, it would be interesting to continue the establishment of the protocol, started during the course of my doctorate, on preparation of root sectioned tissues to use for LMD, in order to perform the successive specific-cells hormones extractions.

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