Polar lipids are linked to nanoparticles in xylem sap of temperate angiosperm species

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21

22 **Abstract**

23 Xylem sap of angiosperm species has been found to include low concentrations of
 24 polar lipids and nanoparticles, including surfactant-coated nanobubbles. Although the
 25 nanoparticles have been suggested to consist of polar lipids, no attempt has been made
 26 to determine if nanoparticle and lipid concentrations are related. Here, we examined
 27 concentrations of nanoparticles and lipids in xylem sap and contamination control
 28 samples of six temperate angiosperm species with a NanoSight device and based on
 29 mass spectrometry. We found (1) that the concentration of nanoparticles and lipids
 30 were both diluted when an increasing amount of sap was extracted, (2) that their
 31 concentrations were significantly correlated in three species, (3) that their
 32 concentrations were affected by vessel anatomy, and (4) that concentrations of
 33 nanoparticles and lipids were very low in contamination-control samples. Moreover,
 34 there was little seasonal difference, no freezing-thawing effect on nanoparticles, and
 35 little seasonal variation in lipid composition. These findings indicate that lipids and
 36 nanoparticles are related to each other, and largely do not pass interconduit pit
 37 membranes. Further research is needed to examine the formation and stability of
 38 nanoparticles in xylem sap in relation to lipid composition, and the complicated
 39 interactions among the gas, liquid, and solid phases in xylem conduits.

40 **Key words:** xylem sap, polar lipids, vessels, pit membranes, nanoparticles,
 41 surfactants, angiosperms

42 **Abbreviations:**

43 DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; LPG,
 44 lysophosphatidylglycerol; LPC, lysophosphatidylcholine; LPE,
 45 lysophosphatidylethanolamine, PA, phosphatidic acid; PC, phosphatidylcholine; PE,
 46 phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS,
 47 phosphatidylserine; TAG, triacylglycerol.

48 **Introduction**

49 One of the long-standing research questions in plant biology is how vascular plants
 50 transport water across xylem tissue over long distances (Hales, 1727). The question
 51 persists as to why plants are able to transport sap under negative pressure through
 52 dead xylem conduits without constant formation of large bubbles (embolism), which
 53 could reduce the transport efficiency considerably (Jansen and Schenk, 2015;
 54 Venturas *et al.*, 2017). The assumption that high surface tension of xylem sap is
 55 required for a transpiration-driven process under negative pressure was recently
 56 challenged by the rediscovery of amphiphilic lipids in xylem sap (Scott *et al.*, 1960,
 57 Esau 1965, Esau *et al.*, 1966, Schenk *et al.*, 2017, 2018, 2021). Moreover, the
 58 observation of nanoparticles and surfactant-coated nanobubbles in xylem sap raises
 59 questions about how plants are able to deal with a transport system that is subject to
 60 negative pressure but does not rely on pure and completely degassed liquid, as is
 61 typically required for artificial devices that operate under negative pressure (Wheeler
 62 and Stroock, 2008; Boatwright *et al.*, 2015). Hence, more information on lipids and
 63 nanoparticles in xylem sap is needed to strengthen our understanding of their

64 occurrence and functional significance.

65 Xylem sap is well known not to be pure water, but contains ions, sugars, proteins,
 66 lipids, etc. (Buhtz *et al.*, 2004; Gollan *et al.*, 1992; Gonorazky *et al.*, 2012). A major
 67 challenge with chemical analyses of xylem sap is the extraction of sufficient sap
 68 material without contamination from cut living cells, including living fibres or
 69 parenchyma cells (Schurr 1988); this can be largely avoided by testing contamination
 70 control samples from the cut and cleaned surface of the xylem (Schenk *et al.*, 2017,
 71 2021). Very few lipidomic studies have been conducted on xylem apoplast fluid
 72 and/or xylem sap, but the findings so far show the presence of polar lipids, such as
 73 phospholipids and galactolipids, in xylem sap in all species tested to date (Gonorazky
 74 *et al.*, 2012; Schenk *et al.*, 2021). Schenk *et al.* (2021) found that the total polar lipid
 75 concentration varied from 0.18 to 0.63 nmol/mL across species, with
 76 monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG),
 77 phosphatidic acid (PA), and phosphatidylcholine (PC) as the most common lipid types.
 78 These amphiphilic lipids are mostly attached to inner surfaces of conduits, including
 79 conduit walls and pit membranes, and are difficult to completely remove by sap
 80 extraction (Schenk *et al.*, 2017, 2018, 2021). It has also been shown that xylem sap
 81 lipids are highly efficient surfactants, with dynamic changes of the surface tension
 82 depending on the local concentration of lipids per surface area (Yang *et al.*, 2020).

83 Given that polar lipids may strongly affect the behaviour of gas-solid-sap interfaces at
 84 pit membranes, which are important safety valves between embolised and sap-filled

85 conduits (Kaack *et al.*, 2019, 2021), surfactant-coated nanobubbles in xylem sap are
86 speculated to originate from the multiphase interactions at the pit membranes (Schenk
87 *et al.*, 2017; Yang *et al.*, 2020). It is known that bubbles can be stable under negative
88 pressure when their radius remains below a critical threshold, especially with the help
89 of surfactants (Oertli, 1971; Atchley, 1989; Schenk *et al.*, 2015). Evidence supporting
90 this hypothesis includes the observation of nanoparticles in xylem sap based on a
91 NanoSight device for five angiosperm species, and the visualisation of
92 surfactant-coated nanobubbles in sap extracted from *Geijera parviflora* (Rutaceae)
93 based on cryo-freeze fracture transmission electron microscopy (Schenk *et al.*, 2017).
94 While it is essential to test whether or not nanoparticles and nanobubbles occur in
95 xylem sap of other species, it is also important to explore if these structures can be
96 linked to xylem sap lipids. Since not all nanoparticles in xylem sap may represent
97 nanobubbles, and because the NanoSight device is unable to distinguish nanoparticles
98 from gas-core nanobubbles, we will use here the general term nanoparticles with
99 respect to NanoSight experiments, and nanobubbles only if confirmed via electron
100 microscopy.

101 In this study, we collected xylem sap from six temperate angiosperm species to
102 conduct both nanoparticle and lipidomic analyses on the xylem sap extracted. In
103 particular, we aimed to address the following three major questions.

104 (1) Is there any quantitative or qualitative correlation between lipids and
105 nanoparticles in xylem sap? As gas-core nanoparticles coated with a mono-layer

106 surfactant were observed in the xylem sap of *G. parviflora*, and the surfactant was
107 speculated to be mainly amphiphilic lipids (Schenk *et al.*, 2017), we expected a
108 correlation between nanoparticle and lipid concentration. This relationship could
109 also be affected by variation in lipid compositions, seasonal changes, and size
110 distribution of nanoparticles.

111 (2) Does the lipid and nanoparticle concentration depend on the amount of xylem sap
112 extracted? As pore sizes in pit membranes between conduits were reported to be well
113 below 50 nm (Choat *et al.*, 2003, 2004; Zhang *et al.*, 2020), these would be too small
114 for lipid micelles or nanoparticles whose diameter is typically > 150 nm to pass
115 through (Schenk *et al.*, 2017). As such, we predicted that most lipids in the extracted
116 xylem sap would come from cut-open vessels at the cut end of a branch (Fig. 1;
117 Schenk *et al.*, 2021). We consequently hypothesize that there would be a dilution
118 effect on lipid and nanoparticle concentration, with reduced concentrations for an
119 increasing amount of xylem sap extracted. It is also possible that the concentrations of
120 lipids and nanoparticles are affected by species-specific vessel anatomy, such as the
121 cut-open vessel volume, and the total vessel perimeter at the cut xylem surface.

122 (3) Do lipids and nanoparticles occur as artefact-free compounds and objects in
123 xylem sap of temperate angiosperm trees? Do seasonal changes have an impact on
124 nanoparticles and lipids, and are nanoparticle dimensions and concentrations
125 affected by a freeze-thaw cycle? We hypothesized that lipid and nanoparticle
126 concentrations in xylem sap would be much higher than that in the contamination

127 controls. Seasonal variation of nanoparticles and lipids is largely unknown, but clear
128 seasonal variation in lipid composition has been found in *G. parviflora* (Schenk *et*
129 *al.*, 2021). Moreover, we expected that the mean nanoparticle size may vary among
130 seasons and between fresh and defrosted sap samples.

131 **Materials and methods**

132 **Plant material**

133 Six deciduous angiosperm species, including *Betula pendula*, *Carpinus betulus*,
134 *Corylus avellana*, *Fagus sylvatica*, *Liriodendron tulipifera*, and *Prunus avium* were
135 studied. These represented common species at Ulm University (48°25'20.3" N,
136 9°57'20.2" E), Germany. For each species, four healthy and mature individuals were
137 selected and labelled. Fresh branches were sampled and processed for measurements
138 in August 2019, November 2019, February 2020, and July 2020.

139 **Xylem sap extraction**

140 The extraction of stem xylem sap followed the methodology of Schenk *et al.* (2021),
141 which investigated in detail the possibility of xylem sap contamination based on
142 control samples, a lipid tracer experiment, and imaging. The experiments conducted
143 by these authors showed that 95% of the xylem sap lipids did not originate from
144 cut-open parenchyma cells at the basal stem surface.

145 Branch terminals with a length exceeding the maximum vessel length (approximately
146 1.5 to 2 meters) were collected during early mornings to ensure a fully hydrated status

147 of the plants. The cut ends of branches were wrapped with moist tissue, and the
148 branch terminals were covered with black plastic bags to prevent transpiration and
149 transferred to the laboratory within half an hour. Immediately after arriving at the lab,
150 a stem segment of ca. 10 cm was cut from the basipetal stem ends under deionized
151 water to remove any potential embolism. Bark tissue was then removed from the cut
152 end over a length of approximately 5 cm to expose the xylem and eliminate potential
153 contamination from bark tissue. To further eliminate potential contamination, a clean
154 and fresh razor blade was used to trim the cut surface, aiming to have cuts as clean as
155 possible without obstructing the cut-open conduits. Cell debris and cytoplasmic
156 remnants were removed using a high-pressure dental flosser (Ultra WP-100, Water
157 Pik Inc., Colorado, USA) to thoroughly flush the exposed xylem cylinder with
158 deionized water for about 90 seconds. A control sample was then collected for each
159 branch to test for potential contamination prior to xylem sap extraction. The cut end of
160 the xylem was submerged for 60 seconds into a watch glass containing 1 mL nano
161 pure water, and the liquid was then transferred to a glass vial with a glass pipette.
162 Contamination control samples were systematically collected during all experiments,
163 except for the first sap extraction in August 2019.

164 Xylem sap was extracted by applying a vacuum to the basipetal end of a stem segment.
165 The stem was inserted through a rubber flanged stopper, and the basipetal cut end was
166 vertically placed into a glass tube embedded in a Buchner flask with ice. After
167 checking the tightness of the whole system, we applied a vacuum to the flask. When
168 the proximal end of the branch was under vacuum, all leaves were removed, and the

169 branch was then shortened by removing acropetal ends. Therefore, we made ca. 5 cm
170 successive cuts until sap was observed coming out from the basipetal end. Then,
171 approximately 1 cm cuts were made successively until the desired sap volume was
172 obtained. Xylem sap was transferred to glass vials and stored temporarily in a 4 °C
173 refrigerator. The volume of the sap extracted was precisely recorded. The total
174 amount of sap obtained from a single branch varied from 2 to 24 mL, which depended
175 on the size of the branches, seasonality, and the xylem sap volume desired. Also, we
176 paid special attention to working with clean glass vials to avoid contamination and to
177 making the glass as hydrophilic as possible, which decreased the likelihood that
178 amphiphilic lipids would stick to it during the lipid extraction process.

179 **Nanoparticle tracking analysis**

180 Nanoparticles in xylem sap were investigated with a NanoSight LM10 microscope
181 (Malvern Instruments Ltd., Malvern, UK). Nanoparticles show typical Brownian
182 movements, which could be detected under a microscope when a laser beam with a
183 wavelength of 638 nm was applied to the sample chamber. The concentration and size
184 distribution of nanoparticles were calculated based on the number of nanoparticles per
185 sap volume and their Brownian motion speed as detected with a NanoSight NTA 2.3
186 Analytical Software programme (Malvern Instruments Ltd., Malvern, UK).

187 Both fresh and frozen-defrosted xylem sap samples were included in the
188 measurements. Fresh samples were stored at 4 °C immediately after extraction, and
189 measurements were done within a week to avoid nanoparticle dissolution and

microbial proliferation. Earlier work had shown that nanoparticles in xylem sap were stable over several days when kept refrigerated (Ushikubo *et al.*, 2010). Because microorganisms show very different, non-Brownian movements, these could automatically be excluded from our analyses by the NanoSight software programme. Overall, we had very little or no contamination with microorganisms when our samples were stored in a refrigerator. Defrosted samples referred to samples immediately stored in a freezer (-18 °C) after sap extraction, and defrosted at 4 °C one day before measurements were taken. We examined possible seasonal variation in nanoparticles by studying the frozen-defrosted xylem sap samples extracted in August 2019, and fresh xylem sap samples extracted in February 2020 and July 2020. To study the potential effect of freezing-thawing on nanobubble size and concentration, both fresh and defrosted xylem sap samples extracted in November 2019 were investigated and compared to each other.

Mass spectrometry analysis

Mass spectrometry analysis of lipids was conducted on xylem sap and contamination control samples collected in August 2019 and February 2020, following the protocol of Schenk *et al.* (2021). One millilitre of fresh sap or control sample was freeze-dried using a SpeedVac concentrator unit (model Savant SPD121P, Thermo Scientific, Waltham, MA, USA) at 40 °C. It took 2 to 3 h to get the samples completely dry. We then added 1 ml chloroform/methanol/water (5/5/1) solution to isolate lipids in the samples. The mixture was vortexed for 60 s to dissolve the solute

211 fully, and centrifuged at 3,500 rpm for 8 min. The supernatant of the centrifuged
212 sample was collected and transferred to a new 2 mL glass vial. This process was
213 repeated twice, and the three supernatant solutions were merged. The supernatant
214 solutions were put into the SpeedVac concentrator to freeze-dry, and the isolated
215 lipids were then stored at -80 °C. The lipid samples were sent with dry ice to the
216 Kansas Lipidomics Research Center at Kansas State University for mass
217 spectrometry analysis.

218 Direct-infusion electrospray ionization triple-quadrupole mass spectrometry was
219 performed with a Waters Xevo TQS mass spectrometer (Waters, Milford, MA, USA).
220 Lipid samples were dissolved in 1 mL chloroform, and the optimal amount of the
221 lipid-chloroform solution (0.9-1 mL) was determined prior to the measurements. The
222 samples were mixed with an internal standard mixture and 1.2 mL
223 chloroform/methanol/300 mM ammonium acetate in water (300/665/35). The mixture
224 was introduced to the mass spectrometer by continuous infusions with a 1 mL loop.
225 Internal standards and mass spectral methods for the Waters Xevo instrument were as
226 described by Schenk *et al.* (2021) with the addition of a neutral loss of 297.2 scan for
227 18:2-containing triacylglycerol (18:2-TAG) in positive mode as $[M + NH_4]^+$. The
228 TAG internal standard was TAG (17:1/17:1/17:1). The cone voltage for
229 18:2-containing TAG analysis was 40 V and the collision energy was 6 V. Response
230 factors to correct for differences in mass spectral responses of biological lipid
231 molecular species compared to mass spectral responses of the internal standards were
232 applied for lipid classes, except 18:2-containing TAG, which is reported as units of

233 normalized mass spectral intensity, where an intensity of 1 unit is equal to the
234 intensity of 1 nmol of internal standard.

235 **Cryo-freeze fracture transmission electron microscopy**

236 Nanoparticles in xylem sap from *C. avellana* extracted with the method described
237 above in June 2016, were visualised based on Papahadjopoulos-Sternberg (2010).
238 Fresh sap was quenched using the sandwich technique and frozen with
239 nitrogen-cooled propane, which avoided ice crystal formation and potential artefacts
240 during the cryofixation process. The cryofixed sample was mounted on a standard
241 Bal-Tec double replica holder, and inserted in a BAF 300 freeze-etching device
242 (Balzers, Liechtenstein; Walther, 2003). The temperature in this device was raised to
243 -120°C, and the vacuum was ca. 2×10^{-7} mbar. Fracturing was done by opening the
244 double replica holder. The fractures were then coated with Pt for 30 s at an angle of ca.
245 30°. A second coating was done with carbon for 30 s at an angle of 0° (2kV per
246 60-70mA, 1×10^{-5} Torr). Unlike Schenk *et al.* (2017), the replicas were not cleaned by
247 fuming concentrated HNO₃ over the samples. The cleaning step may allow more
248 detailed observations of the lipid coating, but was not required for the identification of
249 nanobubbles. Two samples of xylem sap were prepared, and pure water was taken as a
250 control sample. Observation was done with a JEOL JEM-1400 series 120kV
251 Transmission Electron Microscope.

252 **Does the amount of extracted sap volume affect the lipid and nanoparticle**
253 **concentration?**

To test the potential impact of the amount of sap extracted on nanoparticle concentration, we extracted xylem sap following the steps described above. Instead of collecting sap from a branch in one tube, we collected the 1st, 2nd, 3rd, 4th, and 5th mL of xylem sap separately in different glass tubes. Nanoparticle concentration was measured with a NanoSight LM10. One branch sample per species was used.

The mean nanobubble concentration of the multiple sap samples was calculated as follows,

$$C_{mean} = \frac{\sum_{i=1}^n (C_i \times V_i)}{\sum_{i=1}^n V_i} \text{ (Equation 1),}$$

where C was the original nanobubble concentration, V was the volume of xylem sap extracted, i was the serial number of the sap sample investigated, and C_{mean} was the average nanobubble concentration in the cumulative amount of sap extracted ($\sum V_i$). C_{mean} was plotted against $\sum V_i$ for each species to estimate the potential dilution effect.

The open vessel volume (mL), and the total vessel perimeter at the cut surface (cm) were calculated for each species:

$$\text{Open vessel volume} = \frac{1}{2} \times L_V \times A_V \text{ (Equation 2)}$$

$$\text{Total vessel perimeter at the cut surface} = \pi D \times V_D \times A_S \text{ (Equation 3)}$$

The average vessel length (L_V , cm), average vessel diameter (D , μm), and vessel density (V_D , no mm^{-2}) were retrieved from Guan *et al.* (submitted), which were based on the same tree individuals as studied here. Vessel area at the cut end of a branch

segment (A_v , cm^2) and stem xylem surface area (A_s , cm^2) were determined based on images of transverse sections of the stem segments collected in February 2020. Surface area was measured using the Fiji version of ImageJ (Schindelin *et al.*, 2012).

Because the volume of sap extracted was found to impact the concentration of nanobubbles (Fig. 2A, Fig. S1), we normalised the nanoparticle concentration of each sap sample based on the species-specific logarithmic regressions to nanoparticle concentration per mL sap extracted.

Data analysis

Species specific curves of nanoparticle concentration against sap volume extracted were plotted and fitted using SigmaPlot 14 (Systat Software Inc., Erkrath, Germany). After testing data for normal distribution and homogeneity of variance, normalised nanobubble concentration of sap collected in different seasons were compared using a one-way ANOVA for each species. Lipid compositions were obtained by converting lipid concentrations to percentages for each sample. Comparison of lipid compositions between xylem sap samples and contamination control samples, among sap samples of different species was made based on a principal component analysis (PCA). Changes in lipid compositions between summer and winter were examined using an independent sample t-test. Statistics were performed in SPSS 22 (IBM, Armonk, New York, USA) and R (version 4.0.3, R Core Team, 2016).

Results

293 **Normalisation of nanoparticle concentrations in xylem sap and the dilution effect**

294 The first millilitre of sap extracted from a branch showed the highest nanoparticle
295 concentration for all species studied (Fig. S1). In contrast, lower concentrations of
296 nanoparticles were found in the consecutive sap samples. Nanoparticle concentration
297 in the latter samples was within the same range as the control samples (Fig. S1). We
298 found a logarithmic decrease of mean nanoparticle concentration with increasing
299 cumulative volume of sap extracted for each branch, and the species-specific
300 regressions fitted very well to the measured data ($0.94 \leq R^2 \leq 0.99$, Fig. 2A).

301 Moreover, a significant nonlinear correlation between nanoparticle concentration and
302 extracted sap volume was also found in sap samples collected when analysing all
303 species over the different seasons together ($R^2 = 0.36$, $P = 0.002$, Fig. 2B). This also
304 indicated that there was a dilution effect on the nanobubble concentration when a high
305 amount of sap volume was extracted.

306 **Comparison of nanoparticle characteristics in xylem sap and contamination** 307 **controls**

308 To account for the dilution effect, nanoparticle concentration per mL sap extracted
309 was normalised based on the species-specific regression equations shown in Fig. 2A.
310 The normalised nanobubble concentration in fresh sap samples collected in November
311 2019 varied from $5.51 \pm 2.43 \cdot 10^8$ particles/mL for *B. pendula* to $16.03 \pm 2.32 \cdot 10^8$
312 particles/mL for *F. sylvatica*. In contrast, nanoparticle concentration in the

contamination control samples ranged from $0.60 \pm 0.14 \cdot 10^8$ particles/mL for *C. avellana* to $1.29 \pm 0.61 \cdot 10^8$ particles/mL for *L. tulipifera* (Table 1). Therefore, possible contamination from the cut surface of a stem could have caused 3% up to 35% of the nanoparticle amount detected in sap samples, with an average of 10% for all six species.

Similar results were found for the fresh sap and control samples collected in February 2020, where *B. pendula* showed the lowest normalised concentration of nanoparticles ($5.92 \pm 1.20 \cdot 10^8$ particles/mL) and *C. betulus* showed the highest concentration ($14.44 \pm 4.68 \cdot 10^8$ particles/mL, Table 1). Normalised concentrations of nanoparticles in the control samples varied from 0.54 ± 0.14 to $0.94 \pm 0.32 \cdot 10^8$ particles/mL, which accounted for 3 to 20% of the nanoparticle amount in the sap, with an average of 9% for the six species studied.

The mean diameter of nanoparticles in xylem sap ranged from 171 ± 18 nm for *C. avellana* to 232 ± 13 nm for *C. betulus* for the samples collected in November 2019, with an average of 202 ± 24 nm. The mean diameter of nanoparticles in the control samples showed a narrow range from 217 ± 5 nm (*C. betulus*) to 233 ± 10 nm (*F. sylvatica*), with an average of 225 ± 23 nm (Table S1). Similar results were found for the samples collected in February 2020, with nanoparticle diameter ranging from 189 ± 7 nm to 235 ± 13 nm for xylem sap of *P. avium* and *L. tulipifera*, respectively, and from 209 ± 12 nm to 243 ± 21 nm for control samples of *B. pendula* and *L. tulipifera*. The mean nanoparticle diameter was significantly larger in the control

334 samples than sap samples ($F = 0.047$, $df = 46$, $P = 0.002$ for sap and control samples
335 collected in August 2019; $F = 0.161$, $df = 46$, $P = 0.004$ for sap and control samples
336 collected in February 2020).

337 **Seasonal variation and the effect of a freeze-thaw cycle on nanoparticle** 338 **characteristics**

339 Compared to fresh sap samples, the normalised concentration of nanoparticles in the
340 frozen-defrosted samples collected in November 2019 did not differ in five out of the
341 six species studied. However, a significant lower nanoparticle concentration was
342 found in the defrosted sap of *C. avellana* as compared to the fresh sap (Fig. 3A, Table
343 1). A general increasing trend in nanoparticle diameter after a freeze-thaw treatment
344 was found in all six species studied, but this difference was only statistically
345 significant for *B. pendula* (Fig. 3B).

346 There was no significant difference in normalised nanoparticle concentration among
347 sap samples collected over different seasons for *B. pendula*, *C. betulus*, *F. sylvatica*
348 and *P. avium*. Normalised nanoparticle concentration in fresh samples of *C. avellana*
349 collected in November 2019 was significantly higher than the other samples collected
350 from this species, while the fresh samples of *L. tulipifera* collected in February 2020
351 showed a significantly lower normalised nanoparticle concentration than other
352 samples of *L. tulipifera* (Fig. 3A). No significant variation in nanoparticle diameter of
353 xylem sap was found among all sample groups for *C. avellana*, *F. sylvatica* and *L.*
354 *tulipifera*. The mean nanoparticle diameter was not significantly different between the

355 frozen-defrosted samples collected in August 2019 and November 2019, and between
356 the fresh sap samples collected in November 2019 and February 2020 for five out of
357 the six species studied, indicating a lack of seasonal variation in nanoparticle diameter
358 for these species. Significant seasonal difference in nanoparticle diameter was only
359 found between the fresh sap samples collected in November 2019 and February 2020
360 for *B. pendula* (Fig. 3B).

361 **Lipid concentrations in xylem sap and contamination controls**

362 For samples collected in February 2020, the total lipid concentration in xylem sap
363 varied from 0.137 ± 0.036 nmol/mL (*P. avium*) to 0.376 ± 0.182 nmol/mL (*B.*
364 *pendula*). The total lipid concentration was on average four times larger in sap
365 samples than contamination control samples (Table S2). For samples collected in
366 August 2019, the total lipid concentration varied from 0.071 ± 0.015 nmol/mL (*P.*
367 *avium*) to 0.234 ± 0.071 nmol/mL (*B. pendula*, Table S2). Although the total
368 concentration of lipids was significantly higher in February 2020 than in August
369 2019 ($F = 5.100$, $df = 37.35$, $P < 0.001$), a much smaller amount of sap was extracted
370 in February 2020 (on average 2.88 ± 0.67 mL) than in August 2019 (on average
371 17.10 ± 5.45 mL, Table S1). Similar to nanoparticle concentrations, lipid
372 concentrations in xylem sap samples collected in August were clearly diluted, and
373 the lipid concentration in xylem sap was therefore only three times the lipid
374 concentration in contamination controls (Table S2).

375 Based on the PCA analysis, there was a clear difference in the lipid composition

between the sap samples and the contamination controls, with larger variation in lipid composition in control samples than in sap samples (Fig. S2, S3). Plant species were mainly grouped based on the loading scores of PCA analysis, that both summer and winter xylem sap samples showed similarities in lipid composition among different species (Fig. 5A, B).

Lipid composition of xylem sap and seasonal variation

Polar lipids in xylem sap and contamination controls were mainly galactolipids (i.e., digalactosyldiacylglycerol, DGDG, and monogalactosyldiacylglycerol, MGDG) and phospholipids (Fig. 4A, Table S2). The concentration of phospholipids included mainly phosphatidylcholine (PC), phosphatidic acid (PA), and phosphatidylinositol (PI). In contrast, a small number of other phospholipids was detected, including phosphatidylethanolamine (PE), phosphatidylserine (PS), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE) and lysophosphatidylglycerol (LPG) (Fig. 4A, Table 2).

The neutral 18:2-containing triacylglycerol (TAG) showed 2-4 times and up to 2 times of the normalized mass spectral intensity of polar lipids in the sap of *B. pendula* and *L. tulipifera*, respectively, while the 18:2-containing TAG intensity only represented ca. 20-40% of the total intensity of polar lipids in the other four species (Fig. 4B). A significant difference in the percentage of TAG out of the total normalized mass spectral intensity of all lipid was found between summer and winter xylem sap of *C. avellana* and *P. avium* (Table S3).

The xylem sap samples collected in August 2019 showed that the concentration of DGDG varied from $3.59 \pm 0.89\%$ of the total lipids mass spectral intensity in *B. pendula* to $21.24 \pm 2.48\%$ in *F. sylvatica*, and MGDG varied from $3.61 \pm 1.75\%$ in *B. pendula* to $15.79 \pm 3.03\%$ in *F. sylvatica*. PA concentrations ranged from 2.93 ± 1.17 in *B. pendula* to $18.97 \pm 12.59\%$ in *L. tulipifera*, and PC varied from $6.67 \pm 0.88\%$ in *B. pendula* to $16.83 \pm 3.35\%$ in *C. avellana*, (Table S2). The lipid composition of sap collected in August 2019 was largely similar to the sap samples collected in February 2020, indicating a lack of seasonal change in most lipid compounds (Table S4, S5). Results of an independent sample t-test suggested no difference in lipid composition between summer and winter sap for *C. betulus* and *L. tulipifera* (Table S2). A significantly higher proportion of MGDG was found in the winter sap samples than in the summer ones of *P. avium*, and a higher proportion of PG was found in the winter sap samples than in the summer ones of *B. pendula* and *F. sylvatica*. The percentage of LPC in the winter samples was significantly lower than that in the summer samples for *C. avellana* and *F. sylvatica* (Table S2).

An increasing trend of total lipid concentration with increasing nanobubble concentration was found for several species and across seasons (Fig. S4). A significant and positive correlation between the original nanobubble concentration and lipid concentration was shown for three species, namely *C. avellana*, *L. tulipifera* and *P. avium* (Figure 6D-F). A similar trend was also observed in *B. pendula*, *C. betulus* and *F. sylvatica*, although this relationship was not statistically significant (Figure 6A-C).

419 **Linking lipid and nanoparticle concentrations with vessel anatomy**

420 The nanoparticle and lipid concentrations were tested against vessel anatomical traits
421 for the samples collected in February 2020 (Fig. 7). A significant, nonlinear relation
422 was found between the polar lipid concentration and the open vessel volume, while
423 the nanoparticle concentration was related to the total vessel perimeter at the cut stem
424 surface.

425 **Visualisation of surfactant-coated nanobubbles in the sap of *Corylus avellana***

426 Freeze-fracture electron microscopy revealed coated nanobubbles in xylem sap of *C.*
427 *avellana*. These were circular, but their shape was sometimes deformed, most likely
428 due to sample preparation. The Pt and/or C-free areas appeared as white, inner gas
429 volumes, while surfactant shells were dark due to Pt and C coating (Fig. 2E). The
430 diameter of the nanobubbles varied from 43 nm to 243 nm, with an average size of
431 126 nm. The shells appeared as deflated, and frequently wrinkled structures, which
432 were artificially removed from the bubble depression during sample preparation.
433 Therefore, the shells overlapped the bubble partially in most cases, but some bubbles,
434 especially the smaller ones, were still totally coated by complete shells. The
435 nanobubbles had an isolated position, but some arrangements were observed. We also
436 noticed a fairly dark, fuzzy layer on top of the surfactant shells, surrounding the
437 nanobubble. The thickness of this layer was on average 20 nm. Additional xylem sap
438 material may also stick as electron-dense structures to the nanobubbles.

439 Similar to the xylem sap samples, nanobubbles were found in the control samples (Fig.
440 2F). The diameter of nanobubble ranged from 37 nm to 183 nm, with a mean diameter
441 of 63 nm, which was significantly smaller than nanobubbles in the xylem sap (Fig. 2E,
442 F). Quantifying the number of nanobubbles based on freeze-fracture electron
443 microscopy was not possible.

444 Discussion

445 Although the surfactants of nanoparticles were speculated to consist mainly of
446 amphiphilic lipids in previous studies (Schenk *et al.*, 2017; 2021), a quantitative
447 relation between polar lipids and nanoparticles has been demonstrated for the first
448 time. Our results show that much higher concentrations of nanoparticles and lipids
449 were found in extracted xylem sap of six temperate angiosperm species than in their
450 contamination controls. Nanoparticles and lipids in xylem sap became diluted with
451 increasing volume of xylem sap extracted, suggesting that most lipids extracted in the
452 sap samples were not contaminations from living cells at the cut xylem surface, but
453 largely came from cut-open vessels. Since a positive relationship between
454 nanoparticle and polar lipid concentration was found for the six species studied, with
455 a statistically significant relationship for three species, additional evidence is provided
456 that polar lipids are the major components coating nanoparticles in xylem sap, with
457 proteins possibly being another component. Moreover, the number of nanoparticles
458 and lipid amount that could be extracted was also influenced by the open vessel
459 volume and vessel surface, because lipids are mostly distributed on inner vessel walls.

460 While little seasonal variation was observed in nanoparticle concentration, size, and
461 lipid compositions for most species studied, it remains unclear how nanobubbles in
462 xylem sap behave and are affected by lipid composition and concentration in intact
463 plants under a changing environment.

464 **Polar lipid and nanoparticle concentrations are related to each other**

465 Significantly higher concentrations of nanoparticles lipids were found in xylem sap
466 than in contamination controls, clearly suggesting that nanoparticles and lipids
467 existing in xylem sap were collected by applying a vacuum to the basal end of a
468 branch, and did not represent cell debris or contents from living cells (Schenk *et al.*,
469 2017). The samples collected in November 2019 and February 2020 showed that the
470 normalised nanoparticle concentration of xylem sap was on average 10 times higher
471 than the contamination control samples (Table 1). The lipid concentration in xylem
472 sap was also much higher than in the contamination controls (Table S2). Moreover,
473 lipid compositions in the control samples varied considerably from branches of the
474 same species (Fig S2, S3), suggesting quantitative and qualitative difference between
475 lipids in the xylem sap and the contamination control samples.

476 Polar lipid concentrations generally increased with increasing values of original
477 nanoparticle concentration (Fig. S4), and a positive and significant correlation of the
478 concentration of polar lipids and nanoparticles was found for *C. avellana*, *L.*
479 *tulipifera*, and *P. avium* (Fig. 6), suggesting a potential link between nanoparticles
480 and polar lipids (Schenk *et al.*, 2017). To date, the number of relevant studies on

lipids and nanoparticles in xylem sap is limited. For the first time to our knowledge, we tested if the lipid and nanoparticle concentrations are correlated by using the same xylem sap samples. Interestingly, a mean nanoparticle concentration of $1.36 \pm 0.48 \cdot 10^8$ particles mL^{-1} , and a polar lipid concentration of 0.18 ± 0.12 nmol mL^{-1} was found for xylem sap of *L. tulipifera* (Schenk *et al.*, 2017; 2021). These values are very similar to the results in our study (Table 1, Table S2). Besides, high correlation coefficients between the concentration of nanoparticles and polar lipids were found consistently, and these were higher than the correlation between the concentration of nanoparticles and lipids, including neutral lipids (Figure S5). Although more species may need to be tested, these findings indicate that lipids and nanobubble characteristics could be species specific.

Triacylglycerol (TAG) containing the fatty acid 18:2 contributed more than half of the normalized mass spectra signal of lipids for *B. pendula* and *L. tulipifera* (Table S3). Unlike polar lipids, which are amphiphilic, TAGs are mainly hydrophobic and form lipid droplets in aqueous solutions. *Betula* has been characterized as a fat-storing genus, with tiny fat or lipid droplets detected with a light microscope in parenchyma cells (Sinnott, 1918; Harms and Sauter, 1992), as well as on inner vessel walls (Fig. S6, Westhoff *et al.*, 2008). Increased TAG levels have been observed in plants under abiotic stresses and may serve as a reservoir for free fatty acids to avoid fatty acid-induced cell death in leaves generating free fatty acids (Xu and Shanklin, 2016). Lipid droplets also provide substrates and binding sites for lipid droplet-associated proteins, which synthesize bioactive compounds that can increase tolerance to biotic

503 stresses (Lu *et al.*, 2020). However, we are unaware of any research on neutral lipids
504 in the plant apoplast, their functions, and potential changes under stress conditions.

505 Why are nanoparticle concentrations related to polar lipid concentrations? The
506 presence of lipids in xylem sap, on inner vessel walls, and pit borders has been
507 cross-checked with the help of mass spectrometry, the fluorescent dye FM1-43 and
508 OsO₄ (Schenk *et al.*, 2017; 2018; 2021). Nanoparticles in xylem sap has been
509 observed in more than 10 species from various phylogenetic clades (Schenk *et al.*,
510 2017; Schenk unpublished data and this study). The freeze-fracture electron
511 micrographs of the nanoparticles also suggested that each nanoparticle owns a gas
512 core coated with a surfactant shell (Fig. 2 E, F; Schenk *et al.*, 2017). Therefore, it is
513 plausible to speculate that nanoparticles in xylem sap are mainly coated nanobubbles.
514 Xylem sap is never gas free, and the dissolution of gas in lipid phases may change due
515 to temperature and pressure (Mercury *et al.*, 2003). Embolism spreading from
516 embolized vessels to sap-filled ones includes gas movement through intervessel pit
517 membranes, either by mass flow or diffusion (Zimmermann, 1983; Sperry and Tyree,
518 1988; Kaack *et al.*, 2019). Since pit membranes are confined, and lipid-coated,
519 mesoporous media, nanobubbles would be coated with lipids during the penetration of
520 gas-sap interfaces in pit membranes (Schenk *et al.*, 2021). The coating of nanobubbles
521 with lipids could reduce their surface tension, and consequently limit the size, which
522 helps to prevent vessels from instant embolism (Schenk *et al.*, 2015). The consistent
523 relationship of nanoparticle and lipid concentration for all species was revealed
524 quantitatively in our study (Fig. 6, Fig. S4), which adds new evidence that the

525 surfactant coat of nanobubbles in xylem sap are mainly polar lipids.

526 The finding that polar lipid concentrations in xylem sap did not correlate tightly with
527 nanoparticle concentrations in all species (Fig. 6) suggests that the nanoparticles may
528 contain other components, most likely proteins (Buhtz *et al.*, 2004), which are
529 abundant in xylem sap residue (Schenk *et al.*, 2017). Many proteins bind to lipids,
530 especially in membranes, and non-specific lipid transfer proteins have been found in
531 xylem sap of many plant species (Buhtz *et al.*, 2004; Djordjevic *et al.*, 2007; Krasikov
532 *et al.*, 2011; Ligat *et al.*, 2011). As in lung surfactants (Parra and Pérez-Gil, 2015),
533 proteins could play important roles in the behaviour of lipid surfactant layers in plant
534 xylem. An additional explanation could be that lipids are difficult to extracted from
535 inner conduit walls of cut-open vessels (Schenk *et al.*, 2021).

536 **The concentration of nanoparticles and lipids is affected by the extracted sap**
537 **volume and vessel anatomy**

538 The mean diameter of nanoparticles ranged from 170 to 250 nm based on the
539 NanoSight measurements, which is much larger than the pore sizes of pit membranes
540 below 50 nm (Zhang *et al.*, 2020). Therefore, nanoparticles are unlikely to pass
541 through pit membranes, and the nanoparticles collected would mainly originate from
542 the cut-open vessels at the base of our stem samples (Fig. 1). Since the volume of
543 cut-open vessels ranged from 0.4 to 1.1 mL for the species studied, the highest
544 nanoparticle concentration was always measured in the first mL of xylem sap
545 extracted during the consecutive sap extraction from the same branch (Fig. 7, Fig. S1).

546 The mean nanoparticle concentration decreased rapidly with increasing sap volume,
547 especially within the first several millilitres (Fig. 2A, B). Thus, significantly higher
548 original values of nanoparticle concentration were detected when on average 3 mL of
549 sap was extracted in February 2020 than the ca. 20 mL of sap extracted in August and
550 November 2019 (Table S1). Similar results were also found for the lipidomic analyses
551 based on the same samples collected in August 2019 and February 2020 (Table 2).
552 Instead of seasonal changes in nanoparticle concentration, we identified that variation
553 in the original nanoparticle concentration was mainly due to the sap volume extracted.
554 Indeed, there was no longer any difference in nanoparticle concentration after data
555 normalisation (Fig. 3A).

556 Lipids are insoluble in xylem sap and are found to mainly distribute on inner-conduit
557 walls and pit borders (Schenk *et al.*, 2018). A significant and negative correlation was
558 found between nanoparticle concentration and total vessel perimeter at the cut xylem
559 surface, and between polar lipid concentration and cut-open vessel volume in xylem
560 sap collected in February 2020 (Fig. 7). A similar relation was reported between the
561 polar lipid concentration and the total vessel perimeter at the cut xylem surface for
562 another seven species (Schenk *et al.*, 2021). Our results that fewer lipids could be
563 extracted with larger vessel volume or surface agreed with the suggestion of Schenk *et*
564 *al.* (2021) that polar lipids are difficult to be completely removed from vessel lumen
565 surfaces. Since polar lipids are main components of the coating of nanoparticles, our
566 observation that considerable nanoparticles were detected in the second and third mL
567 of xylem sap extracted (Fig. S1), provides additional evidence to this hypothesis.

568 Further research aiming to quantify lipids based on mass spectrometry imaging of
569 vessels before and after xylem sap extraction could help to understand lipid
570 distribution in vessels and how tightly they attach to vessel walls (Ellis *et al.*, 2013).

571 **How do plants cope with changing seasons with respect to nanoparticles and**
572 **lipids?**

573 Although the normalised concentration of nanoparticles showed little variation in the
574 sap samples collected across different seasons, some variations of the nanobubble
575 diameter were found among seasons and between fresh and defrosted sap samples
576 (Fig. 3). To test if temperature would affect the stability of nanoparticles in xylem
577 sap, a frozen-defrosted treatment was applied to the sap samples collected in
578 November 2019, which simulated winter frost. Generally, larger nanoparticles were
579 observed in the defrosted sap compared to the same fresh sap samples, but this
580 difference was only statistically significant for *B. pendula* (Fig. 3B). Dissolved gas is
581 released from sap and forms bubbles when sap freezes. The stability of these bubbles
582 and their ability to be redissolved into the liquid phase during the thawing process
583 could influence embolism formation (Sperry and Sullivan 1992). Lipid-coated
584 nanobubbles could stabilise the existence of bubbles in the xylem sap, and the
585 area-dependent surface tension also increases with increasing bubble diameter,
586 which allows bubbles larger than the critical bubble size to be stabilised under
587 certain conditions without requiring more lipids (Schenk *et al.*, 2017; Yang *et*
588 *al.*,2020). Although plants live in a much more complex and constantly changing

environment, it is impossible for us to know if (nano)bubbles exist in intact plants, and how exactly they behave under negative pressure. More studies on nanobubble characteristics undergoing a freeze-thaw cycle may help to understand the potential role of lipids in preventing freeze-induced embolism.

An overall similarity in lipid composition was found between sap samples of August 2019 and February 2020. The mass spectra intensity of 18:2 containing TAG in winter sap differed significantly from the summer sap for *C. avellana* and *P. avium* (Table S3), but not for the other four species. This is in line with a previous study showing little seasonal variation of TAG concentration in the wood of *B. pendula* (Piispanen and Saranpää, 2004). Regarding polar lipids, MGDG, DGDG, and PA are the major components that consist of more than half of the total polar lipid concentrations (Fig. 4A). A significant change in the proportion of MGDG between summer and winter sap samples was only found in *P. avium*, but no significant change was found in the proportion of DGDG for all species studied (Table S3).

In a previous study (Schenk *et al.*, 2021), the lipid composition in xylem sap was found to be very similar to the composition of lipids in the entire xylem, suggesting that there is no selective transport of lipids into vessels from surrounding cells. Instead, it seems likely that lipids in vessels originate from the living vessel content (Scott *et al.*, 1960; Esau 1965; Esau *et al.*, 1966; Schenk *et al.* 2021), and therefore do not change much in composition over time once the vessel becomes functional. Seasonal change in lipid composition has yet only been tested for *G. parviflora*, where

a significant difference in the concentrations of DGDG and MGDG was found between xylem sap sampled in spring and summer. This could be due to sampling at different stages of xylem development (Schenk *et al.*, 2021). Moreover, seasonal changes in the electron density of pit membranes have been observed (Schmid and Machado, 1968; Sorek *et al.*, 2021), suggesting a potential lipid accumulation in membranes over time. Other experimental techniques, such as matrix-assisted laser desorption/ionisation MS imaging (MALDI), may help to visualise lipid distribution in xylem directly (Saito *et al.*, 2012), and determine if there are any changes related to season or ageing of vessels.

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Author contributions

630 X.G. conducted experiments, performed formal analysis, curated all data, and wrote
 631 the original draft; R.W. and M.R. conducted lipidomic experiments and data analysis.
 632 H.J.S. and S.J. conceived and supervised the project. J.W., L.K. and C.T. performed
 633 part of the experiments. R.W., M.R., H.J.S. and S.J. edited and finalised the
 634 manuscript. All authors read and approved the final manuscript.

635 **Data availability**

636 The data supporting the findings of this study are all included in figures, tables and
 637 supplementary material files

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Table 1. Normalised nanobubble concentration (10^8 particles per mL) of xylem sap samples and control samples. Values indicate means $\pm$ standard deviation (n = 4 individuals per species).

	2019 August	2019 November		2020 February		
	Defrosted sample	Fresh sample	Fresh control	Defrosted sample	Fresh sample	Fresh control
<i>B. pendula</i>	6.31 $\pm$ 1.98	5.51 $\pm$ 2.43	1.18 $\pm$ 0.31	6.35 $\pm$ 2.22	5.92 $\pm$ 1.20	0.57 $\pm$ 0.41
<i>C. betulus</i>	14.90 $\pm$ 1.47	13.90 $\pm$ 0.83	0.68 $\pm$ 0.20	15.06 $\pm$ 1.01	14.44 $\pm$ 4.68	0.54 $\pm$ 0.14
<i>C. avellana</i>	6.15 $\pm$ 1.15	9.50 $\pm$ 0.81	0.60 $\pm$ 0.14	6.73 $\pm$ 1.19	10.60 $\pm$ 6.05	0.72 $\pm$ 0.19
<i>F. sylvatica</i>	16.01 $\pm$ 3.00	16.03 $\pm$ 2.32	1.06 $\pm$ 0.28	14.45 $\pm$ 1.41	10.66 $\pm$ 3.30	0.94 $\pm$ 0.17
<i>L. tulipifera</i>	11.46 $\pm$ 0.94	12.57 $\pm$ 1.29	1.29 $\pm$ 0.61	11.13 $\pm$ 1.11	6.82 $\pm$ 0.70	0.90 $\pm$ 0.26
<i>P. avium</i>	12.04 $\pm$ 1.03	15.39 $\pm$ 5.54	0.62 $\pm$ 0.22	16.29 $\pm$ 4.62	12.97 $\pm$ 3.56	0.94 $\pm$ 0.32

Figure legends

Figure 1. Diagrams of (A) xylem vessels in a stem subject to sap extraction by applying a vacuum at the acropetal end, and (B) the filtering capacity of an intervessel pit membrane in an end wall, which allows passage of sap, while lipid coated nanoparticles are largely prevented. Blue arrows indicate the direction of sap flow when applying a vacuum at the acropetal end of the stem. The orange dotted lines on the inner conduit walls (A) represent lipids, and also (B) coated nanoparticles. It is hypothesised that the lipids extracted in xylem sap originate only from the cut-open vessels (blue colour) at the acropetal end.

Figure 2. (A) Impact of the cumulative volume of extracted sap (mL) on the mean nanoparticle concentration (10^8 particles per mL) based on consecutive sap extraction from a single branch per species in July 2020. Species are shown with different symbols and species-specific logarithmic regressions are calculated for further normalisation of sap samples per volume extracted. (B) Relationship between the volume of extracted sap (mL) and the nanoparticle concentration (10^8 particles per mL) for sap samples extracted from different species and in different seasons. Data are presented as mean values $\pm$ standard deviation ($n = 4$ individuals per species) for samples collected in August 2019, November 2019 and February 2020; a single branch per species was collected in July 2020. The total sap volume and its corresponding mean nanoparticle concentrations in (A) were included in (B). Typical NanoSight images showing nanoparticles in the 1st (C) and 4th (D) sap sample during consecutive sap extraction from *Fagus sylvatica*. Freeze-fracture electron

micrographs of surfactant-coated nanobubbles in xylem sap (E) and a control sample (F) of *Corylus avellana*. Nanobubbles are visible as white gas bubble cores (Pt/C-free areas) and dark, Pt/C decorated surfactant shells, which frequently shifted away from the bubble, and wrinkled during sample preparation. Scale bars: 20 μm for NanoSight images and 100 nm for ff-EM images.

Figure 3. Variation in (A) normalised nanoparticle concentration (10^8 particles per mL) and (B) nanoparticle diameter (nm) in xylem sap samples of different treatments and/or seasons. Data are presented as mean values $\pm$ standard deviation ($n = 4$ individuals), and different letters indicate a significant difference between samples ($P < 0.05$).

Figure 4. (A) Composition of polar lipids, and (B) 18:2-containing TAG in sap samples of six temperate angiosperm species extracted in August 2019 (summer) and February 2020 (winter), respectively. Galactolipids: DGDG = digalactosyldiacylglycerol, MGDG = monogalactosyldiacylglycerol. Phospholipids: LPG = lysophosphatidylglycerol, LPC = lysophosphatidylcholine; LPE = lysophosphatidylethanolamine, PA = phosphatidic acid, PC = phosphatidylcholine, PE = phosphatidylethanolamine, PG = phosphatidylglycerol, PI = phosphatidylinositol, PS = phosphatidylserine. Neutral lipid: 18:2-containing TAG = 18:2-containing triacylglycerol. The sequence of lipids in the bars is the same as the sequence of lipid compounds shown in the caption.

Figure 5. Scores plots of principal component analysis (PCA) based on lipid composition in percentage of (A) 2019 summer and (B) 2020 winter xylem sap of six

angiosperm species. Dots were grouped by plant species, and the elliptical areas are 95% confidence regions.

Figure 6. Relationship of original nanoparticle concentration (10^8 particles/mL) and original polar lipid concentration (nmol/mL) for six angiosperm species. Each dot indicated one sample of xylem sap, which was used for both NanoSight and mass spectrometry measurements. The solid and open circles are xylem sap extracted in summer 2019 and winter 2020, respectively. The black solid and blue dashed lines indicated the linear regression and 95% confidence interval, respectively. The coefficient of determination and *P*-value was shown, when the correlation between the original nanobubble concentration and lipid concentration was significant.

Figure 7. Relationship of vessel anatomy (open vessel volume, mL and total vessel perimeter at the cut xylem surface, cm) and nanoparticle/lipid concentration (original nanoparticle concentration, 10^8 particles/mL; normalised nanoparticle concentration, 10^8 particles/mL; polar lipid concentration, nmol/mL; total lipid concentration, nmol/mL). Each symbol represented the mean value of a species and its standard deviation. Solid lines showed a logarithmic regression.

Figure 1

Figure 2

Figure 3

Figure 4

(A) Summer samples

(B) Winter samples

Col.

Figure 5

Figure 6

Figure 7