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1 Gradients and dynamics of inner bark and needle osmotic potentials in Scots
2 pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* (L.) Karst.)

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10

11 ABSTRACT

12 Preconditions of phloem transport in conifers are relatively unknown. We studied the variation of
13 needle and inner bark axial osmotic gradients and xylem water potential in Scots pine and Norway
14 spruce by measuring needle and inner bark osmolality in saplings and mature trees over several
15 periods within a growing season. The needle and inner bark osmolality was strongly related to xylem
water potential in all studied trees. Sugar concentration

48 Turgor pressure is vital for living cells to maintain functionality and achieve growth (Lockhart 1965).
49 Turgor pressure of the phloem tissue is driven by its osmotic potential and is most likely impacted by
50 xylem water potential (Thompson & Holbrook 2003; Hölttä et al. 2006). The water potential in xylem
51 practically consists of the pressure potential created by water since the osmotic potential in that
52 tissue is assumed to be small (Scholander et al. 1965).

53
54 The osmotic potential (and osmolality) is driven by osmotically active
55 solutes, e.g. carbohydrates and ions in an aqueous solution. In most tree species, sucrose is the vast
56 majority of transported carbohydrates in phloem sap, and therefore, it is expected to have a
57 significant effect on the changes of osmotic potential in phloem (Zimmermann 1957; Pate 1976;
58 Turgeon & Wolf 2009).

59 The foundation of phloem transport theory has for long been the hydrostatic (turgor) pressure
60 difference between the relative solute input and output locations, source and sink, which is also
61 referred to as the 'Münch hypothesis' (Münch 1930; Kaufmann & Kramer 1967; Christy & Ferrier
62 1973; Knoblauch & van Bel 1998; Thompson & Holbrook 2003; Minchin & Lacombe 2005; Knoblauch
63 & Peters 2013; An et al. 2014; Knoblauch et al. 2016). The estimations of phloem osmotic potential
64 and xylem water potential enable the turgor pressure to be estimated since phloem tends to
65 equilibrate rapidly with the water potential of the surrounding tissues (Thompson & Holbrook 2003).
66 The phloem turgor pressure has been very difficult to study since direct measurements change the
67 conditions inside the pressurized sieve cells (e.g. Sovonick-Dunford et al. 1981; van Bel 2003; Turgeon
68 & Wolf 2009). That is why today there is still a lack of measured data on turgor pressure and a lack of
69 support for the theory behind the phloem transport (An et al. 2014; Ryan & Asao 2014; Steppe et al.
70 2015), although a very few studies have successfully measured phloem turgor pressure differences
71 directly on smaller plants (Gould et al. 2006; Knoblauch et al. 2016). Data about gymnosperms or
72 data collected in the field conditions are especially scarce (Turgeon 2010; Knoblauch et al. 2016).

73 As phloem and xylem equilibrate rapidly in water potential (Molz & Klepper 1973;
74 Sovonick-Dunford et al. 1981; [Hölttä et al. 2006](#)) it has been hypothesized that water potential in
75 xylem impact the turgor pressure in phloem and more so higher in the tree (Sevanto et al. 2002;
76 Woodruff et al. 2004; Hölttä et al. 2006). Low xylem water potential might hinder phloem transport
77 by causing an accumulation of sugars that affects the phloem loading from the leaf mesophyll (Hölttä
78 et al. 2006; Lieshce et al. 2011; Nikinmaa et al. 2013). Both the sink and source dynamics are this way
79 connected to stomatal control and tree water relations (Paul and Foyer 2001; Nikinmaa et al. 2013).

80 One key question concerning phloem transport in trees is whether the turgor pressure gradient over
81 the whole transport pathway alone, can explain phloem transport over long distances such as those
82 that are found in taller trees. It is not known how plants could regulate the turgor gradient over long
83 distances (Thompson 2006; Ryan and Asao 2014). Mencuccini & Hölttä (2010) predicted, based on
84 the scaling between plant height and phloem transport rates from different studies that the turgor
85 pressure varies with plant height. This mechanism could also explain the long-distance phloem
86 transport with the aid of structural adjustments along the transport pathway from canopy to roots.
87 The phloem conduit lumen size has been found to impact phloem conductivity significantly (Jensen
88 et al. 2012; Woodruff 2014). Several studies have reported gradual changes in conduit size from the
89 stem apex towards the tree base (Rosner et al. 2001; Petit & Crivellaro 2014; Woodruff 2014; Jyske &
90 Hölttä 2015). The ratio of phloem conductivity to xylem conductivity has also been found to decrease
91 towards the base of the tree (Jyske & Hölttä 2015). These studies indicate that structural
92 adjustments do exist as a function of tree height and they are bound to affect the turgor pressure
93 gradient through the phloem and xylem conductivity. It is also possible that leakage and retrieval of
94 sugars along the phloem transport pathway is aiding in maintaining a sufficient turgor gradient
95 especially in larger trees (Thompson and Holbrook 2003; Epron et al. 2016).

96 Axial osmotic pressure gradients have been studied and reported since the 1930s to find support for
97 the Münch hypothesis in natural conditions (Münch 1930; Zimmermann 1960; Kaufmann & Kramer

1967; Hammel 1968; Rosner et al. 2001). Axial osmotic gradients can be expected to counteract the xylem water potential gradient and maintain a turgor pressure gradient from the source to sink. In addition, the osmotic gradient may be expected to be steeper near the apex since the conduit size at the apex is smaller for both xylem and phloem (Anfodillo 2006; Woodruff 2014; Petit & Crivellaro 2014; Jyske & Hölttä 2015). The phloem transport velocities and allocation of sugars in trees are also affected by seasonality (Dannoura et al. 2011). The sink strength of growing tissue is dependent both on temperature and stage of phenological development, which theoretically should impact upon the magnitude and perhaps even the direction of the turgor gradient. The previous studies on axial osmotic gradients have almost exclusively detected decreasing osmotic gradients from leaves to the stem base. However, an osmotic gradient translates to a turgor pressure gradient only when it is larger than the xylem water potential gradient (Kaufmann & Kramer 1967). Some support for a turgor pressure gradient has been obtained from studies involving osmotic pressure measurements in angiosperms but with differing methods (Hammel 1968; Rogers & Peel 1975). Temporal studies of phloem pressure have been conducted at least on white ash (*Fraxinus americana* L.) (Lee 1981). The osmotic gradients of conifers have been studied mainly using Norway spruce (*Picea abies* Karst.) (Rosner et al. 2001). Temporal studies of water and sugar content in the phloem have also been carried out on Norway spruce and spatial studies on Douglas-fir (*Pseudotsuga menziesii*) (Gall et al. 2002; Woodruff et al. 2004; Woodruff 2014). Osmotic gradient studies on Scots pine (*Pinus sylvestris* L.) are scarce or have not been carried out.

Temporal estimations of turgor pressure *in situ* also seem to be missing with Scots pine.

Our aim in this study was to measure the osmolality gradients in Scots pine and Norway spruce to find preconditions of turgor gradient driving phloem transport in conifers. We measured osmolality in needles and inner bark of branches, upper stem and stem base in mature trees. The osmolality in Scots pine and Norway spruce saplings were also measured in needles, stem bark and coarse roots. Additionally, we measured xylem water potential and estimated its dynamics with point

dendrometer measurements to understand how the water potential equilibrium is maintained between xylem and inner bark. Water and solute contents as well as sugars were examined to understand the drivers of osmotic potential and turgor pressure.

METHODS

Measurement site and plant material

The measurements of the mature Scots pine were carried out at the Station for Measuring Forest Ecosystem – Atmosphere Relations (SMEAR II) in southern Finland (61° 51' N, 24° 17' E, 181 m above sea level) (Hari et al. 2013). The mean growing season at that location is from the end of April to mid-October (Vanhatalo et al. 2015). Mean annual rainfall is approximately 700 mm (Pirinen et al. 2012). The forest stand is dominated by 50-year-old Scots pine trees and it represents a typical managed sub-xeric heath forest of Finland. The mean height of the trees was 18 m and the mean diameter at breast height was 18.5 cm during the sampling periods in 2014 and 2015. The mean soil depth is approximately 0.5 m above the bed rock (Vanhatalo et al. 2015). Measurements of the mature Scots pine were carried out in the early autumn of 2011, in July and September 2014 and in June 2015. The mean temperatures over these measurement periods and some other environmental variables are shown in Table 1. Measurements of the mature Norway spruce were conducted during the growing season in 2012 in a spruce dominated forest site in Haapastensyrjä, Southern Finland (60° 4' N, 24° 3' E, 120 m above sea level). The spruce trees were 30 years old and rooted from cuttings (clone no. 255) planted in a fertile former agricultural land (Jyske et al. 2015). The sun rises approximately at 4:00, 4:30 and 6:30, and sets around 23:00, 22:30 and 20:00 in June, July and mid-September, respectively, in Southern Finland (University of Helsinki Almanac Office).

Scots pine saplings were collected in the beginning of May 2012 from a forest opening that is situated close to SMEAR II station in a similar vegetation type to mature Scots pine trees in this study. The initial height of the saplings was about 40 cm. The saplings were carefully dug up with a shovel,

avoiding damage to the roots, and planted into pots of 16.5 cm in diameter and 18.0 cm in depth. The soil used for planting was taken from the growing site. Planted saplings were kept in the greenhouse during the winter and outside, under a transparent roof, during summer. Norway spruce saplings were obtained from a greenhouse experiment in Haapastensyrjä, Loppi, Southern Finland, during the growing season of 2012. The saplings were grafted in 2009 by using cuttings (clones E330, E2833, and K8051) of mother trees originating from central Finland. The mean height of the grafts was 63 ± 11 cm (from the top to the grafting point). The saplings had been grown outside until the autumn of 2011, and then planted in pots. The potted grafts were stored over winter in a plastic-covered greenhouse in ambient temperature but without snow cover. The roots in the pots were covered with moss to avoid frost damage. The potted grafts were transferred into greenhouse on the 15th of March 2012 into two departments. One department had temperature elevated to +1°C above the ambient (outdoors) conditions; the temperature was always kept above freezing point. The other department had temperature +4°C above the ambient. The temperature differentiation started on 10th April.

Stem and xylem diameter

The stem and xylem diameter variations were measured by linear displacement transducers (LVDT, Solartron AX/5.0/S, Solartron Inc. West Sussex, UK). Both the stem and xylem sensors were attached side-by-side to a metal frame around the tree through holes and tightened with a screw at 1.5 m height. The xylem sensor rested against a screw that was attached to the xylem, whereas the stem sensor rested against the bark where the rhytidome had been removed (e.g. Mencuccini et al. 2013). The data received of the diameter changes occurred at a one-minute frequency.

Bark and needle sampling

173 The numbers and timings of sample collections are shown in Table 2. The sample positions are
174 illustrated in Figure 1. The osmolality and water content samples of mature Scots pine from the
175 needles, the branches, the upper stem and the stem base were collected at 7:00 and 15:00 resulting
176 in two samples per sampling position per day for five consecutive days in 2011. In total 6–8 samples
177 per position for two consecutive days were collected between 9:00 and 19:30 with approximately
178 three hours between the samplings in July and September in 2014. The sampling procedure in June
179 2015 was in other ways similar except that an additional sample was collected at 7:00, which
180 amounted to between 7–10 samples per position for two consecutive days. The measurements of
181 the Scots pine were carried out on two mature trees and the sampling involved only one tree per
182 measurement period. A tree with (LVDT) diameter measurements was used for the sample
183 collections in July 2014 and June 2015. Samples taken from August to September in 2011 and in
184 September 2014 were collected from the tree without the diameter measurements. The needle and
185 branch samples were collected from the canopy that was exposed to light at 16 m height. Branches
186 of a mean length of 40 cm were first cut from the tree and samples were immediately collected. The
187 stem samples were collected from the upper stem, which located at the lower canopy, and from the
188 stem base, 10–12 m and 1.5–2.5 m in height, respectively. Bark samples including tissues of the
189 cambium and the cork cambium were detached using a scalpel after the cork was removed. We
190 henceforth refer to these samples as ‘inner bark’ in this study. The sample dimensions varied from
191 0.5 to 1 cm in width and 1.5–2 cm in length. The samples were immediately placed in 5 ml cryo tubes
192 (Nunc, Thermo Scientific, Massachusetts, US), which were then frozen in liquid nitrogen. Samples for
193 the osmolality analysis were stored in a freezer at -80 °C. Two bark pieces with similar dimensions
194 that were adjacent to osmolality samples were collected for water content and sugar concentration
195 measurements. The sugar concentration samples were collected only in July and September 2014. All
196 the sugar concentration samples were first frozen and stored in -80 °C. The needle samples for water
197 content were collected from the same group of needles in the same branch as the osmolality

198 samples. One sample collection, included osmolality, sugar and water content samples, took
199 approximately 45 to 60 minutes.

200

201 Samples were taken from two mature Norway spruce trees that were 23 meters in height.
202 Collections were carried out once every month between 13:00 and 15:00 from May to August in
203 2012. The sampling heights of the needles and the branches were the same and both varied from 13
204 to 17 m. The upper stem and stem base heights were similar to those with Scots pine being 11–13 m
205 and 1.3 m, respectively. In addition, samples from the stem at the ground level were collected.
206 Sampling of all positions were carried out in July and August. All samples in August were taken at
207 predawn. At other times samples were collected only from upper stem and stem base. Only
208 osmolality and water potential was measured for Norway spruce.

209 The data of Scots pine saplings has already been published previously (Aaltonen et al. 2017). Samples
210 from Scots pine saplings were collected from needles, inner bark of stem and coarse roots with a
211 scalpel. This procedure, carried out in August 2013, was similar to sampling of mature trees. The
212 stem samples were collected from the middle of the stem. Samples were placed in cryo tubes and
213 stored in liquid nitrogen until measuring. Stem inner bark samples from Norway spruce saplings were
214 collected from 6 individuals each time, which represented one clone per treatment (i.e., one graft
215 each of clone E330, E2833, and K805 from both greenhouse departments) once or twice a month
216 from April to August and once on October. Needles and inner bark of roots were collected in mid-
217 May, mid-June, late July, and mid-October with the stem bark sampling. The samples were collected
218 between 12:00 and 15:00. The sampling locations for needles, stem inner bark, and root inner bark
219 were within the upper 1/3 of the crown, between the whorls of years 2010 and 2011, and the upper
220 coarse roots, respectively. After sampling, the outer bark was immediately removed and all samples
221 placed into cryo tubes, frozen with dry ice (-78°C), and then stored in -80°C until analysis.

222

223 *The osmolality analyses*

224 The sap from inner bark and needles was extracted using a method similar to those previously
225 described by Irvine et al. (1998) and Devaux et al. (2009). We measured the mean osmolality of the
226 needles and inner bark of samples that had undergone the freezing and mechanical sap extraction.
227 The samples were kept at room temperature for 15 to 30 min inside cryotubes to thaw before the
228 sap extraction. After this, a fresh cut was made in the bark samples with a scalpel. Each needle
229 sample included five pairs of needles cut in two. Needle and bark samples were set in silica-based
230 membrane collection tubes (GeneJET Plasmid Miniprep Kit, Thermo Scientific, Massachusetts, USA)
231 the fresh cut downwards against the membrane. Sap was extracted with a centrifuge (Heraeus
232 Fresco 17 Centrifuge, Thermo Scientific, Massachusetts, USA) in 14000 g 10 min prior to osmolality
233 measurement (Devaux et al. 2009). The extracted liquid was immediately moved to osmometer
234 tubes with a pipette and measured with a freezing point osmometer (Osmomat 030 cryoscopic
235 osmometer, Gonotec, Berlin, DE). The exposure to room air was avoided during the sample
236 preparation. Samples were ready for osmometer measurement within an hour from thawing.

237 The osmolality value was measured as the mean of two repetitions in mature Scots pine in 2011 and
238 2014. At other times and in other studied trees, only one repetition was used. The cell membranes
239 are expected to be damaged and to have released their contents into the apoplast after rapid
240 freezing in liquid nitrogen (Steponkus 1984; Fuller 2004). The mechanically extracted sap from the
241 needle and inner bark samples therefore consists of a mixture of phloem sap and sap from the
242 surrounding cells and from the apoplast. The phloem cells in particular are expected to die as a result
243 of this kind of procedure (Ball 2004). However, the advantage of mechanical sap extraction with a
244 centrifuge is that no solvents are added, and the results describe actual circumstances in the samples
245 (Devaux et al. 2009). The unit of osmolality measurement is molar concentrations of solutes per mass
246 of water and is expressed as mol kg^{-1} . Osmolality is temperature independent because it is related to
247 mass and not the volume (Lord 1999).

248

249 *Effects of sampling procedure on results describing the phloem dynamics*

250 The inner bark samples, consisting of tissues from the cambium to cork cambium, are a
251 heterogeneous unit and the actual sieve cells, through which the phloem transport takes place, is
252 only a small part of the unit. Measurements targeted accurately at the conducting phloem are
253 difficult to execute in practice. They contain a large risk of error due to the evaporation from samples
254 that occurs during the protocol. Therefore, our approach is less precise but is also supported by
255 previous studies (Rosner et al. 2001; Thompson & Holbrook 2003). The osmotic potential values
256 obtained from the conductive phloem when measured alone, have been reported to be higher but
257 they follow similar dynamics to those obtained in the conducting and non-conducting phloem when
258 measured in combination (Rosner et al. 2001). The *in situ* osmolality seems not to be very different
259 between these two compartments in Norway spruce (Rosner et al. 2001).

260 *Water content of tissue*

261 The fresh weight for water content was measured immediately after sample collection. Then, the
262 samples were stored in a freezer in -20 °C for less than three weeks before they were dried in an
263 oven at 80 °C for 72 hours, after which their dry weight was measured. The water content (WC) of
264 samples was calculated as

$$265 \quad WC = (FW - DW)/DW \quad (1)$$

266 where FW is fresh weight and DW is dry weight.

267

268 *Relative water content*

269 The turgid weight of samples was measured for relative water content calculations. The turgid
270 weight was measured after the samples had been saturated in closed tubes with milli-Q water in a

271 | cold room at 5 °C for 48 hours. Samples were preserved in a cold room at 5 °C for 24 hours before
272 | the saturation procedure began. Before weighing, the extra water on the surface of samples was
273 | carefully wiped with a tissue. The relative water content (RWC) was calculated as

274 |
$$RWC = (FW - DW) / (TW - DW) \quad (2)$$

275 | where TW is the turgid weight.

276 |

277 | *Osmolality at full saturation*

278 | The relative water content (RWC) was used for calculating the osmolality at full saturation as
279 | follows (Takami et al. 1981):

280 |
$$osMol_{full \text{ saturation}} = osMol_{in \text{ situ}} * RWC \quad (3)$$

281 | where $osMol_{in \text{ situ}}$ is the *in situ* osmolality in $mol \text{ kg}^{-1}$.

282 |

283 | *Sugar concentrations*

284 | The soluble sugar concentrations in the needle and inner bark samples were measured at the Natural
285 | Resources Institute Finland laboratory. The samples were freeze-dried and milled into powder in a
286 | ball mill while frozen. About 50 mg of the powder was weighed into glass test tubes. The soluble
287 | sugars were extracted with an 80% aquatic ethanol solution to which meso-erythritol (Sigma) was
288 | added as an internal standard. The tubes were placed in an ultrasonic water bath for 45 minutes, and
289 | then eluted overnight. The suspension was then shaken and centrifuged at 3000 rpm for 3 min. A
290 | 450 μ l volume of the supernatant of each sample was pipetted into a separate autosampler vial for
291 | three times (three repetitive subsamples). The samples were dried with N_2 flow, placed into a
292 | vacuum oven for at least 15 min, and then silylated with 0.5 ml of N-trimethylsilyl imidazole (TMSi in
293 | pyridine, Sigma-Aldrich) for at least two hours or overnight, and analysed by gas chromatography–

mass spectrometry (GC–MS; Agilent Hewlett-Packard 6890 GC, fitted with a Zebron ZB-SemiVolatiles column (30 m 9 0.25 mm i.d. 90.25 lm df) and Hewlett-Packard 5973 MSD, EI-MS 70 eV), in which helium was used as the carrier gas (flow 1.5 ml/min). The chromatographic conditions were as follows: an initial temperature of 110 °C; rate of temperature increase 10 °C min⁻¹; a final temperature of 320 °C that was maintained for 14 min; an injector temperature 260 °C, and a split ratio 1:20. The MS-interface temperature was 300 °C and the ion source temperature was 230 °C. The results were calculated using an internal standard and the following external standards: D-fructose (Merck), myo-inositol (Merck), D-glucose (BDH AnalaR, VWR International Ltd., Poole, UK), sucrose (BDH AnalaR), D-raffinose pentahydrate (Fluka), and D-pinitol (Sigma-Aldrich). The results per each sample were calculated as the arithmetic mean of three repetitive subsamples. The sugar concentrations (g_{sugars} / g_{dry weight}) are calculated to sugar osmolality as

$$\text{sugar osmolality} = \text{sugar concentration} / M / WC \quad (4)$$

where M is the molar mass of the sugar in question and the fresh and dry weights are measured from the same bark sample as the osmolality.

A recent study by Quentin et al. (2015) demonstrated that it is very difficult to obtain reliable absolute values for sugar contents as the results from sugar analysis were shown to vary considerably from one method to another, and from one lab to another. Therefore, we focus here only on the temporal and spatial variation of sugar contents all carried out with the same method in the same lab.

Stem and needle water potential

Stem water potential was measured by sealing the needles of mature trees in small aluminum foil bag for 20 min to equilibrate with the xylem water potential (Scholander et al. 1965; Boyer 1967). Samples were taken for water potential measurements just before each collection of osmolality

samples. The water potential measurement was performed immediately after the sampling cycle using Pump-Up Chamber (PMS Instrument Company, Albany, US). The stem water potential values are the mean of three to five repetitions collected around the canopy that is exposed to light. The time lag between the sampling of stem water potential and osmolality from the needles, branches, upper stem and stem base were on average 5, 7, 20 and 45 minutes, respectively. The water potential in Scots pine saplings was measured as the mean of three individual needles for each sapling that were not equilibrated with xylem water potential and therefore represented the needle water potential. Needles were collected from the saplings around 12:00 and each needle was measured immediately. In Norway spruce saplings, the water potential was measured from branch tips c. 5 cm in length. These samples were collected from the same branch from which the needles for osmolality were collected. The branch tips were sealed by gently wrapping them in aluminum foil inside plastic zipper bags at a minimum of 20 minutes before collection, thus representing the stem water potential. Water potential was measured in a similar way as described above. Water potential of mature Norway spruce represented the needle water potential.

Turgor pressure

The turgor pressure was estimated using the assumptions of xylem and phloem equilibrium

$$\Psi_{\text{xylem}} = \Psi_{\text{phloem}} \quad (5)$$

where Ψ_{xylem} is the total water potential in xylem and Ψ_{phloem} is the total water potential in phloem.

With this assumption, the turgor pressure can be estimated as

$$\Psi_{\text{pph}} = \Psi_{\pi\text{p}} + \Psi_{\text{px}} \quad (6)$$

where Ψ_{pph} is the pressure potential in phloem, $\Psi_{\pi\text{p}}$ is the osmotic potential in phloem and Ψ_{px} is the pressure potential in xylem (e.g. Sovonick-Dunford et al. 1981). The xylem osmotic potential is assumed to be zero in this study. Osmolality is converted into osmotic potential as follows

$$\Psi_{\pi p} = RTc \quad (7)$$

where $\Psi_{\pi p}$ is the osmotic potential (MPa), R is the universal gas constant $8.314 \text{ (J mol}^{-1} \text{ K}^{-1}\text{)}$, T is the
air temperature (K) at the time of sample collection and c is osmolality (mol g^{-1}).

Statistical analyses

Linear regression analysis was used for statistical testing. The statistical testing was performed using R (v. 3.2.1, 2015, R Foundation for Statistical Computing, Vienna, Austria). Results were considered to be significant when the p-value was lower than 0.5.

RESULTS

The coupling of needle and inner bark osmolality with water potential was clearly seen in both saplings and mature Scots pine. Osmolality correlated with the stem water potential in needles, in branches and the upper stem in mature Scots pine (Fig. 2A). The connection with stem water potential was diminished at the stem base. Osmolality was also found to be a function of water potential in saplings in both Scots pine and Norway spruce. The relationship between osmolality and needle water potential in Scots pine saplings was slightly weaker in comparison to the mature trees (Fig. 2C). In contrast, the correlation with stem water potential was strong in needles and in stem bark in Norway spruce saplings (Fig. 2E). The correlation diminished towards the lowest sampled tree positions in all the studied trees, stem base in mature trees and roots in saplings. The low number of observations prevented firm conclusions about the relationship between osmolality and stem water potential in mature Norway spruce.

364 The osmolality gradient between higher sample positions and the stem base was significantly
365 correlated with water potential in Scots pine (Fig. 2B and 2D). The gradient between the stem
366 positions diminished when the stem water potential was approximately -0.2 MPa, which
367 approximately corresponded to pre-dawn water potential in the mature Scots pine as soil water
368 potential was very close to zero during all periods (not shown). A small osmotic gradient remained
369 between the branches and stem base at the pre-dawn water potential.

370

371 Inner bark osmolality at the two stem positions was negatively correlated with the xylem diameter
372 change measured at the stem base in Scots pine (Fig. 3). Needle osmolality was similarly negatively
373 correlated with xylem diameter but this correlation was much weaker than those of the stem
374 positions (not shown). The correlation between the xylem diameter and inner bark osmolality was
375 the strongest in the stem base where the xylem diameter was measured.

376

377 When the daily dynamics of osmolality were examined, osmolality was almost always the highest in
378 the needles and always the lowest at the stem base (Fig. 4). The changes in osmolality were the most
379 consistent from the end of August to September when osmolality increased towards the afternoon
380 for all the sample positions (Fig. 4C & 4D). The distance between needles and bark at the branch
381 sampling position was less than 20 cm while the difference in osmolality was as large as amongst the
382 other positions. This indicates a large transport resistance in xylem and/or phloem over this short
383 distance. The gradient between needles and branch was often close to zero or reversed, which
384 indicated that branch osmolality was sometimes larger than that in the needles. Moreover, the
385 gradient between branches and upper stem seemed to have a large daily variation and was
386 occasionally close to zero or even reversed.

387

The mean osmolality was the highest in the needles and decreased gradually towards the stem base in every measurement period in mature trees of both species and in the Scots pine saplings (Fig. 5A, 5D and 5E). The Norway spruce saplings had a less clear pattern as it often had higher values in the roots than in the stem (Fig. 5D). The mean water content mainly showed an opposite pattern to that of osmolality (Fig. 5C). The mean water content was high and very similar in both stem positions in July.

Stem water potential and branch turgor pressure correlated at all times. The correlation was highly significant in July and September. The regression line of branch turgor pressure and stem water potential approached zero turgor between -1.7 and -1.6 MPa in all the measurement periods (Fig. 6). The slope of the regression between the turgor pressure and stem water potential differed in July compared to other measurement periods. The mean estimated turgor pressure in Scots pine branches was 0.3, 0.5 and 0.7 MPa for June, July and September, respectively.

The osmolality is a function of the amount of water and the concentration of osmotically active solutes; therefore, we compared osmolality with these two components. Neither water content nor relative water content correlated with osmolality, except in the needles in June for water content (not shown). The mean relative water content decreased gradually from needles to stem base, with only small change in levels of given sample position between the sampling periods.

The osmolality at full saturation was used to assess the influence of osmotically active solutes that drive the variation in osmolality (Fig. 7). Osmolality at full saturation varied with the *in situ* osmolality with similar significant regressions between inner bark samples and with highly significant regression in needles. Osmolality at full saturation, i.e. changes in the molar amount of osmotically active substances, explained 36% to 50% of the changes *in situ* osmolality in the inner bark samples and more than 90% in the needles. The mean osmolality at full saturation

showed a much more distinct pattern between the positions compared to mean *in situ* osmolality (Fig. 5A and 5B).

The calculated osmolality of sucrose in the inner bark was axially less variable within a tree compared to those of glucose, fructose and pinitol (Fig. 8A). The ratio of sucrose to monosaccharides decreased from the stem base towards the branch. The amounts of pinitol in the samples were in similar or larger quantities compared to glucose in all bark positions (not shown). The calculated total sugar osmolality was in strong correlation with the measured osmolality (Fig. 8B).

DISCUSSION

Dynamics of inner bark and needle osmolality and water potential

Osmolality is clearly associated with water potential in mature Scots pine and in saplings of Scots pine and Norway spruce. The exceptions to this are the lowest measurement points, i.e. stem base in mature Scots Pine and the roots in saplings of both species. However, the stem base osmolality positively correlate with the xylem diameter changes, which reflect the water potential in the xylem (Irvine and Grace 1997). These results suggest that xylem water potential is driving a significant portion of inner bark osmolality dynamics. Modelling studies suggest that the daily dynamics of transpiration are most likely to influence phloem turgor and its gradients and thus phloem sugar transport (Hölttä et al. 2006; Steppe et al. 2006; Nikinmaa et al. 2014; Steppe et al. 2015). Moreover, De Schepper & Steppe (2010) and De Swaef et al. (2013) combined existing models of coupled xylem and phloem transport with measured stem diameter data, and reported estimations for osmotic pressures in phloem that support the strong interaction between xylem and phloem. Additionally, Mencuccini et al. (2013) and Chan et al. (2016) estimated, based on stem diameter change measurement and a hydraulic model, the osmotic concentration in Scots pine trees to peak during

the afternoon and be at its lowest when transpiration ceased. The examples of modelling and data analyses cited above indicate that models with coupled xylem and phloem transport seem to predict the osmotic concentration in phloem realistically, but they ~~had~~have not been validated against measured data. On the contrary, an MRI study by Windt et al. (2006) conducted on smaller herbaceous plants and poplar saplings showed rather constant phloem transport velocities during daytime while the transporting phloem area was found to vary diurnally. It must be emphasized that measured value of phloem osmotic potential in the present study is for the bulk tissue, and the osmotic potential of sieve cells involved in phloem transport could differ from this. Nevertheless, changes in xylem water potential were shown to affect the osmolality of the bulk phloem tissue, and at least the changes in total water potential can be expected to change in a similar way in the sieve cells as in the other cell types in the phloem (Thompson & Holbrook 2003). The osmotic potential of the needles in our study also describe a heterogeneous bulk since majority of the needle is e.g. mesophyll and transfusion tissue, and xylem and phloem tissues represent only the minority (Liesche et al. 2011). However, also the needle osmolality follows xylem water potential dynamics.

The weaker correlation between stem water potential and osmolality measured at the stem base might be because of time lag. Time lag in xylem diameter changes between the upper stem and stem base has been reported to be approximately 30 minutes for a similar distance in the same species at the same site as our measurements (Sevanto et al. 2002). Further, the bark water potential has a time lag with xylem water potential (Pfautsch et al. 2015). However, the time lag in our sampling procedure (see Materials and Methods) more or less cancels out the time lag between the xylem water potential and osmolality for different tree positions when comparing the dynamics of stem water potential and bark osmolality.

The relation between the osmolality gradients and the stem water potential is evident in mature Scots pine. When the stem water potential approached pre-dawn conditions, there was only a small

osmolality gradient between branch and stem base. If the turgor gradient is to be driving the phloem transport, then the osmotic potential gradient plus gravitational potential would need to exceed the xylem water potential gradient (Taiz [et al.](#) 2015). Our Scots pine trees were c. 18 meters in height, thus the gravitational potential is close to 0.2 MPa at the canopy. Our results indicate that at pre-dawn conditions the osmotic gradient between the upper stem and stem base is zero, which would leave a driving force ~0.2 MPa for phloem transport because of gravity. Therefore, in pre-dawn and rainy conditions the gravitational potential may have a significant role in phloem transport in the stem (Hölttä [et al.](#) 2006).

The gravitation could also compensate for some of the resistance that increases with the increasing axial pathway length in taller trees (Hölttä [et al.](#) 2013; Ryan & Asao 2014). Small phloem pressure gradients have also been predicted in previous studies (Thompson 2006). Recently, a study by Knoblauch [et al.](#) (2016) showed that turgor pressure difference between source and sink was up to 1.65 MPa in 14 meter high herbaceous vine. However, Turgeon (2010) reported that trees tend to have lower turgor gradients compared to herbaceous plants. Additionally, the resistance to phloem sap flow may be expected to be larger in conifers judging from the small size of the sieve cells in relation to sieve tubes in angiosperm trees (Schulz 1992; Jensen [et al.](#) 2012; Liesche [et al.](#) 2015).

Turgor pressure in branches

Branch turgor pressure followed stem water potential very closely especially in July and September in Scots pine. The trend of regression in our study indicate that inner bark turgor pressure estimates for branches approach zero turgor between stem water potentials -1.7 to -1.6 MPa in all the measurement periods. Generally, stomatal control has been reported to be sensitive in Scots pine (Irvine [et al.](#) 1998; Zweifel [et al.](#) 2007) to preserve the needle water potentials above -1.5 MPa to prevent widespread xylem embolism in a mature Scots pine stand (Irvine [et al.](#) 1998). Although, in

very dry conditions needle water potentials less than -2 MPa have been reported (e.g. Poyatos et al. 2013). Salmon et al. (2015) reported xylem vulnerability curves for Scots pine where the observed embolism increased considerably at needle water potentials below -1.5 MPa. Our results are in agreement with these observations and a loss of turgor seems to occur below this threshold in branches unless osmoregulation prevents it (Morgan 1984; Nikinmaa et al. 2014). The turgor pressure calculated with stem water potential might be a slightly rough estimate because of the radial time lag between the xylem and phloem (Sovonick-Dunford et al. 1981; An et al. 2014). However, we are not aware of any turgor estimates reported for Scots pine branches in any previously published studies.

Axial osmotic gradients and turgor pressure gradients

The mean osmolality values during the daytime were the highest in the needles and the lowest in the stem base in all measurement periods in mature trees and saplings of both species. The spruce saplings differed from other studied trees by having a high osmolality in the stem and the roots in relation to the needles at low water potentials. Decreasing axial osmotic gradients towards the stem base have been reported before, but those studies for the most part have been conducted on Norway spruce or other species than pine trees (Zimmermann 1957; Kaufmann & Kramer 1967; Hammel 1968; Rosner et al. 2001). The axially increasing xylem resistance can be assumed to affect the axial osmolality gradient because xylem and phloem are thought to equilibrate rapidly (Thompson & Holbrook 2003; Hölttä et al. 2006). Furthermore, the decreasing xylem water potential towards the apex has been proposed to be a partial explanation for the decrease in conducting cell size towards the apex (Woodruff 2014). Structural properties of the conducting tissue of both the xylem and phloem are reported to vary axially in conifers (Petit & Crivellaro 2014; Jyske & Hölttä 2015). The decreasing conduit size towards the apex, in both xylem and phloem, is in accordance with our finding of increasing osmotic potential gradient towards the apex (Jensen et al. 2012). The

511 daily dynamics of osmolality in the canopy positions of mature Scots pine momentarily overlapped in
512 June and July, and the gradients were the most consistent with osmolality decreasing towards the
513 stem base in both autumn periods. This could indicate to seasonal differences, and the brief
514 overlapping of branch and upper stem osmolality that phloem transport could temporarily reverse
515 the direction towards the needles during daytime (Hölttä et al. 2006; Nikinmaa et al. 2014). Turgor
516 profiles for the whole tree would be possible with our osmolality measurements, if the xylem water
517 potentials along the tree axial gradient were also known. It can be estimated from our results that
518 the mean xylem water potential in the stem base would have to be higher than -0.8 MPa in June and
519 July, and higher than -0.6 MPa in September for the mean turgor gradient of inner bark to be in the
520 direction towards the roots and not towards the branch during the daytime.

521

522 *Osmolality values compared to literature*

523 The osmolality of the needles is similar in magnitude as that reported earlier by Irvine et al. (1998)
524 for mature Scots pine, who reported values between 0.6 and 0.9 mol kg⁻¹. Fu et al. (2011)
525 reported mean leaf osmolality in species considered as passive loaders to be c. 0.8 mol kg⁻¹, which is
526 similar to the mean osmolality in our dataset for July and September. The mean osmolalities in the
527 upper stem and stem base in Scots pine are higher than that previously reported for Norway spruce.
528 Rosner et al. (2001) reported *in situ* upper stem osmolalities from c. 0.4 to 0.5 mol kg⁻¹ for the
529 conductive and the non-conductive phloem, respectively, in Norway spruce in July. Kraemer (1953)
530 reported osmolality c. 0.3 to 0.5 mol kg⁻¹ in the stem in Norway spruce.

531

532

533

534 *Influence of water and solutes on osmolality*

In theory, both an increase in transpiration and photosynthesis should affect the osmolality in phloem because the transpiration decreases the water content and photosynthesis increases the amount of sugars. Our osmolality data was also compared against photosynthetically active radiation (PAR) but no clear association there was seen (not shown). Relative water content decreased from the tree top to base. This is reasonable as the turgor pressure is also expected to follow the same trend. Water content per dry weight shows an opposite pattern, but in addition to water status, it reflects also the anatomical properties of the tissue (Rosner et al. 2001).

Significant temporal and spatial patterns have been reported for water status in the vertical profile of trees (Rosner et al. 2001; Gall et al. 2002; Woodruff 2014). Additionally, in study by Lintunen et al. (2016) the water content had a significant role in generating the osmolality levels of branch inner bark in different latitudes around Europe. Osmolality at full -saturation explain *in situ* osmolality with 30% to 50% regression in branches and stem positions when all the sampling periods are examined together in our study. The relationship between the *in situ* osmolality and osmolality at full -saturation in the needles is different in June compared to other studied periods, which could be due to active shoot growth during that time (Hansen & Beck 1994).

Phloem osmotic concentration has been reported to be mainly driven by its sugar concentrations, especially the sucrose concentration (Pate 1976; Rennie & Turgeon 2009). The mean sucrose concentration remained temporally and spatially constant compared to monosaccharides at all the sampling positions in our study. The relation of sucrose to the sum of glucose, fructose and pinitol increased from the branches towards the stem base. These observations indicate that the variations in inner bark osmolality are driven more by the monosaccharides and pinitol than by sucrose. Additionally, Woodruff (2014) reported that the sugar content increased as the result of increases in monosaccharides when the water potential decreased in Douglas-fir. Thus, sucrose had no significant spatial trend unlike that for the monosaccharides. Other studies have also found sugar

concentrations to increase with decreasing water potential (e.g. Weatherley [et al.](#) 1959). The measured osmolality strongly correlated with the total sugar osmolality calculated from the sugar concentrations in a daily scale, in both July and September. Devaux et al. (2009) reported that total sugar contents changed on a monthly scale and that significant daily variations could not be observed in phloem sap of maritime pine. [However, our sugar concentrations represent the sugar content of the bulk needle and inner bark in addition to the phloem sap.](#)

CONCLUSIONS

Our results indicate that xylem water potential considerably influences the needle and inner bark osmotic potentials and their gradients in addition to branch turgor pressures. These findings are in line with theory and the models that emphasize a close relationship between xylem and phloem transport in trees. Gravitation seems to have a considerable effect on the driving force of phloem transport in mature trees. This seems to be especially the case in the stem where the osmotic concentration gradients and predicted pre-dawn turgor pressure gradients were found to be very small. The axial osmotic gradients from the needles to stem base are evident in all studied periods but seem to be coherent at the whole tree level in the autumn. Both water and sugars seem to be significantly involved in the changes in osmolality in the short term. Our data on sugar concentrations, although limited, suggest that sucrose concentration remains rather constant on both the temporal and spatial scales whereas monosaccharide concentrations vary much more.

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587

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Table 1. Environmental variables at the measurement site of the Scots pine stand

	August 2011	July 2014	September 2014	June 2015
Monthly mean air temperature, °C	15.2	18.6	10.1	11.9
Mean air temperature in the measurement period, °C	10.3	23.2	12.7	9.4
Maximum air temperature in the measurement period, °C	16.5	29.3	18.3	14.5
Monthly precipitation, mm	83.1	44.1	22.1	81.5
Volumetric soil water content in the measurement period (depth 2-36 cm), m ³ m ⁻³	0.279	0.211	0.213	0.222

790 The mean monthly air temperature and precipitation for the site was obtained from the Finnish
791 meteorological institute weather archives. Other variables are calculated with the data from Smart
792 Smear data service (Junninen et al. 2009).

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805 **Table 2.** Number of samples collected in measurement periods

	Scots pine mature trees				Norway spruce mature trees	Scots pine saplings	Norway spruce saplings
	2011	2014	2014	2015	2012	2013	2012
	Aug. 29th-Sep. 2nd	Jul. 22nd-23rd	Sep. 11th-12th	Jun. 16th-17th	May-Aug.	Aug.	May-Aug.
	tree A	tree B	tree A	tree B	tree A and B		
Needles	10	7	6	9	4	31	10
Branches	10	8	6	9	4		
Upper stem	10	6	6	8	6	33	31
Stem base	10	8	6	8	6		
Ground level					4		
Roots						31	4
Water potential	10	8	6	9	6	33	32
Sugar concentration		3-4 per pos. ^b	3-4 per pos. ^b				
Water content	osm. nr ^a	osm. nr ^a	osm. nr ^a	osm. nr ^a			
Relative water content		osm. nr ^a	osm. nr ^a	osm. nr ^a			

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807 ^a The number of samples was the same as the collected osmolality samples.808 ^b Number of the sugar samples per position during the sampling period.

Figure 1. Illustration of sampling positions of mature Scots pine where N, I, II and III stand for needles, branches, upper stem and stem base, respectively.

91x121mm (150 x 150 DPI)

Figure 2. Osmolality in situ (A, C, E) and osmolality difference (B, D, F) as a function of stem water potential in mature Scots pine (A, B) and Norway spruce saplings (E, F) and as a function of needle water potential in Scots pine saplings (C, D). Statistically tested p-value is in symbols; *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$. The linear regression equations of osmolality and water potential are in mature Scots pine (A) $y = 0.62 - 0.15x$ (needles); $y = 0.48 - 0.13x$ (branch); $y = 0.42 - 0.14x$ (upper stem); $y = 0.44 - 0.04x$ (stem base); and in Scots pine saplings (C) $y = 0.64 - 0.19x$ (needles); $y = 0.41 - 0.19x$ (stem bark); $y = 0.39 - 0.09x$ (root); and in Norway spruce saplings (E) $y = -0.14 - 0.57x$ (needles); $y = 0.35 - 0.21x$ (stem bark). The linear regression equations of osmolality difference and water potential are in mature Scots pine (B) $y = 0.04 - 0.1x$ (branch to stem base); $y = -0.02 - 0.11x$ (upper stem to stem base); and in Scots pine saplings (D) $y = 0.25 - 0.1x$ (needle to root); $y = 0.02 - 0.1x$ (bark to root).

147x189mm (148 x 150 DPI)

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Figure 3. Osmolality in situ of stem positions (A & B) and xylem diameter change measured in 1.5 m height in mature Scots pine in July 2014 and June 2015. Statistically tested p-value is in symbols; *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$.

225x106mm (98 x 101 DPI)

Figure 4. Osmolality in situ in needles, branches, upper stem and stem base, and stem water potential in daytime in Scots pine in June 2015 (A), July (B) and September (C) 2014, and early autumn 2011 (D). In figure D, the measurement points at the date ticks are measured around 7 a.m. whereas points between the ticks are measured around 3 p.m.

218x185mm (131 x 133 DPI)

Figure 5. Mean of osmolality in situ (A), osmolality at full saturation (B), mean relative water content (C) and mean water content (F) in mature Scots pine. Osmolality in situ in Scots pine and Norway spruce saplings (D) and in mature Norway spruce (E). The mean stem water potential (MPa) during the measurement period is shown as values in figures A and E. The error bars are standard deviations.

262x163mm (129 x 132 DPI)

Figure 6. Inner bark turgor pressure estimate in Scots pine branches in July and September 2014, and in June 2015 as a function of stem water potential. Statistically tested p-value is in symbols; *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$.

145x132mm (104 x 104 DPI)

Figure 7. Osmolality in situ and osmolality at full saturation in needles (A), branches (B), upper stem (C) and stem base (D) in mature Scots pine in July and September 2014, and in June 2015. Statistically tested p-value is in symbols; *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$.

185x173mm (147 x 149 DPI)

Figure 8. The mean of calculated sucrose compared to sum of fructose, glucose and pinitol in branches, in upper stem and stem base (A) in mature Scots pine. Together all these four sugars represented 97 % or more of the total sugar osmolality. The measured osmolality in situ as the function of calculated total sugar osmolality in the stem positions (B). The error bars are standard deviations (Fig. 8A). The statistically tested p-value is in symbols (Fig. 8B); *, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$.

250x117mm (128 x 131 DPI)