Physiologia Plantarum
An International Journal for Plant Biology

Involvement of lignin and hormones in the response of woody poplar taproots to mechanical stress

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Received 7 September 2011; revised 28 September 2011

doi:10.1111/j.1399-3054.2012.01601.x

Mechanical stress is a widespread condition caused by numerous environmental factors that severely affect plant stability. In response to mechanical stress, plants have evolved complex response pathways able to detect mechanical perturbations and inducing a suite of modifications in order to improve anchorage. The response of woody roots to mechanical stresses has been studied mainly at the morphological and biomechanical level, whereas investigations on the factors triggering these important alterations are still at the initial stage. *Populus* has been widely used to study the response of stem to different mechanical stresses and, since it has the first forest tree genome to be decoded, represents a model woody plant for addressing questions on the mechanisms controlling adaptation of woody roots to changing environments. In this study, a morphological and physiological analysis was used to investigate factors controlling modifications in *Populus nigra* woody taproots subjected to mechanical stress. An experimental model analyzing spatial and temporal mechanical force distribution along the woody taproot axis enabled us to compare the events occurring in its above-, central- and below-bending sectors. Different morphogenetic responses and local variations of lignin and plant hormones content have been observed, and a relation with the distribution of the mechanical forces along the stressed woody taproots is hypothesized. We investigated the differences of the response to mechanical stress induction during the time; in this regard, we present data referring to the effect of mechanical stress on plant transition from its condition of winter dormancy to that of full vegetative activity.

Introduction

Environmental stresses have a great influence on plant life and specific factors, such as gravity, touch, wind, soil-resistance to root penetration (impedance) and slope of the rooting terrain, act by means of mechanical forces eliciting morphological, physiological and biochemical modifications. Various studies on the effect of mechanical impedance on herbaceous species growth (Okada and Shimura 1994, Masle 2002, Braam

Abbreviations – ABA, abscisic acid; ABS, above-bending sector; ACCox, 1-aminocyclopropane-1-carboxylate oxidase; BBS, below-bending sector; BS, bending sector; GA_3, gibberellic acid 3; GA_4, gibberellic acid 4; IAA, indole-3-acetic acid; PDAD, photo diode array detector.

2005, Ditegout et al. 2008, Richter et al. 2009) and of mechanical forces generated by wind or slope on the stems, branches and roots of trees (Rees and Grace 1980a, 1980b, Fredericksen et al. 1994, Telewski 1995, Stokes and Guitard 1997, Stokes et al. 1997, Lindstrom and Rune 1999, Watson 2000, Peltola et al. 2000, Chiatante et al. 2001, 2003a, 2003b, Cucchi et al. 2004, Di Iorio et al. 2005, Dupuy et al. 2005, 2007, Lu et al. 2005, Azri et al. 2009) have been accomplished. They have been generally conducted in the field and involve expensive and time-consuming plant excavation to examine the morphology of the root apparatus; most importantly, they have shown that these environmental stresses are responsible for various morphogenetic modifications regarding, in particular, orientation and density of lateral roots and production of reaction woods (Chiatante et al. 2001, 2003a, 2003b). These morphogenetic variations involve a very complex series of events at molecular, cellular and tissue level, with the participation of endogenous hormones (jasmonic acid, ethylene, abscisic acid and auxin) and the alteration of major cytological events (growth, elongation, lateral root emission, vascular cambium activity), metabolic and signaling pathways (Braam and Davis 1990, Sistrunk et al. 1994, Xu et al. 1995, Bostock 2005, Fujita et al. 2006). Often, they become clearly apparent only a long time after the beginning of stress perception (Jaffe 1973, Scippa et al. 2006) and change depending upon plant-age (Kus et al. 2002), type of organ (Taylor et al. 2002) and stage of the plant life cycle (Wareing 1969). The occurrence of an interaction between these morphogenetic modifications and other environmental factors (temperature, day-length condition, mineral nutrition) has also been suggested. However, no investigation has been published to date that presents a complete sequence of physiological events determining the mechanical stress response in woody roots (Di Michele et al. 2006, Scippa et al. 2006, 2008) and proposes the associated activation of specific molecular mechanisms in plants.

We have recently devised a simple experimental system, which is able to induce a mechanical stress on the woody taproots of plant species grown into containers (Scippa et al. 2008). Preliminary results obtained after 6 months of stress induction showed the apparent occurrence of morphological, biomechanical and molecular alterations similar to that obtained in the field and determined by natural environmental factors (Scippa et al. 2008). In order to have a complete picture of this response over the time, which may also include slow morphogenetic modifications present in roots (Jaffe 1973, Scippa et al. 2006), and ascertain therein the occurrence of some associated metabolic events, such as accumulation of lignin and concomitant variation of endogenous plant hormones, this experimental model was further investigated. Thus, woody unstressed and stressed roots were collected after 12, 13 and 14 months of growth and their morphological traits and mechanical stress modeling were evaluated; content in lignin, indole-3-acetic acid (IAA), gibberellins ($GA_3 + GA_4$) and abscisic acid (ABA) was also determined, together with 1-aminocyclopropane-1-carboxylate oxidase (ACCox) expression. This study reports the results obtained in this contest, together with data referring to plant transition from its condition of winter dormancy to that of full vegetative activity.

Materials and methods

Plant material, growth conditions and stress treatment

One-year-old seedlings of *Populus nigra* were selected, cleansed of soil and freed from all laterals by cutting the first-order lateral roots at 4–5 cm from the taproot junction (Scippa et al. 2008). Before transplanting, pruned taproots were scanned with a calibrated LA1600+ flatbed scanner (Epson America Inc., Long Beach, CA) coupled with a lighting system for image acquisition. Root diameter and length were measured on the scanned images by using the WhinRhizo software v. 2003b (Regent Instruments Inc., Quebec, Canada). These measurements were referred to T_i (Time initial) corresponding to the time, February 15–20, 2006, when mechanical stress treatment began.

To simulate mechanical perturbations, the taproot of 30 seedlings (26.47 ± 0.69 cm long) were bent by using a right-angle curved steel net of 90°, where the point of maximum bending was located at 19.60 ± 0.52 cm from collar (see Fig. S1 in Supporting Information). These plants, together with the same number of unstressed ones linked to a vertical steel net, were planted in pots (35 wide × 45 high cm) filled with a mixture 6:3:1 of soil, peat and pumice, and grown under controlled water regime and natural photoperiod and temperature (for minimum, maximum and average temperature values see Fig. S2 in Supporting Information). Roots were excavated after 12 (T_0), 13 (T_1) and 14 (T_2) months of plant growth; in fact, a period of 12 months before the analysis was necessary to exclude any artifact deriving from transplantation operations. All the determinations were carried out along the taproot, starting at 10 cm from the root-collar, where a secondary structure was well developed (see Fig. S1 in Supporting Information). In the case of the stressed roots, the bent region was divided into three sectors (each 5 cm long; see Fig. S1B

in Supporting Information) corresponding to: (1) the region just above the bending zone, named above-bending sector (ABS; 12.09–17.09 cm distant from the collar); (2) the region representing the bending zone, named bending sector (BS; 17.10–22.10 cm distant from the collar, with a middle point at 19.60 cm); (3) the region just below the bending zone, named below-bending sector (BBS; 22.11–27.11 cm distant from the collar). In the case of unstressed taproots (C), the region between 10 and 25 cm was randomly sampled (see Fig. S1A in Supporting Information). Sampled taproots from identical sectors, freed of all laterals, were cut and stored in liquid N_2 for successive analysis of their lignin, indole-3-acetic acid, gibberellic acid 3 and 4, abscisic acid content and ACCox expression.

Modeling bending stress along the woody taproot

A simple modeling for mechanical forces distribution in the bent root was performed by the Impact – Explicit Dynamic Finite Element Program software (Impact, free explicit Finite Element Program Suite). Measurement of taproot diameters at the beginning of stress treatment (T_i) and at the end of the bending stress treatment (T_2) were considered. Plant material was considered as elasto-plastic (Fourcaud 2008). Taproot diameters of plants at time T_i and T_2 were computed and corresponding meshes were constructed with four node elements. Bending was performed through the application of a forced displacement at the narrow end of the taproot. Average values of tension and compression forces were calculated as the average, in the considered portion, of mesh node points (corresponding to the vertices of cells).

Morphological analysis

First-order lateral emission's architecture measurements were carried out at the end of the bending stress treatment (T_2). To facilitate measurements, first-order lateral roots of control and stressed plants were pruned again at 4–5 cm from the taproot junction. Plants were suspended and their orientation fixed by means of small strings. The three-dimensional position coordinates (x, y, z), diameter and topology (i.e. the branching hierarchic structure) of the taproot and the first-order laterals junction point were measured by using a 3D digitizer with a Long Ranger transmitter (3 SPACE Fastrak, Polhemus Inc., Colchester, VT). All first-order lateral roots with a proximal diameter larger than 0.5 mm were digitized. Data from the digitizer and root topology were logged by using the DIPLAMI software (Sinoquet et al. 1997) modified for root topology as described by Danjon et al. (1999). Because of their small size, lateral roots were assumed to be circular in cross section. Taproots occasionally had an elliptical shape; in this case, we recorded the largest diameter and its orientation, as well as the diameter perpendicular at the largest diameter. The output data file was analyzed by using the AMAPMOD software (Godin et al. 1997), which handles topological structures at several scales and provides 3D graphical reconstruction for data checking.

Within each growth condition and the three sectors examined, the clustering tendency of the first-order lateral emission points was evaluated by using circular statistical methods and, in particular, the Rayleigh's Uniformity test (Mardia and Jupp 2000); calculations were performed by using the ORIANA software v. 2.01 (Kovach Computing Services, Anglesey, Wales; Kovach 1994). The null hypothesis was that data were uniformly distributed. A probability value <0.05 was taken to indicate that data were not distributed uniformly, i.e. there was evidence of a preferred direction of lateral emission. The Z value was calculated as $Z = nm^2$, where n is the number of observations and m is the length of the mean vector. A greater mean vector length (and the resulting larger Z value) means a greater concentration of data around the mean, and thus less likelihood of the data being uniformly distributed.

Lignin content measurement

Lignin content within samples from ABS, BS and BBS regions and control was measured according to a previous protocol (Doster and Bostock 1988), with minor modifications. For lignin extraction, 1 g of root tissue was boiled in ethanol for 30 min, pulverized in N_2 and homogenized in 10 ml of 50 m*M* Tris–HCl, 0.01% Triton X-100, 1 *M* NaCl, pH 8.3 (extraction buffer). The suspension was stirred, centrifuged at 10 000 *g* for 10 min, washed twice with 4 ml of extraction buffer, 2 ml of 80% (v/v) acetone, 2 ml of acetone and then dried in a concentrator. Each pellet was then treated with 0.4 ml of thioglycolic acid and 2 ml of 2 *M* HCl, for 4 h, at 95°C, centrifuged at 15 000 *g* for 10 min and washed three times with distilled water. Lignothioglycolic acid from each pellet was then extracted with 2 ml of 0.5 *M* NaOH, understirring for 18 h, at 25°C. The supernatant was acidified with 0.4 ml of 37% (v/v) HCl. Lignothioglycolic acid was precipitated at 4°C, for 4 h, recovered by centrifugation at 15 000 *g* for 20 min, and dissolved in 1 ml of 0.5 *M* NaOH. Lignin amount within each sample was calculated by measuring the absorbance at 280 nm, using a specific absorbance coefficient of $6.0\ l{\cdot}g^{-1} \times$ cm. Because this specific absorbance coefficient provides only an approximate conversion (Doster and Bostock 1988a), the specimen

with the highest lignin content was also used as a standard in relative measurements of lignin content of other samples. The results of 20 independent assays were used for statistical analysis ($P < 0.01$).

Hormones extraction and analysis

IAA, gibberellin A_3(GA_3) plus gibberellin A_4(GA_4) and ABA content within samples from ABS, BS and BBS and control regions was measured by using a chromatographic procedure. To this purpose, weighed frozen samples (about 0.5 g) from taproots were ground with methanol (2.5 ml g^{-1} of fresh weight) in a mortar and pestle. Naphthalene acetic acid (Sigma) was added to each sample as internal standard (10 nmol g^{-1} of fresh tissue). Each extract was cleared by centrifugation at 16 000 *g*, for 10 min, at 4°C. Supernatant was then concentrated under vacuum to reach a one-tenth of the initial volume. A volume of pure water adjusted to pH 9 was then added to each sample, which then was extracted with an equal volume of ethyl acetate. Aqueous and organic phases were separated by centrifugation at 16 000 *g*, for 2 min. The lower aqueous phase was transferred to a new tube adjusting the pH of the solution below three to maintain all hormones in a protonated form; it was partitioned against ethyl acetate and cleared by centrifugation. The upper organic phase was then recovered, completely dried under vacuum and then dissolved in 30 µl of methanol before its further analysis by reversed-phase HPLC.

HPLC analysis was performed on a LC-20 Prominence HPLC system (Shimadzu, Japan) equipped with a LC-20AT quaternary gradient pump, a SPD-M20A photo diode array detector (PDAD) and a SIL-20 AH autosampler (20 µl injection volume). Plant hormones were separated on a Gemini-NX C_{18} column (250 × 4.5 mm, 5 µm particle size) (Phenomenex, Torrance, CA), assembled with a Security Guard® pre-column (Phenomenex) by using a gradient of acetonitrile containing 0.1% (v/v) trifluoroacetic acid in aqueous 0.1% (v/v) trifluoroacetic acid, at 45°C; acetonitrile ramped from 15 to 30% over 5 min, from 30 to 50% over 5 min, from 50 to 80% over 2 min, and then restoring the starting elution conditions, at a flow rate of 1.5 ml min^{-1}. Separated compounds were identified through their retention times, UV spectra and relative literature data by comparison with IAA (12886, Sigma, St Louis, MO) GA_3 (G7645, Sigma) and GA_4 (G7276, Sigma) and ABA (A1049, Sigma) standards. These standard compounds were also used to build up calibration curves (in the range 5–200 µg ml^{-1}) at specific wavelengths (λIAA = 254 nm; λABA = 254 nm; λGA_s = 205 nm). For quantitative analysis, two different extract amounts from unknown samples were injected in triplicate. Reported values represent the concentration (expressed as µg of hormone per gram of fresh tissues). Gibberellin concentration was reported as the sum of GA_3 and GA_4 content. The results of independent assays were used for statistical analysis; the mean value ± SD of three independent extractions is provided. In addition, we evaluated the correlation between spatial and temporal amount changes of each variable (IAA, ABA and $GA_3 + GA_4$) by Pearson's *r* correlation (Pearson 1957). Pearson's r correlation reflects the degree of linear relationship between two variables (ranging from +1 to −1 value). A correlation of +1 or −1 means that there is a perfect positive or negative linear relationship between variables, respectively. The correlation was significant at $P < 0.01$ and $P < 0.05$ level.

ACCox expression measurements

To evaluate spatial and temporal ethylene accumulation changes, we measured the expression of ACCox gene, an important enzyme involved in final steps of ethylene biosynthesis (Yang and Hoffman 1984, Kende 1993); experiments were performed by using RT-PCR analysis of control and ABS, BS and BBS regions of stressed taproots at T_0, T_1 and T_2. RNA was extracted from 0.1 g of taproots samples by using the Qiagen RNeasy Mini kit (Qiagen, Valencia, CA), according to manufacturer's suggestions. RNA concentration was measured by using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE); RNA integrity and quality was checked on 1.5% (w/v) agarose Tris–acetate EDTA ethidium bromide gels and by the ratio of absorbancies at 260 and 280 nm, respectively.

cDNA was synthesized by using 1.0 µg of total RNA, the poly(A) oligonucleotide primer and Superscript III reverse transcriptase (Invitrogen Co., Carlsbad, CA, USA). Gene-specific primers were used for PCR amplification of ACC oxidase gene (F5′-TT CAGGTTGAGAACCATGGAC-3′; R5′-GGGATCTTTAT CCATCCTCCA-3′). Conditions for RT-PCR reactions were as follows: 95°C for 4 min, followed by 38 cycles of 95°C for 35 s, nucleotide annealing at 50°C for 35 s, 72°C for 50 s, then followed by 1 cycle of 72°C for 7 min. PCR reactions were performed in 25 µl vol by using Taq recombinant polymerase (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer's protocol.

Three independent biological replicates were run for each sample, each with two technical replications. Images of gels were acquired by Chemidoc [BIO-RAD, Segrate (Milan), Italy] using QUANTITY ONE software (BIO-RAD, Hercules, CA). Gels were analyzed using IMAGEJ 1.41o software (Wayne Rasbanb, National

Institute of Health, Bethesda, MD; http//rsb.info.nih.gov/ij). To account for small differences in RNA loadings, data were normalized to cyclophilin gene expression (F 5′-GGCTAATTTTGCCGATGAGA-3′; R 5′-ACGTCCATCCCTTCAACAAC-3′).

Results

Distribution of mechanical forces along the root axis

A simple modeling of mechanical forces distribution in the bent root was performed on the basis of two main factors, namely root diameter and wood mechanical property variations. According to the results obtained in our experimental system, the distribution of mechanical forces applied on the taproot of poplar seedling changed considerably from the beginning (T_i) to the end (T_2) of the stress treatment. In particular, the stress level was lower and homogeneously distributed at T_i, whereas it changed at T_2, showing at this time a maximum value of compression and tensile stress of 38 and 32 MPa, respectively. Concerning the ABS, BS and BBS regions along the taproot, our model indicates that central sector (BS) was always characterized by the highest stress, as demonstrated by the compression and tension force values (Fig. 1). At T_i, compression force values were 12, 23 and 17 MPa for ABS, BS and BBS, respectively. At the same time, corresponding tensile force values resulted 11, 22 and 15 MPa, respectively. At T_2, compression force values varied to 14, 27 and 21 MPa for ABS, BS and BBS, respectively, while corresponding tensile forces were 12, 24 and 15 MPa, respectively.

Morphogenetic modifications of roots

The overall root architecture of control and stressed root apparatus was also compared at T_2; at this time, root differences became sufficiently apparent to be measured. In this regard, we observed a significant difference between stressed and control taproots (two-sample Kolmogorov-Smirnov test, $P < 0.001$). In fact, the number of first-order lateral roots in stressed taproots was generally higher than in control (Table 1); stressed roots also showed a smaller basal diameter than control ones (Table 1). In particular, stressed taproots presented a complex situation, with specific distribution frequency variations in ABS, BS and BBS (Fig. 2). An increase of the distribution frequency was observed for ABS and BS; conversely, BBS showed a distribution frequency very similar to the one present in control plants.

When we examined the three-dimensional distribution of the first-order laterals along the root axis, we also found a considerable difference between the control and stressed plants (Fig. 3A, B). In fact, an evaluation of the mean distribution demonstrated that the majority of first-order lateral roots in the bent taproots were emitted in the convex side (0° centered) (Fig. 3A), whereas in control they were distributed in various directions (contained within a 120° angle; Fig. 3B); this measurement was obtained by examining the taproot (in acropetal direction) at regular intervals (having a length of 5 cm) in a portion comprised between 10 to 25 cm from the root-collar. When we focused on BS, which includes

Table 1. Root parameters. Reported values are the mean of 30 replicates (±SE). *P* value refers to Student's *t*-test (parametric) and median test (nonparametric) depending on the fulfillment of the requirements of parametric analysis, respectively, at a significance level of $\alpha = 0.05$.

Trait	Control	Bending	*P*	Test
First-order laterals number (no.)	44.90 (1.71)	53.47 (1.82)	<0.001	*t* Student
First-order laterals basal diameter (mm)	4.2	3.9	<0.001	Median

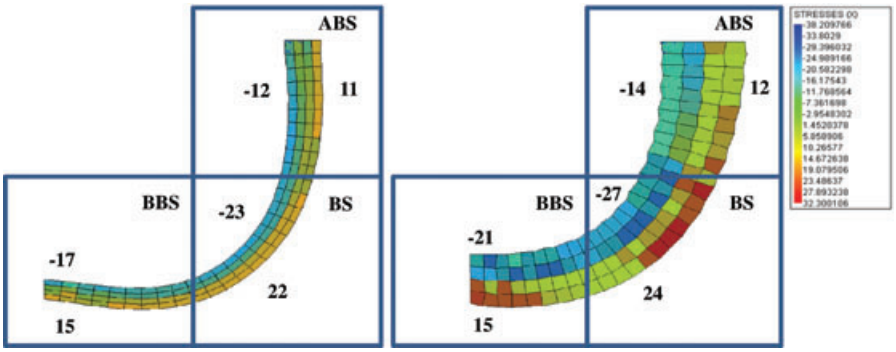

Fig. 1. Model of the mechanical forces distribution. Distribution of longitudinal mechanical forces along the *Populus nigra* taproot at time T_i (left) and T_2 (right). Average value of the mechanical force for the three defined sectors (ABS, BS, BBS) are indicated for both the areas being under tension- (positive values) and compression-condition (negative values).

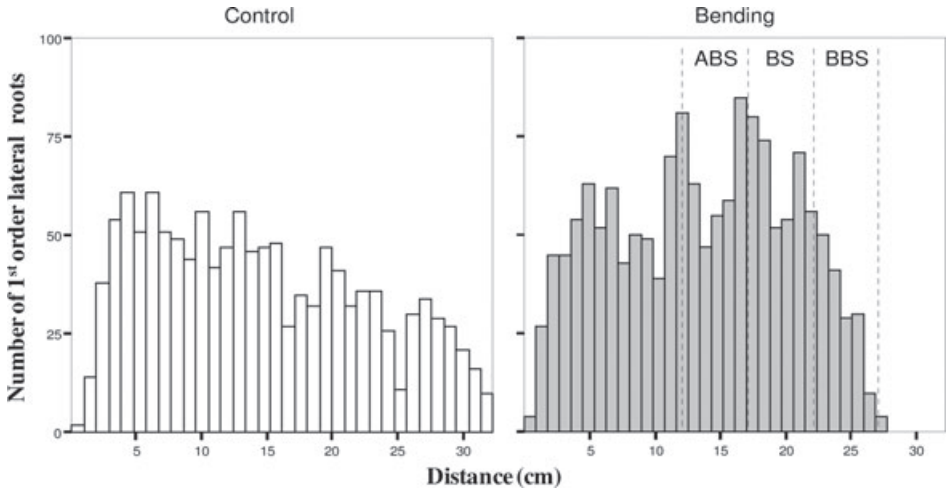

Fig. 2. Frequency distribution of first-order lateral roots along the *Populus nigra* taproots in acropetal direction (from 0 to 35 cm) after 14 months of stress treatment (T_2). For each treatment, data are referred to 30 replicates. Significant differences were evidenced by two-sample Kolmogorov-Smirnov test ($P < 0.001$).

only the curvature, the distribution differences between control and stressed taproots became even more evident, as demonstrated by the confidence interval reported in Fig. 3C, D, with a significant first-order laterals clustering present in the convex side of stressed taproots. These results were confirmed by the Rayleigh's test (Table 2).

Distribution of lignin along the root axis

Lignin concentration was measured in extracts from control and stressed (ABS, BS and BBS) taproots at T_0, T_1 and T_2, respectively. At T_0, the value ascertained in control roots was slightly higher than that determined in stressed ones (Fig. 4); in control taproots, this value did not change significantly during the time. Conversely, higher temporal variations in lignin content were observed in stressed taproots; these changes varied differently depending on the sector analyzed. In particular, ABS, which at T_0 showed the lowest lignin concentration between samples, reached a value at T_1 and T_2 generally comparable with those measured in control (at T_0, T_1 and T_2) and in BS and BBS (at T_1 and T_2), respectively. Conversely, BS and BBS showed a slight increase in lignin content at T_1 and a significant rise at T_2, reaching the highest values measured in this study. This phenomenon was more evident for BS.

Distribution of endogenous hormones along the root axis

Endogenous concentration of different plant hormones (IAA, $GA_3 + GA_4$ and ABA) was also measured in extracts from control and stressed tap roots at T_0, T_1 and T_2, respectively. In control taproots, the concentration of $GA_3 + GA_4$ and ABA decreased during the time, whereas the amount of IAA showed a slight increase at T_1 and T_2 (Fig. 5). For stressed taproots, these values differed depending on the sector (ABS, BS and BBS) and the time considered.

In particular, all hormones showed a significant content increase in ABS, with the highest value being reached at T_2. In BS, IAA concentration showed a progressive decrease passing from T_0 to T_2; an opposite trend was observed for $GA_3 + GA_4$, which showed its maximum value at T_2. Diversely, ABA concentration showed an increase from T_0 to T_1 then a decrease from T_1 to T_2 reaching values lower than T_0.

In BBS, IAA content increased passing from T_0 to T_2, whereas ABA and $GA_3 + GA_4$ showed unappreciable variations (Fig. 5).

To verify if the content of the three hormones was related, all measured values were analyzed by means of the Pearson's r Correlation test (Pearson 1957). Results demonstrated the existence of a significant relationship between all three hormones (Table 3), although the strongest correlation was found between the values of ABA and IAA.

ACCox gene expression along the root axis

As ACCox catalyzes the production of ethylene, its expression is widely used as an indicator of the site where ethylene is actually produced. Thus, we measured the ACCox gene expression in control and three (ABS, BS and BBS) portions of stressed taproots at T_0, T_1 and T_2, respectively. As reported in Fig. 6, results showed

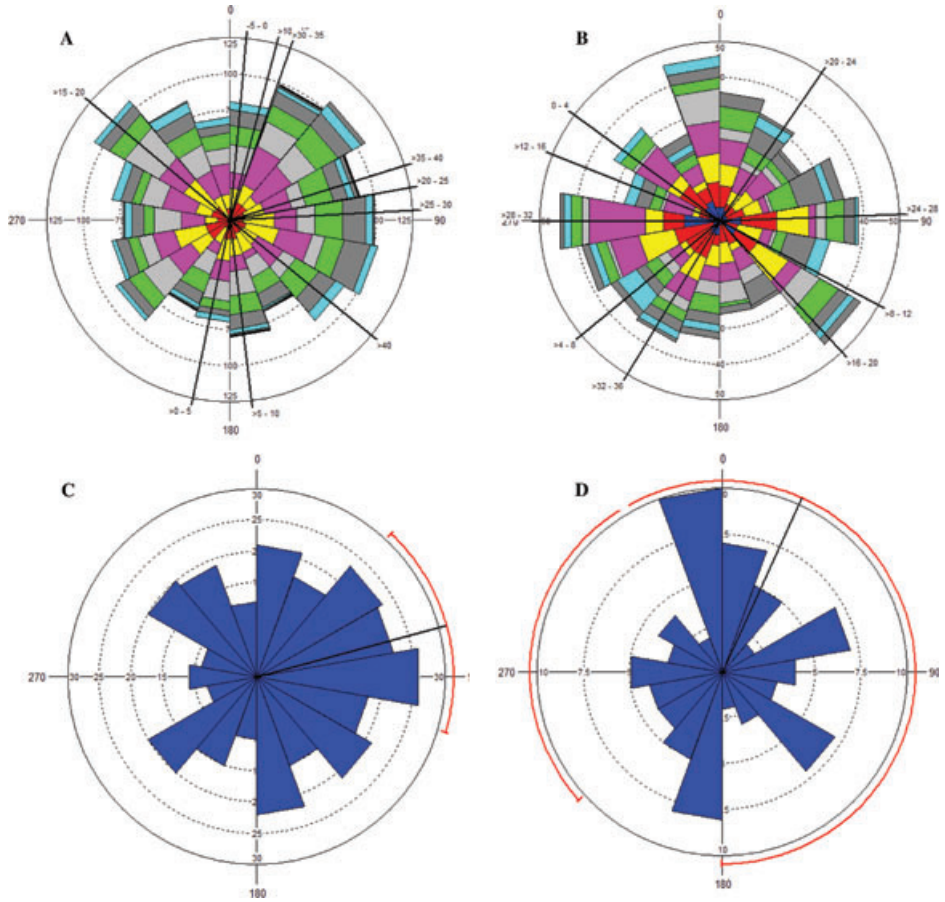

Fig. 3. Stacked rose of frequency of first-order lateral emission directions for stressed (A) and control (B) *Populus nigra* taproots measured along the taproot at 5 cm length intervals. The black lines originating from the center indicate the average emission direction within each 5 cm interval. Average lateral direction in BS (within the 17.1–22.1 cm taproot length) for stressed (C) and control (D) plants. The black lines originating from the center indicate the average emission direction, whereas the arc red line highlights the 95% confidence interval. 0° coincides with the convex side for stress treatment.

Table 2. Parameters of the vectorial circular analysis of the first-order lateral emissions in BS (within the 17.1–22.1 cm taproot length). The eccentricity vectors are significantly clustered at $P < 0.05$.

	Control	Bending
Mean vector (μ)	24.308°	74.672°
Length of mean vector (r)	0.055	0.142
Standard error of mean	79.591°	16.189°
95% Confidence interval (−/+) for μ	228.278°	42.935°
Rayleigh test (Z)	0.259	6.2
Rayleigh test (P)	0.772	0.002

that ACCox gene expression in control taproots resulted constant from T_0 to T_1, whereas it decreased at T_2. At T_0, ACCox expression levels measured in stressed regions were lower than control; in particular, the lower amounts were detected in ABS and BS. At T_1, ACCox expression increased in ABS, BS and BBS portions of the bent taproot, although the levels remained lower than control. At T_2, ACCox gene expression decreased differently in the three sectors; in BS, the value was similar to that of control, whereas in ABS and BBS it was higher (Fig. 6).

Discussion

Previous observations have established that the root apparatus of woody species grown on slope conditions presents a variation in the number and the spatial distribution (generating an asymmetric architecture) of lateral roots, with respect to plants present on plane. These morphogenic adaptations have been interpreted as an attempt of the roots to counteract active slope terrain forces or windy conditions, which menace the uprooting of the plant (Di Iorio et al. 2005, Chiatante and Scippa, 2006). A simple experimental system

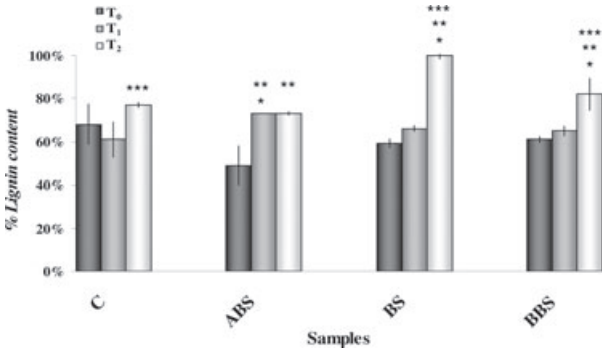

Fig. 4. Lignin content. Lignin content within control and three sectors (ABS, BS and BBS) of stressed *Populus nigra* taproots were measured from T_0 to T_2, according to the Doster and Bostock (1988) protocol. Lignin content is expressed as percentage of the value measured in BS bent roots at T_2 (considered 100%). Bars represent the mean of twenty biological replicates (±SD). $P < 0.01$ was used as level of significance. * indicates significant differences between ABS, BS, BBS and the control at T_0, T_1 and T_2. ** and *** indicate significant differences between $T_0 - T_1/T_0 - T_2$ and $T_1 - T_2$, respectively, for each sample (C, ABS, BS, BBS).

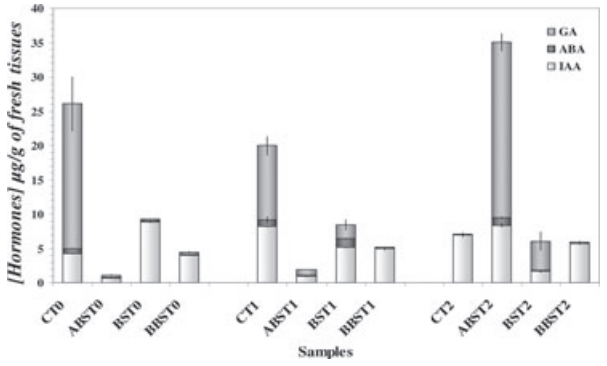

Fig. 5. Endogenous hormones concentration. Taproots of control and three regions (ABS, BS, BBS) of stressed *Populus nigra* samples were collected at T_0, T_1 and T_2 time points and extracted by methanol procedure. Extracted samples were then analyzed by HPLC. Separated compounds were identified by comparison with authentic standard derivatives based on their retention times, UV spectra and relative literature data. For quantitative analysis, calibration curves were constructed as described in the experimental section. Reported values are the mean of hormone content per weight of fresh tissues (µg g^{-1})± SD, as determined for three independent extractions.

was recently proposed by us to investigate long-term mechanical stresses affecting woody roots (Scippa et al. 2008). It highly resembles the situation/forces occurring in nature but, at the same time, it allows the organisms growing in container and greenhouse environments, without the need of expensive and time-consuming plant excavations in the field. *Populus nigra* woody taproot response to a static non-destructive long-term mechanical forces was studied by analyzing root morphology and mechanical properties, together

Table 3. Pearson Correlation values. Pearson's r Correlation values revealed a significant ($P < 0.01$** or $P < 0.05$*) relationship between all variables (IAA, ABA, GA_s and ACCox). 'r' reflects the degree of linear relationship between two variables (ranging from +1 to −1 value). '*P*' represents the probability to have the r-value result. *Correlation is significant at the 0.05 level (two-tailed). **Correlation is significant at the 0.01 level (two-tailed).

Pearson correlations				
		GAs	ABA	IAA
ACCox	r	0.062	−0.167	−0.133
	P	0.718	0.331	0.440
IAA	r	0.423*	0.630**	
	P	0.010	<0.0001	
ABA	r	0.488*		
	P	0.003		

with variations in the levels of plant hormones. In order to investigate mechanisms controlling woody root mechanical stress responses at the beginning of the growth resumption, lignin content and concentrations of auxin, gibberellins and abscisic acid, as well as levels of ACCox gene expression, were measured during three plant life times, T_0, $T_1$and T_2. Due to the lack of information on poplar woody root's annual life cycle we based our time course on shoot phenology observations, cross-related to yearly average temperatures recorded at the experimental site and literature data (Howe et al. 1995, Chen et al. 2002; see Fig. S2 in Supporting Information). On this basis, we assumed that T_0, T_1 and T_2, corresponded to the plant dormancy time, the beginning of vegetative growth and active growth, respectively.

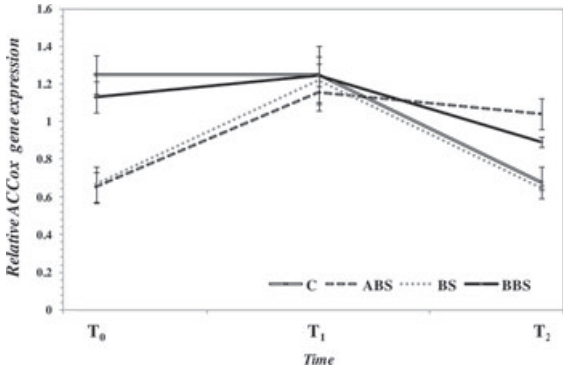

Fig. 6. Relative ACCox gene expression. Relative ACCox gene expression was measured by RT-PCR in control and ABS, BS, BBS portions of stressed taproots at T_0, T_1 and T_2, as reported in the experimental section. Three independent biological replicates were run for each sample, each with two technical replications. Results were analyzed by using Image J 1.41o software (Wayne Rasbanb National Institute of Health, USA; http//rsb.info.nih.gov/ij). Data were normalized to cyclophilingene expression values ± SD.

The morphological data presented here clearly indicate that total number and diameter of first-order lateral roots in stressed plants present a significant variation with respect to control ones (Table I). In particular, morphogenetic inductions were correlated to the distribution of mechanical forces, as revealed by our mechanical model (Fig. 1). In fact, an higher number of lateral roots occurred in ABS and BS (Fig. 2). An asymmetric distribution of lateral roots was also observed, with the highest concentration of new lateral roots occurring in the convex side of BS. The mechanisms of lateral root emission by parental root have been extensively investigated at molecular level in the model herbaceous plant *Arabidopsis thaliana* (De Smet et al. 2007, Laskowski et al. 2008, Lucas et al. 2008a, 2008b, Peret et al. 2009). Particularly, two recent studies, have shown that artificial bending induces the emission of lateral roots on the convex site of the curved taproot (Ditengou et al. 2008, Richter et al. 2009), similar to what we observed in this study. These data suggest that, despite the anatomical differences existing in the secondary growth compared to primary taproot structure, it is reasonable to hypothesize that stress perception and consequent morphogenetic response elicited by root bending may follow similar physiological mechanisms.

Angiosperm trees constantly develop specialized woody tissues termed 'reaction wood', which are thought to be part of a stress-sensing mechanism that leads to an increased mechanical support and corrects bent stem growth (Sinnott 1952, Barnett 1981, Timell 1986, Wu et al. 2000). Reaction wood is composed of tension wood (TW), induced in the upper side of the bent tree stem, and opposite wood (OW), which accumulates in compression stressed xylem tissue, in the stem wood opposite to TW (Wu et al. 2000). An increase of lignin content has been reported to characterize OW, where it provides a mechanical support in the side opposite to the direction of the tension force (Timell 1986, Hu et al. 1999, Wu et al. 2000).

While most of the literature refer to the angiosperm stem, very few data are available about mechanically stressed roots and, in particular, about reaction wood development and composition. Although not investigating the anatomy of the bent taproots, here we demonstrate that lignin content is spatially and temporally altered in the sectors subjected to bending, with the highest levels of lignin occurring in BS and BBS at T_2. Hence, based on force distribution modeling (Fig. 1), it is reasonable to hypothesize that the increase of compression forces in BS and BBS (from T_i to T_2) may trigger cell wall stiffening by means of new lignin deposition. In this context, future studies have to be realized to investigate whether lignin accumulation may occur asymmetrically in BS, with the aim of accounting for the prevalent localization of lateral roots in the convex side. We have recently shown that the emergence of new lateral roots originating from the parenchyma ray initial cells involves the degradation of secondary tissues (Chiatante et al. 2010). In this respect, an higher lignin concentration in the concave side could hamper emission of new lateral roots. Increase of lignin accumulation started in T_1 phase, thus suggesting that mechanical stress induces an early metabolism reactivation as compared to what physiologically occurs in T_2 phase.

It is well-known that hormones, such as IAA, GAs, ABA and ethylene, are involved in modulating mechanical stress responses (Okada and Shimura 1994, Sundberg et al. 2000, Bostock 2005, Braam 2005, Fujita et al. 2006), and behave as key factors in regulating dormancy/vegetative growth (Rinne et al. 1997, 1998, Welling et al. 1997, 2002, Li et al. 2002, Ruonala et al. 2006, Druart et al. 2007). On the basis of this knowledge, we measured their concentration (or levels of ACCox gene expression) in control and stressed taproots at different times (from T_0 to T_2). At the beginning of the time course (T_0), concentration of ABA and ACCox transcript level, as measured in ABS, BS and BBS of stressed taproot, was significantly lower than in the unstressed taproot. This result may be indicative that vegetative growth resumption in the bent taproot occurs earlier compared to the unstressed root, in accordance to what was observed for lignin deposition (see above). Processes as flowering, dormancy and senescence have been reported to be influenced by mechanical perturbation (Anten et al. 2005), and a stem radial growth increase has been observed in Scots pine (*Pinus sylvestris* L.) seedlings subjected to bending during winter dormancy (Valinger et al. 1995). Although the ecological meaning remains to be elucidated, evidence reported in the above-mentioned literature seems to support the hypothesis that the long-term bending stress is perceived by the woody taproot as an internal signal for anticipating dormancy breaking and growth resumption. More difficult is to rationalize the levels of GAs and IAA we measured in ABS, BS and BBS of stressed taproot at T_0, since both hormones have been reported to be involved in regulating growth cycle (Schrader et al. 2003, 2004, Druart et al. 2007). However, the decrease of GA-20 oxidase transcript, as associated with GAs level after cambium growth resumption in poplar stem (Druart et al. 2007), may be an additional evidence for the earlier dormancy break proposed in the bent taproot.

With the progression of time, hormone levels and ACCox gene expression showed a different distribution

in ABS, BS and BBS. It is well established that in the primary root, plant hormones and environmental signals co-ordinately regulate lateral root formation (reviewed in Casimiro et al. 2003, Lopez-Bucio et al. 2003, Malamy 2005), and that auxin plays the major role in lateral root emission, formation and development (Himanen et al. 2004, Aloni et al. 2006, Ditegout et al. 2008, Ivanchenko et al. 2008, Richter et al. 2009). Moreover, other hormones seem to affect lateral root formation at various stages, by positively or negatively interacting with auxin (De Smet et al. 2003, Aloni et al. 2006, Stepanova et al. 2007, Ivanchenko et al. 2008, Fukaki and Tasaka 2009, Gou et al. 2010). The involvement of abscisic acid in lateral root formation has been studied mainly using ABA signaling mutants in *Arabidopsis*. These studies showed that ABA has distinct roles at different stages in lateral root development (De Smet et al. 2003, 2006). In fact, the balance between auxin-promoting and ABA-repressive signaling pathways activation appears to control lateral root development during primordium development and emergence. The upregulation of genes involved in ABA biosynthesis during lateral root initiation (Taylor et al. 2000, Vanneste et al. 2005) suggests that auxin-induced ABA biosynthesis taking place in cells surrounding the lateral root initiation site is essential to define the organ boundaries for future lateral roots (De Smet et al. 2006). Experimental data showed that ABA suppresses lateral root meristem emergence (De Smet et al. 2003). Although no direct evidence for long-term lateral root dormancy was produced, it was suggested that under unfavorable conditions ABA might impose to the lateral root primordia a 'dormant' state (De Smet et al. 2003).

The role of gibberellins in lateral root formation is poorly understood (Osmont et al. 2007, Fukaki and Tasaka 2009). However, some authors suggested that the growth-promoting effect of auxin on taproots and lateral root seems to be mediated by the GAs either in *Arabidopsis* (Aubert et al. 1998), tomato (Shi and Olszewski 1998), rice (Furukawa et al. 2006), petunia (Ben-Nissan et al. 2004) and maize (Zimmermann et al. 2010). As far as ethylene, recently it has been reported that this hormone either regulates lateral root development in *Arabidopsis* by partial crosstalk with auxin, and might also interact according to different mechanisms with auxin in lateral root primordia initiation and emergence (Ivanchenko et al. 2008).

We verified that the bending sector (BS) is subjected to the highest compression and tension forces that, correspondingly, bring about the most prominent alteration of cell wall mechanical properties and lateral root emission, among the three sectors investigated. In the BS sector, interestingly, auxin levels at T_0 were unusually higher than that measured in the unstressed root or to the ABS and BBS sectors. On the other hand, IAA levels showed a slight reduction at the T_1 phase, which was accompanied by a significant increase in the GAs and ABA content, as well as in ACCox transcript level. At T_2, IAA level was decreased and likewise the ABA content and ACCox gene expression, whereas the GAs level was at maximum. This hormone profile and its evolution during time appears peculiar of the BS sector, where the highest mechanical stress occurs. In particular, the occurrence of precociously high levels of auxin may be a strong indication that this hormone can trigger the primary response to the applied stress and can influence the subsequent variation of ABA, ethylene and GAs levels. This profile is maintained in the T_1 phase (when the stress response is still to be completed); in the later phase, IAA (as well as ABA and ACCox expression) levels decline, leaving place to a GAs maximum, which contribute to full development of the new organs. In fact, it is well-known that auxin can regulate GAs biosynthesis (Frigerio et al. 2006) whereas ethylene seems to control auxin biosynthesis (Ivanchenko et al. 2008). Furthermore, IAA is involved in the regulation of mechanical stress response (Mitchell 1977) and, together with GAs, regulates wood formation from cambium (Aloni et al. 2006). Experiments on mechanical stimulated-induction of reaction wood in the stem of several tree species showed that the frequency of cambial cell divisions is increased at early developmental stages (Ko et al. 2004, Telewski 2006). Auxin has been reported to control the extent of cambial growth both in gymnosperm and angiosperm trees (Sundberg et al. 2000) and induce GA biosynthesis (Taiz and Zeiger 2006). Both gibberellin and auxin also regulate lignin biosynthesis (Aloni et al. 1990). On the basis of these preliminary pieces of evidence auxin and GAs may play apivotal role in controlling reaction wood formation. At the beginning of growth resumption (T_0–T_1), auxin could promotes cambial cell division; later on (T_2), the increase of GAs may control lignin accumulation.

In BBS, where compression forces increased with growth and a gravitropic response is occurring, hormone levels were almost unchanged during timecourse; conversely, in ABS, where a reduced mechanical stress is occurring, interpretation of hormonal profile was less clear. BBS presented an increase of lignin accumulation, whereas ABS was characterized by an high lateral roots production. These results might indicate the presence in the three stressed regions of multiple hormones signaling pathways differentially modulated during time (Baba et al. 2011).

Considering that a very few data are available on woody plants, the results reported in this work 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, such as lateral root emission and reaction wood formation. Our results also suggest that in woody root, under mechanical stress conditions, a complex interplay among IAA, GAs, ABA and ethylene may take place. These data will promote future investigations at molecular level with the aim to decipher hormonal variations in single tissues of root sectors, which are involved in the response to mechanical stress.

Acknowledgements – This work was supported by the Italian MIUR (Ministero Istruzione Università e Ricerca; Progetti di Rilevanza Nazionale, PRIN 2005, 2008) and it was carried out in the context of the COST Action E38, financed by the European Commission.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Experimental system.

Fig. S2. Seasonal climatic changes.

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Edited by Y. Helariutta