The pitfalls of *in vivo* imaging techniques: evidence for cellular damage caused by synchrotron X-ray computed micro-tomography

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Summary

- Synchrotron X-ray computed micro-tomography (microCT) has emerged as a promising noninvasive technique for *in vivo* monitoring of xylem function, including embolism build-up under drought and hydraulic recovery following re-irrigation. Yet, the possible harmful effects of ionizing radiation on plant tissues have never been quantified.
- We specifically investigated the eventual damage suffered by stem living cells of three different species exposed to repeated microCT scans. Stem samples exposed to one, two or three scans were used to measure cell membrane and RNA integrity, and compared to controls never exposed to X-rays.
- Samples exposed to microCT scans suffered serious alterations to cell membranes, as revealed by marked increase in relative electrolyte leakage, and also underwent severe damage to RNA integrity. The negative effects of X-rays were apparent in all species tested, but the magnitude of damage and the minimum number of scans inducing negative effects were species-specific.
- Our data show that multiple microCT scans lead to disruption of fundamental cellular functions and processes. Hence, microCT investigation of phenomena that depend on physiological activity of living cells may produce erroneous results and lead to incorrect conclusions.

Introduction

In plants, long-distance water transport relies on transmission of transpiration-induced negative pressure (= tension) via the xylem conduits connecting root tips to leaf cells (Jensen *et al.*, 2016). Such a fascinating mechanism has the important drawback to be metastable and vulnerable to liquid-to-vapour transition, leading to the blockage of water transport (Zimmermann, 1983). Most frequently, this happens when air is aspirated through interconduit pit membranes into water-filled conduits experiencing critical tensions (Shen *et al.*, 2015; Zwieniecki & Secchi, 2015). Increased frequency of drought and heat waves is accelerating plant mortality rates worldwide (Hember *et al.*, 2017), and hydraulic failure has emerged as the main cause (Anderegg *et al.*, 2011). A detailed knowledge of species-specific vulnerability to xylem embolism (Maherali *et al.*, 2004) and of the eventual capacity for hydraulic

recovery (Mayr *et al.*, 2014; Secchi *et al.*, 2017; Klein *et al.*, 2018) is crucial to improve projections of forest and crop resistance/resilience under future climate scenarios.

Techniques used to measure plant hydraulic conductance upon drought and recovery are generally destructive (Cochard *et al.*, 2013). Stems, roots, petioles and even leaves are excised from plants during or after drought stress and connected to hydraulic systems to measure flow rates across samples under known pressure differences (Sperry *et al.*, 1988). Alternatively, tissues can be infiltrated with dyes to distinguish functioning vs embolized or otherwise nonconducting conduits (Ewers & Fisher, 1989). Due to negative pressure in functional xylem conduits, samples' excision might cause air entry in the xylem, producing artefactual embolism (Wheeler *et al.*, 2013). The magnitude of this artefact may depend on xylem pressure at sampling time and on conduit length (Beikircher & Mayr, 2016). Although several studies found no striking evidence for artefacts associated with classical hydraulic techniques (Jacobsen & Pratt, 2012; Trifilò *et al.*, 2014; Fukuda *et al.*, 2015; Hacke *et al.*, 2015; Scoffoni & Sack, 2015; Venturas *et al.*, 2015; Ogasa *et al.*, 2016; Nardini *et al.*, 2017; Nolf *et al.*, 2017), it is conceivable that estimates of xylem vulnerability to embolism and restoration of xylem functionality via conduit refilling (Nardini *et al.*, 2018) are biased by destructive sampling protocols.

These controversies have contributed to a move forward in the field of plant hydraulics (Jansen *et al.*, 2015; Venturas *et al.*, 2017) and stimulated the use of nondestructive techniques for *in vivo* monitoring of xylem function, like magnetic resonance imaging (Zwieniecki *et al.*, 2013), X-ray computed micro-tomography (microCT; Brodersen *et al.*, 2010), and the optical method applied to leaf venation (Brodribb *et al.*, 2016). In particular, microCT has emerged as a very promising technology, due to relatively ease of use, high spatial and temporal resolution, good contrast between air-filled and water-filled spaces, and fast scan times (Dhondt *et al.*, 2010; Pajor *et al.*, 2013; Cochard *et al.*, 2015). Due to its supposed noninvasive nature, microCT has been suggested to represent a reference technique to determine xylem vulnerability to embolism (Cochard *et al.*, 2015), and the eventual refilling of embolized conduits which supposedly relies on the activity of living xylem parenchyma cells (Tyree *et al.*, 1999; Brodersen & McElrone, 2013; Secchi *et al.*, 2017; Nardini *et al.*, 2018). While some studies demonstrated the occurrence of conduit refilling (Brodersen *et al.*, 2010, 2018), others failed to detect hydraulic recovery following drought and re-irrigation (Choat *et al.*, 2015; Knipfer *et al.*, 2015; Charrier *et al.*, 2016; Hochberg *et al.*, 2016).

These contrasting findings raise questions about possible factors affecting the reliability of microCT observations (Pratt & Jacobsen, 2018). An obvious but often overlooked drawback of microCT is the use of X-ray sources and the potential tissue damage caused by the ionizing radiation (Han & Yu, 2009; Daly, 2012). Although this has been considered a minor issue because of short scan times, some studies on animal organisms indicated irreversible cellular damage even by exposure to very low X-ray doses (Rothkamm & Löbrich, 2003; Nguyen *et al.*, 2015). However, respective X-rays effects on plant samples have never been investigated in detail, although previous studies indicated damage of plant tissues after microCT scans. As an example, Charrier *et al.* (2016) used vital staining to assess the functional status of stem parenchyma cells after exposure to X-rays, showing that several cells were damaged. Similarly, Savi *et al.* (2017) reported shrinkage and brownish scar formation in sunflower stems exposed to X-rays. Hence, it is very important and urgent to test eventual negative or otherwise undesired effects of X-rays on the observed samples, considering the raising importance of microCT as a tool for studies on plant hydraulic functioning. Here, we discuss the results from an experiment specifically designed to assess eventual damage to stem living cells during repeated microCT scans.

Materials and Methods

Plant material

Experiments were performed on three species: *Helianthus annuus*, *Coffea arabica* cv Pacamara and *Populus tremula* × *alba*. *Helianthus annuus* plants were 6-wk old, with a height of *c.* 20 cm and a stem diameter of 2–3 mm at root collar. *Coffea arabica* plants were part of a collection of coffee cultivars hosted by the University of Trieste. Experimental plants were 3-yr-old, with a height of 20 to 30 cm, and a stem diameter of 3 to 4 mm. Plants of *P. tremula* × *alba* were 3 months old, *c.* 50 cm tall with a stem diameter of 3 to 4 mm.

All plants were maintained in a glasshouse at the University of Trieste for 4 wk before experiments (end of September 2017), and regularly watered. Air temperature and relative humidity averaged 16.5°C and 60%, respectively. Mean daily photosynthetic photon flux density (PPFD) was 150 μmol m^{-2} s^{-1} (maximum 400 μmol m^{-2} s^{-1}).

Experimental setup

Experiments were performed at the SYRMEP beamline, Elettra Sincrotrone Trieste (www.elettra.trieste.it). Two silicon filters (0.5 mm each) were used to obtain an average X-ray source energy of 25 keV, resulting in an entrance dose rate in water of 47 mGy s^{-1}. X-ray window was 4 mm in height with horizontal opening up to 120 mm. Initial experiments were performed on intact plants of *H. annuus* and *P. tremula* × *alba* ($n = 3$) to test for eventual over-heating of stems as a possible factor inducing damage during scans. A type T thermocouple connected to a datalogger (1000 Series Squirrel, Eltek) was inserted in the stem at half height. The plant was placed on the sample holder and the stem was aligned with the beam. The beam was turned off to allow temperature equilibration for 5 min. Then, the stem was irradiated for 10 min at a position located 8 mm above the thermocouple insertion point, while temperature was continuously recorded. After a 5 min interval without beam, the stage was moved upward and the stem was irradiated 3 mm above the thermocouple, for another 10 min. The procedure was repeated by directly irradiating the thermocouple insertion point.

The cellular damage caused by microCT scans was assessed by measuring cell membrane integrity estimated by relative electrolyte leakage (REL), and level of RNA degradation on irradiated stem

tissues. Stem segments with a length of 1 cm ($n = 5$) were obtained from the mid portion of stems of well-hydrated plants and immediately wrapped in Parafilm® in groups of five (sampled from five different plants). This allowed to prevent desiccation during storage (see later) and to irradiate more samples during each scan. For each species, 14 sample sets (each with five stem pieces) were prepared (total of 70 stem samples per species).

Samples were subjected to microCT scans while horizontally oriented to assure that all cells were exposed to X-rays during the 360° rotation (the position was checked via real-time visualization). The exposure time was set at 100 ms, at an angular step of $2° \, s^{-1}$ resulting in 3 min scan. Samples were then used to measure REL (seven sets) and RNA quality (seven sets), according to experimental design presented in Fig. 1. Exposed samples were tested after one, two or three consecutive scans at 90-min intervals (E1, E2 and E3). Controls (C0, C1, C2, C3) were never exposed to irradiation. Time of exposure and beam energy level were similar to previously reported experiments (e.g. Charrier *et al.*, 2016), although not all experiments are provided with energy level parameter.

The integrity of cell membranes was estimated via REL measurements. C or E samples were placed in 1.5 ml vials (one segment per vial) with 1 ml of deionized water. In the case of *C. arabica* and *P. tremula* × *alba*, segments were split longitudinally immediately before immersion to favour contact between stem cells and the solution, as preliminary experiments showed that the bark delayed solute diffusion. The tubes were shaken for 30 min at laboratory temperature. The initial electrical conductivity (C_i) of the solution was measured (Twin Cond B-173; Horiba, Kyoto, Japan) using a 10 µl aliquot. Samples were then subjected to three freezing–thawing cycles (1 min in liquid nitrogen followed by 30 min at laboratory temperature), shaken for 5 min, and the final electrical conductivity was measured (C_f) on another 10 µl aliquot. REL was finally calculated as $(C_i/C_f) \times 100$ (Savi *et al.*, 2016).

For RNA analysis, frozen stems for each species and treatment were pooled and ground in sterile mortars using liquid nitrogen followed by tissue lysing (TissueLyser II; Qiagen, Hilden, Germany). Total RNA was extracted following Chang *et al.* (1993), and RNA quantity and quality were determined spectrophotometrically by NanoDrop (Thermo Fisher Scientific, Waltham, MA). RNA integrity (expressed as RNA integrity number, RIN; Schroeder *et al.*, 2006) was finally inspected using the RNA 6000 Nano kit and the Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA), according to manufacturer's instructions. The RNA integrity number (RIN) is a standard reference for RNA quality assessment, specifically introduced in routine RNA quality control processes to avoid subjective interpretation of results. The RIN values resulting at the end of each Bionalyser run provide a classification of total RNA quality, based on a numbering system ranging from 1 (poor quality and high level of degradation) to 10 (high quality and high integrity levels). As RNA degradation proceeds, there is a decrease in the 18S to 28S ribosomal RNA band ratio and an increase in the background noise between the 18 and 28 ribosomal peaks (see https://www.agilent.com/cs/library/applications/5989-1165EN.pdf).

Statistical analysis

One-way parametric analysis of variance (ANOVA) was run separately for each species to test differences between REL values measured in C and E samples through 'aov' function in 'STATS' package for R software. Data were log transformed to meet assumptions of normality and homoscedasticity of variance. Post hoc Tukey's Honestly Significant Differences comparisons were run through 'TukeyHSD' function in 'STATS' package for R.

Results

Exposing stems of *H. annuus* or *P. tremula* × *alba* to X-rays did not result in biologically significant changes in tissue temperature (Fig. 2). Stem temperature oscillated between 25 and 26°C with no beam, and no change was detected at a distance of 3 or 8 mm from the irradiated point even under prolonged exposure (10 min).

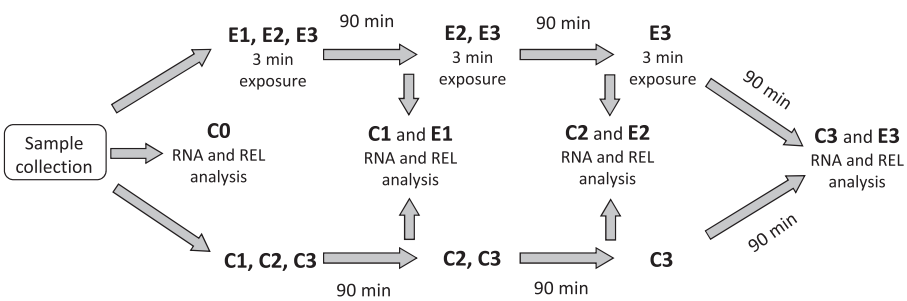

Fig. 1 Experiment time line. C0, control samples (1) immediately used for relative electrolyte leakage (REL) measurements or (2) frozen in liquid nitrogen and kept at −80°C until RNA analysis. C1, samples excised from stems and stored at laboratory temperature until segments from group E1 were ready, then processed for REL or RNA analyses (see earlier). Samples C1, C2 and C3 were prepared to check eventual time-related trends in cellular damage not associated to X-ray exposure. E1, samples exposed to a single 3-min microCT scan (see later), maintained for 90 min at laboratory temperature to allow eventual damage build-up, then processed for REL or RNA analyses. C2, samples excised from stems and stored at laboratory temperature until samples from group E2 were ready, then processed for REL or RNA measurements. E2, samples exposed to two successive microCT scans (90 min between each exposure and after the last one), then processed for REL or RNA measurements. C3, samples excised from stems and stored at laboratory temperature until samples from group E3 were ready, then processed for REL or RNA measurements. E3, samples exposed to three successive microCT scans (90 min between each exposure and after the last one), then processed for REL or RNA measurements.

Temperature in stem section directly exposed to radiation rose by *c.* 1°C in both tested species.

Cell membrane integrity, quantified via REL measurements, was affected by X-ray exposure in analysed species (Fig. 3). In all plants, REL of C0 samples was *c.* 25%, and this value did not change significantly as a function of time from excision in C1, C2 and C3 samples (Fig. 3). In the case of *H. annuus*, the first scan did not induce changes in REL, however, these became apparent in E2 and E3, when REL peaked to 70%, with some samples reaching values as high as 90%. In *P. tremula* × *alba*, REL increased to 35% in E2 and remained similar in E3. In *C. arabica,* an increase of REL to 45% (albeit not significant due to large data variability) was already observed in E1 samples, reaching values > 50% in E3.

Total RNA quality was estimated by the RIN value, as this is a reliable proxy to compare the integrity of RNA in different samples. Analyses based on this metric confirmed the results obtained by REL measurements, showing similar variability and resistance of species to radiation (Fig. 4). In the case of *H. annuus*, the first scan did not affect RNA quality (E1, RIN 7.5). However, RNA degradation increased after the second exposure, and the RNA after the third exposure (E3) was almost fully degraded (Fig. 4). In this species, the controls (C0–C3) showed no degradation of RNA quality. There was no effect of time or X-ray exposures on RNA quality of samples collected from *P. tremula* × *alba* stems, although E3 samples had slightly lower RNA quality (RIN 6.2; Fig. 4). After one scan, RNA extracted from *C. arabica* was partially degraded (E1; RIN 4.1), in comparison with controls (C1, RIN 7). However, both X-ray exposures and time from excision influenced the RNA quality in this species (see C2–C3 and E2–E3; Fig. 4) confirming that *C. arabica* was sensitive to both manipulation and X-rays.

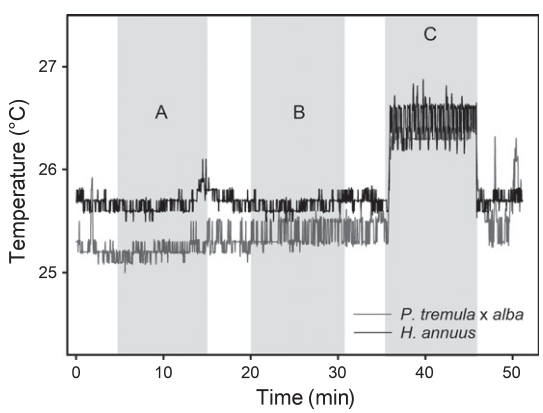

Fig. 2 Temperature changes over time in irradiated stems of *Populus tremula* × *alba* (grey line) and *Helianthus annuus* (black line) at 8 mm (A), 3 mm (B) and at the same position (C) of irradiation point.

Discussion

X-Ray microCT is emerging as an important new tool for the visualization and quantification of xylem embolism (Cochard *et al.*, 2015). Based on its supposed noninvasive nature, microCT has also been used to visualize eventual post-drought recovery. In both cases, plants are generally exposed to successive microCT scans to check embolism build-up during plant dehydration (Choat *et al.*, 2016), or conduit refilling following re-watering (Charrier *et al.*, 2016).

Xylem conduits are frequently considered as inert pipelines, but long-distance water transport relies on the activity of phloem and parenchyma, for example for the regulation of xylem sap ionic content (Zwieniecki *et al.*, 2001; Nardini *et al.*, 2011), modulation of xylem sap surface tension (Losso *et al.*, 2017), release of sugars and water during the refilling of embolized conduits (Secchi *et al.*, 2017) and production of conduit-filling exudates as a response to wounds (Jacobsen *et al.*, 2018). Hence, any eventual damage to living cells can be suspected to alter xylem function, thus casting doubts on the reliability of techniques inducing harmful effects on phloem or parenchyma. Our data clearly show that microCT scans produce severe cellular damage and call for renewed caution in the interpretation of findings based on this technique (Pratt & Jacobsen, 2018). The X-ray energy level and scan times in our experiment were similar to or even lower than those used in several recent studies (e.g. Charrier *et al.*, 2016; Choat *et al.*, 2016; Knipfer *et al.*, 2017). Yet, the X-ray dose was high enough to induce damage to both cell membranes and RNA.

Samples exposed to microCT scans showed significant increases in REL, indicating serious alterations to cell membranes. This is not surprising, as several studies have shown that X-rays produce

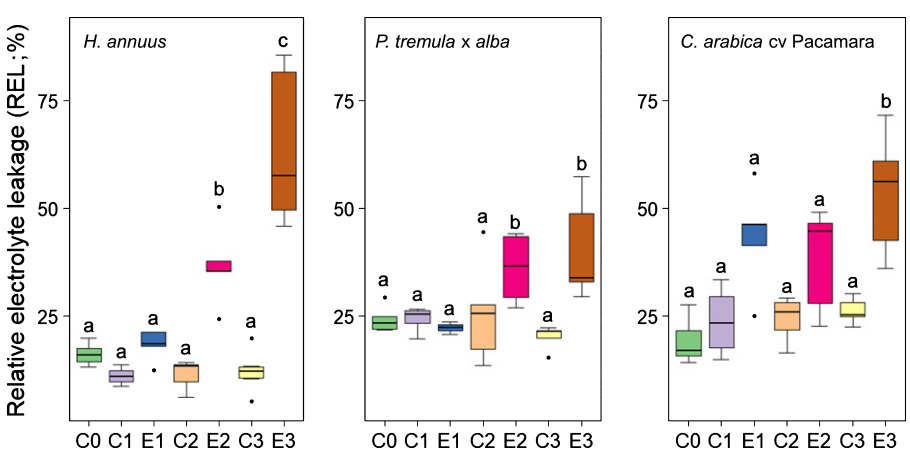

Fig. 3 Median values, 25th and 75th percentiles of relative electrolyte leakage (REL) in control (C) and exposed (E) sample groups in *Helianthus annuus*, *Populus tremula* × *alba* and in *Coffea arabica* cv Pacamara. Different letters indicate statistically significant differences among groups ($P < 0.05$).

irreversible damage to membrane lipid bilayers due to phase transformation and lamellar stacking (Köteles, 1982; Cheng & Caffrey, 1996; Cherezov *et al.*, 2002), with consequent effects on membrane permeability (Cao *et al.*, 2015). Cherezov *et al.* (2002) reported that membrane damage is not associated to temperature effects during sample irradiation at synchrotron light sources, as also confirmed by the lack of over-heating recorded in our samples, but it rather depends on generation of free radicals. Most importantly, Cherezov *et al.* (2002) evidenced that the damage was independent on the source energy in a 9–17 keV range, suggesting that the risk of membrane damage is intrinsic to the technique and cannot be reduced by modifying X-ray energy level without losing image quality and resolution.

In addition to disruption of cell membranes, our data indicate that microCT scans negatively affect RNA quality. This is also not unexpected, as ionizing radiation is known to induce significant alterations on nucleic acids, often resulting in DNA double-strand breaks (Rothkamm & Löbrich, 2003; Han & Yu, 2009) and reactive oxygen species (ROS)-mediated DNA/RNA disruption (Van Huystee *et al.*, 1968; Tominaga *et al.*, 2004), finally leading to severe RNA and/or protein damage (Daly, 2012). Our data from plants are in line with findings obtained on animal cell models and suggest that, as a consequence of RNA damage, protein synthesis can be impaired in stem parenchyma cells after microCT scans.

Both membrane damage and RNA degradation were observed in three studied species, but the susceptibility to X-ray damage was species-specific. *Coffea arabica* was damaged by a single scan, while two-to-three scans were necessary to produce significant effects in the other two species. It is possible that not all microCT experiments performed on different species and reported in the literature are affected to the same extent by harmful radiation effects, potentially explaining the observed range of hydraulic recovery in different species (Choat *et al.*, 2015; Knipfer *et al.*, 2015; Charrier *et al.*, 2016; Brodersen *et al.*, 2018). Our findings call for a careful reassessment of previous conclusions, based on dedicated experiments to evaluate the susceptibility of individual species to the specific experimental conditions adopted.

Our data show that multiple microCT scans lead to disruption of fundamental cellular functions and processes. Hence, microCT investigation of phenomena that depend on physiological activity of living cells may produce erroneous results and lead to incorrect conclusions. This probably applies to conduit refilling, which has been suggested to occur via secretion of sugars into embolized conduits by phloem and vessel-associated parenchyma cells to generate the osmotic forces necessary to counterbalance eventual residual tension in still functioning elements (Secchi & Zwieniecki, 2012). Such a mechanism requires the activation of genes encoding key proteins involved in carbohydrate metabolism pathways and membrane transport of inorganic ions, sugar molecules and water (Secchi *et al.*, 2011; Perrone *et al.*, 2012; Chitarra *et al.*, 2014; Secchi & Zwieniecki, 2016). Thus, failure in detecting conduit refilling in microCT experiments, when involving repeated scans of the same plant subjected to drought stress and then re-irrigated (Choat *et al.*, 2015; Knipfer *et al.*, 2015, 2017; Charrier *et al.*, 2016), might arise from X-ray induced damage to living cells. We

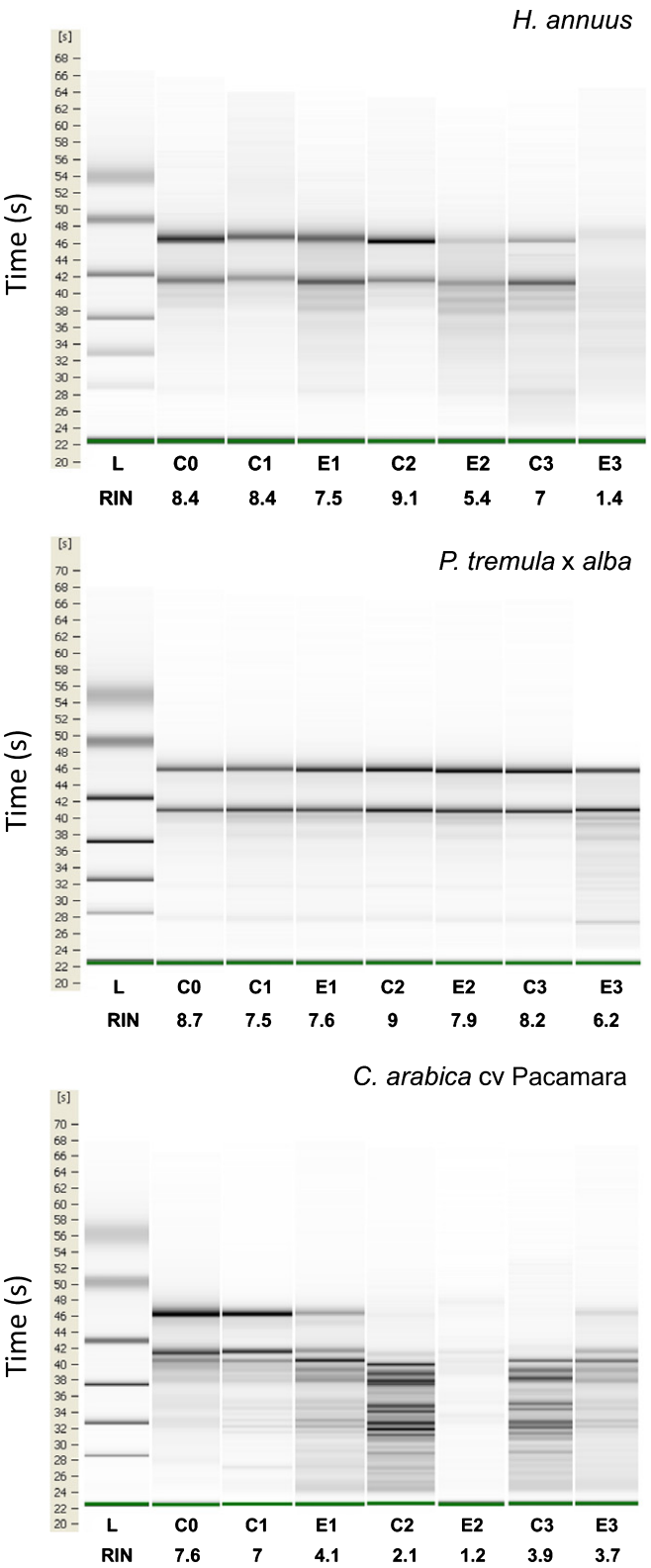

Fig. 4 Evaluation of RNA integrity by Bioanalyser assay. For each RNA sample, extracted from control (C) or exposed (E) plant stems (*Helianthus annuus*, *Populus tremula* × *alba* and *Coffea arabica* cv Pacamara), the related RIN (RNA integrity number) is provided below each lane of the gel. L, ladder.

strongly suggest that such evidence should be re-evaluated in the light of our findings and also recommend that xylem vulnerability or recovery curves generated by microCT should be based on sets of different plants, so that a single plant at any given water status or physiological stage is scanned and observed only once if the species is susceptible to applied radiation levels. This approach might raise doubts on the advantages of microCT as an alternative or complementary technique with respect to classical hydraulic measurements. However, we believe that microCT is very important to provide information or confirmation on the functional status of conduits with no risk of bias due to cutting procedures, and with a three-dimensional (3D) resolution which could not be obtained with hydraulic techniques.

MicroCT observations are increasingly used to observe xylem embolism build-up during plant dehydration. It is assumed that embolism spreads by aspiration of air through the pores of pit membranes. Thus, the validity of observations of embolism spread is seemingly not challenged by our findings, unless X-rays disrupt and alter the structure and porosity of pit membranes, leading to erroneous estimates of embolism vulnerability. Also, damage to living cells might result in wound responses leading to rapid filling of xylem conduits with gels (Crews *et al.*, 2003; Soukup & Votrubová, 2005; Marañón-Jiménez *et al.*, 2017; Che-Husin *et al.*, 2018; Jacobsen *et al.*, 2018). Such an effect would cause conduits to appear filled with a liquid phase even though nonconducting (Pratt & Jacobsen, 2018), leading to overestimate plant resistance to xylem embolism. The occurrence and relevance of these effects should be evaluated by future studies.

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Author contributions

A.N., F.S., M.A.Z. and S.M. planned and designed the research. F.P., C.P., T.S., A.L., S.C., G.T., C.D., A.B., A.G., A.M., S.M., M.A.Z., A.N. and F.S. contributed to perform experiments and analyse data. F.P., A.N. and F.S. wrote the manuscript, with contribution and revision by all other authors.

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