QUANTIFYING PLASTICITY IN VESSEL GROUPING –
ADDED VALUE FROM THE IMAGE ANALYSIS TOOL ROXAS

Georg von Arx[1,*], **Christoph Kueffer**[2] and **Patrick Fonti**[1]

[1]Swiss Federal Institute for Forest, Snow and Landscape Research WSL, Birmensdorf, Switzerland
[2]Swiss Federal Institute of Technology ETH, Zurich, Switzerland
[*]Corresponding author; e-mail: georg.vonarx@wsl.ch

ABSTRACT

The functional role of the connectivity of the xylem network, especially the arrangement of solitary and grouped vessels in a cross section, has often been discussed in the literature. Vessel grouping may improve hydraulic integration and increase resilience to cavitation through redundancy of hydraulic pathways. Alternatively, a high degree of hydraulic integration may facilitate the spread of cavitations among neighboring vessels. Here we show how automated image analysis tools such as ROXAS (see www.wsl.ch/roxas) may greatly enhance the capacity for studying vessel grouping while avoiding some methodological limitations of previous approaches. We tested the new analysis techniques by comparing the xylem network of two populations of the herbaceous species *Verbascum thapsus* collected at a dry and moist site on Big Island (Hawaii, USA). ROXAS accurately, objectively and reproducibly detected grouped and solitary vessels in high-resolution images of entire root cross sections, and calculated different and partly novel vessel grouping parameters, *e.g.* the percentage of grouped (*vs*. solitary) vessels among different vessel size classes. Individuals at the dry site showed a higher degree of vessel grouping, less solitary vessels, greater maximum vessel sizes and an increase of the percentage of grouped vessels with increasing vessel size. The potential, but also some limitations of automated image analysis and the proposed novel parameters are discussed.

Keywords: Hydraulic pathways, automated image analysis, grouped vessels, hydraulic integration, solitary vessels, spreading of embolism, spatial vessel arrangement, vessel grouping index.

INTRODUCTION

The connectivity among vessels, also referred to as vessel grouping in a cross-sectional view (Carlquist 1984; Loepfe *et al*. 2007; Carlquist 2009), is increasingly attracting research interest as a potentially important hydraulic trait in angiosperms, particularly in a context of changing climate. This is because of the outstanding importance of vessels for water-transport. It is widely known, for example, that efficiency potentially increases with the fourth power of vessel radius according to the Hagen-Poiseuille law (Tyree & Ewers 1991), but also that wider vessels are arguably more susceptible to hydraulic

failure by drought-induced cavitation, *i.e.* the rapid phase change of liquid water to vapor ("safety *vs*. efficiency trade-off"; *e.g.* Hacke & Sperry 2001; Hacke *et al*. 2009; Cai & Tyree 2010). The importance of the topology of vessel networks for plant water transport and plant-water relationships in general lies in the water-permeable pit pores formed in the cell walls between neighboring vessels. Previous studies observed an evolutionary trend towards an increase of vessel grouping with increasing water limitation (Carlquist 1966; Lopez *et al*. 2005; Lens *et al*. 2011; Carlquist 2012), although some studies also found inconsistent patterns or no relationship (Baas & Carlquist 1985; Baas & Schweingruber 1987). Similarly, a consistent increase of vessel grouping in response to water limitation and wounding was observed within species (Robert *et al*. 2009) and individuals (Arbellay *et al*. 2012), respectively.

However, the functional role of vessel grouping remains controversial. Several scientists suggested that a high degree of vessel grouping may provide alternative pathways when water transport through a vessel is blocked by drought-induced embolism but bypassed through one or more still functional vessels from the same vessel group. (Baas *et al*. 1983; Zimmermann 1983; Carlquist 1984; Tyree *et al*. 1994). In this case vessel grouping improves the hydraulic redundancy and reduces the potential loss of water transport capacity associated with cavitation. Another benefit of vessel grouping is related to the observed increasing permeability of intervessel pit membranes upon changes in the ionic concentration of the xylem sap which can occur during water limitation ("ionic effect", Jansen *et al*. 2011; Nardini *et al*. 2012). Finally, cavitations may be more easily removed in clustered than in solitary vessels, although this has so far only been postulated theoretically (Hölttä *et al*. 2006). In contrast, an increase of vessel grouping can also bring disadvantages. For example, vessels in a dense network have more contact surface, which enhance the risk of cavitation spreading from one vessel to the next by the aspiration of air through the pit pores (Sperry & Tyree 1988; Brodersen 2013; Brodersen *et al*. 2013) known as air-seeding hypothesis (Alder *et al*. 1997; Wheeler *et al*. 2005; Loepfe *et al*. 2007). Furthermore, vasicentric tracheids, where present, potentially offer a subsidiary conductive tissue that would lower the value of hydraulic integration by vessel grouping (Carlquist 1984, 2001), but likely not invalidate it (Sano *et al*. 2011). The empirical evidence from the relatively few quantitative studies investigating vessel grouping is insufficient to clarify the controversy about its functional role under water limitation.

Vessel grouping has been often quantified visually from the xylem cross section (see Mencuccini *et al*. 2010; Martínez-Vilalta *et al*. 2012 for some exceptions). However, this approach bears several potential limitations. First and most importantly, the coverage is usually restricted to a rather small sub-area of the entire sample and is not representative of the whole variability (see Arbellay *et al*. 2012 for an exception). Secondly, the results are hardly reproducible because there is usually some interpretation involved as to whether two vessels are truly connected through pit pores or not. Thirdly, the possibilities to quantify different aspects of the connectivity of the xylem network beyond grouped *vs*. solitary vessels and group size are limited. These methodological shortcomings can be significantly reduced through the use of image analysis tools for automatic detection and quantification of conduits. Advancements in digital imaging and improved com-

puter performance allow nowadays to perform analysis of large samples while using objective criteria for the definition of vessel grouping. In this article, we present a new approach for quantifying several and partly novel parameters of vessel grouping in entire cross sections and tree cores using the image analysis tool ROXAS (von Arx & Dietz 2005; Fonti *et al.* 2009; Wegner *et al.* 2013). We test the potential of ROXAS for whole-sample grouping analysis with data of the forb *Verbascum thapsus* collected at two sites with contrasting climate, and suggest some directions for future studies into vessel grouping.

MATERIALS AND METHODS

Test dataset

To test the potential of whole-sample grouping analysis, and to investigate the plasticity of several aspects of vessel grouping within a species, we collected roots of 14 individuals of *Verbascum thapsus* L. (Scrophulariaceae) at two contrasting sites on Big Island, Hawaii, USA, between March 28th and April 24th 2008. The two sites represent the dry and wet margin of the distribution of *V. thapsus* in the selected study area (C. Kueffer, unpublished data). The first site ('dry' site), was at 600 m asl on a north-westerly, leeward slope (mean annual temperature: 20.5 °C, total annual precipitation: 790 mm, n = 6). The second site ('moist' site) was at 1700 m asl on an easterly, windward slope (14.5 °C, 2380 mm, n = 8). Plants at both sites grew on lava gravel characterized by poorly developed soil and sparse vegetation cover. The two sites were 60 km apart. The study species *V. thapsus* is a stout, erect herbaceous species that generally produces a rosette in the first and a flowering stalk in the second growing season, after which it dies. *Verbascum thapsus* is considered a problematic invasive species (Kueffer *et al.* 2010), and it is known to show very high plasticity in aboveground growth patterns in response to environmental variability (Parker *et al.* 2003; Seipel *et al.* 2013). All selected individuals were flowering when roots were collected. Plants from the dry site were 1.30 ± 0.15 m tall and plants from moist site 0.64 ± 0.08 m.

After collection, the proximal part of the main root of each individual was conserved in a 50 % ethanol solution. Complete 30–50 μm thick cross sections near the proximal end (root collar) were produced using a sledge microtome and stained with phloro-glucinol/HCl causing reddish coloration of lignified structures (here the cell walls of secondary xylem vessels and lignified parenchyma cells). Stained cross sections were then photographed through the oculars of a transmitting microscope (Olympus BX51, 20× magnification) using a standard digital camera (Nikon Coolpix 990). Multiple overlapping images were taken from each sample and stitched together using PTGui (New House Internet Services BV, Rotterdam, NL) to obtain high-resolution images (0.477 pixels/μm) of the entire cross sections (Fig. 1a, b; von Arx *et al.* 2012)). Digital images of cross sections were then analyzed using ROXAS, and automatic grouping results were compared to manual inspection of the digital images in three randomly chosen individuals. Manual inspection was performed by systematically counting the vessel groups of different sizes in the digital images. Only vessels from the root sections representing the flowering year in 2008 according to herb-chronological analysis were considered for analysis (von Arx & Dietz 2006), and from these only vessels having a
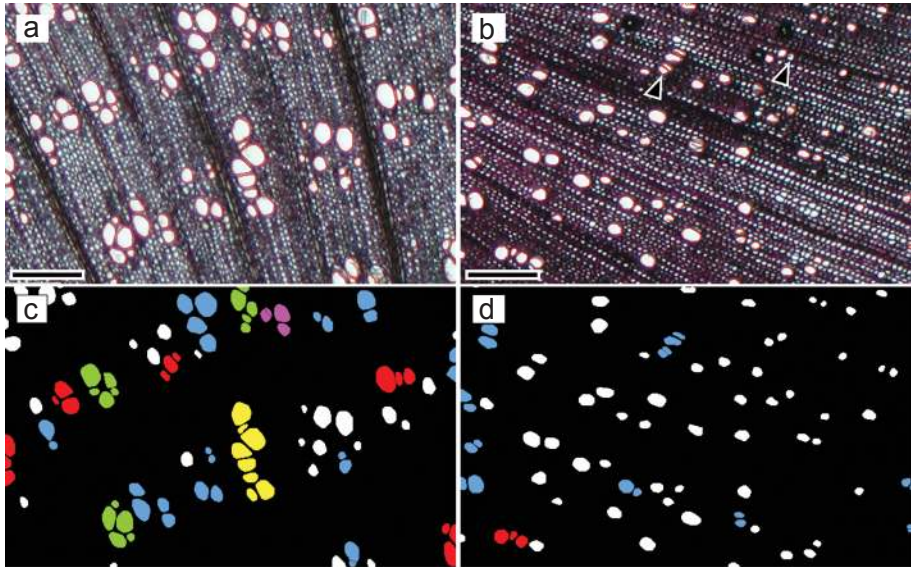
	V_G	1.19
1.90		
0.29	V_S	0.70
3.00	V_M	2.09

Vessel group size FD_G (%):

29.0	☐ - 1	72.3
25.8	■ - 2	24.1
16.1	■ - 3	3.6
4.3	■ - 4	-
16.1	■ - 5	-
8.6	■ - 8	-

Figure 1. Root cross sections (cut-out images) of *Verbascum thapsus* from a dry (a, c) and moist site (b, d) showing how the image analysis tool ROXAS automatically quantifies vessel grouping patterns. – a & b: Original images with outlines of considered vessels in red. – c & d: Binary images of considered vessels with vessels belonging to different vessel group sizes depicted in different colors and values of several vessel grouping parameters given below. The left arrow in b indicates a paired vessel that might actually be overlapping ends of two vessels. The right arrow in b shows a vessel that was excluded because of the lower cut-off vessel size. See Table 1 for explanations of acronyms of vessel grouping parameters. — Scale bars in a and b = 200 μm.

cross-sectional lumen area ('vessel size') ≥ 250 μm^2, because below this size vessels could not be unambiguously distinguished from parenchyma cells.

Parameters to quantify vessel grouping

From a functional perspective, there is no unique best parameter to quantify vessel grouping, because several properties become relevant depending on the ecophysiological processes considered. We therefore considered five different parameters to quantify vessel grouping (Table 1). The first parameter was the vessel grouping index V_G proposed by Carlquist (2001), *i.e.* the mean number of vessels per vessel group (counting a solitary vessel as 1, a pair of vessels in contact as 2, etc.), which also corresponds

Table 1. Parameters of vessel grouping calculated with the image analysis tool ROXAS.

Acronym	Definition
V_G	Vessel grouping index; mean number of vessels per group (counting a solitary vessel as 1, a pair of connected vessels as 2, etc.; Carlquist 2001)
V_S	Vessel solitary fraction; ratio of solitary vessels to all vessels
V_M	Mean group size of non-solitary vessels
FD_G	Frequency distribution of different group sizes (absolute or relative)
RG_{VA}	Percentage of grouped (non-solitary) vessels per vessel size class

to the most commonly used parameter in the literature. While V_G provides a good general estimate of vessel grouping, it misses information about variation. Three other parameters were thus defined: the fraction of solitary vessels (V_S), the mean group size of non-solitary vessels (V_M), and the frequency distribution of vessel groups (FD_G). The rationale behind using V_G, V_S, and V_M is that two individuals may have the same value of V_G, but one of them may have smaller vessel groups (V_M) and thus fewer solitary vessels (V_S), than the other (Fig. 2). Compared to V_G and V_M, FD_G better represents the range of vessel grouping and the relative importance of different group sizes for overall connectivity. Finally, the functional meaning of vessel grouping may not be the same for wide vessels (expected to cavitate first) as for narrow vessels (expected to provide an hydraulic safety net). We therefore calculated the percentage of grouped (non-solitary) vessels for different vessel size classes (RG_{VA}) as a fifth parameter.
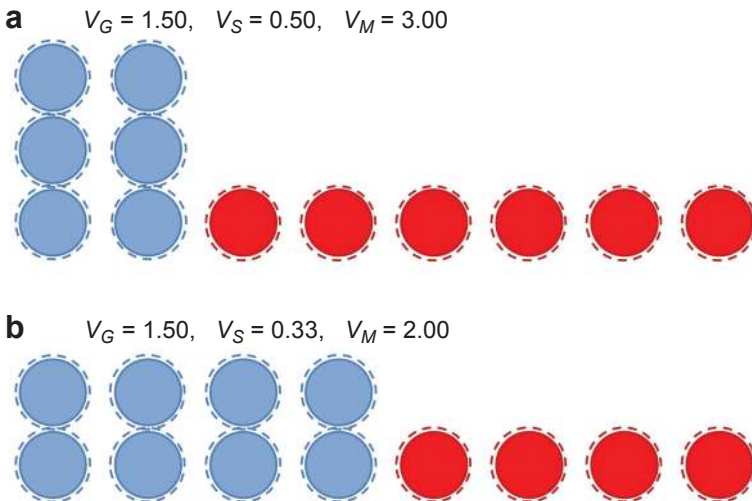
a $V_G = 1.50$, $V_S = 0.50$, $V_M = 3.00$

b $V_G = 1.50$, $V_S = 0.33$, $V_M = 2.00$

Figure 2. The same vessel grouping index V_G can be attained by a setup of differently large vessel groups V_M and a different vessel solitary fraction V_S. In the example of panel **a** with totally 12 vessels, 6 vessels belong to two groups of 3 vessels each, while 6 vessels are solitary. In the example of panel **b**, 8 vessels belong to four groups of 2 vessels, while only 4 vessels are solitary. The hydraulic integration in **b** is therefore greater than in **a**. Vessel lumen area is depicted by filled areas while the outlines of vessel walls are schematically represented by dashed lines.

Image analysis tool ROXAS

ROXAS is an image analysis tool for quantifying the xylem anatomy in cross-sectional samples of trees (angiosperms and conifers), shrubs and herbaceous plants. It is based on the image manipulation and registration capabilities of Image Pro Plus ≥ v.6.1 (Media Cybernetics, Silver Spring, Maryland, USA), and adds own code and algorithms to automatically detect conduits and, with some limitations, tree-ring boundaries (von Arx & Dietz 2005; Fonti *et al.* 2009; Wegner *et al.* 2013). The user can manually edit the automatically-generated results, and obtains a large set of output parameters for the entire sample including ring width, conduit lumen area, and vessel grouping parameters. ROXAS is, under certain conditions, free of charge for the research community (see www.wsl.ch/roxas for more details and a download link).

The identification of grouped *vs.* solitary vessels in the considered plane of the xylem cross section is based on distance criteria: to decide whether two neighboring vessels are touching each other, ROXAS first calculates the Euclidean distance d between their centroids and then subtracts the radius of each vessel to obtain the distance d' between the outlines of the vessel lumina (Fig. 3). The radius of the vessels is thereby adjusted for elliptical vessel shape. If d' is equal or smaller than the empirically determined threshold d_{thr} that approximately corresponds to the double-cell-wall thickness of the two neighboring vessels, they are considered as touching and hydraulically con-
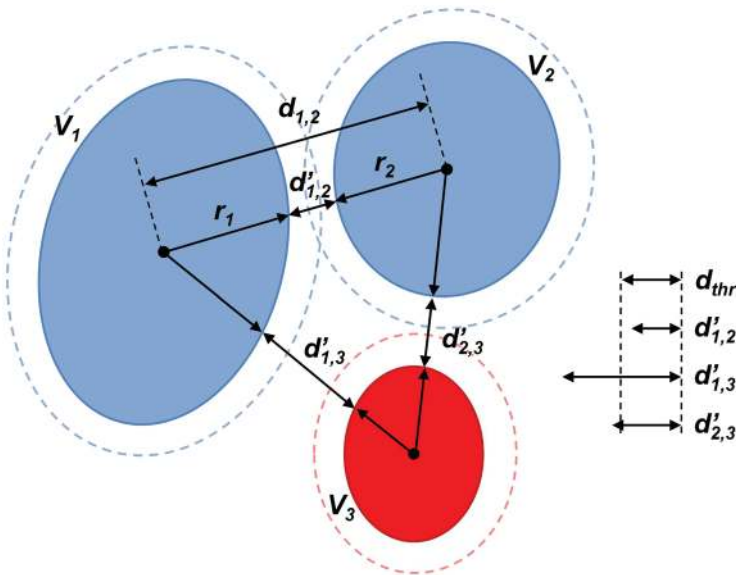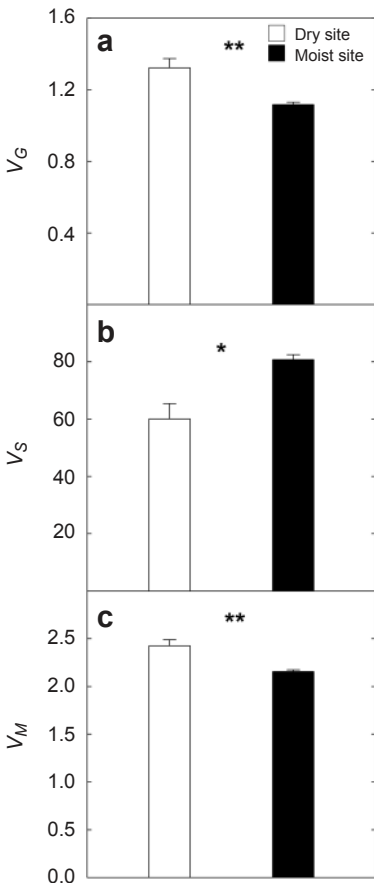

Figure 3. Conceptual model of how ROXAS distinguishes grouped from solitary vessels. The vessels are first expressed as their area-equivalent ellipses. Taking the example of vessels #1 (V_1) and #2 (V_2), the distance $d_{1,2}$ between the centroids of V_1 and V_2 is then calculated. From $d_{1,2}$, the radii r_1 and r_2 along the connection between the centroids is then subtracted to yield the distance $d'_{1,2}$ between the outlines of the two ellipses. If $d'_{1,2}$ is smaller than a threshold d_{thr}, which approximately corresponds to twice the thickness of average vessel walls, the two vessels are assumed to be grouped. Vessel lumen area is depicted by filled areas while the outlines of vessel walls are schematically represented by dashed lines.

nected through pits. This approach does not discriminate different intervessel contact length, *i.e.* the length of vessel wall in contact with a touching vessel as seen in the cross section (Jansen *et al.* 2011; Scholz *et al.* 2013); however, a longer contact length will indicate more intervessel pits and therefore potentially better hydraulic integration of the touching vessels.

RESULTS AND DISCUSSION

Plasticity of vessel grouping within species

Overall, 32,000 vessels were measured in the roots of the 14 individuals (mean number per individual: dry site: 3,800, moist site: 1,150). The individuals from the two sites differed significantly in the vessel grouping index (V_G), vessel solitary fraction (V_S), and the mean size of grouped vessels (V_M; Fig. 4). Individuals growing at the dry site had more grouped and fewer solitary vessels, and mean group size was also larger than in plants at the moist site. Similarly, the relative frequency of different group sizes (FD_G) also differed substantially, with plants growing at the dry site having larger maximum group sizes and a higher representation in all group sizes except the solitary ones (Fig. 5). Wider vessels were more often grouped than narrower vessels in the individuals at the dry site, while at the moist site, vessel grouping was independent from vessel size (Fig. 6). An example of how ROXAS detected vessel groups is shown in Figure 1c,d.

These results are consistent with other studies finding a positive relationship between vessel grouping and habitat dryness (Carlquist 1966; Robert *et al.* 2009; Lens *et al.* 2011; Carlquist 2012). Our results are also in accordance with a previous study by Robert *et al.* (2009) where considerable plasticity in vessel grouping patterns was found within species. Our findings suggest that a higher degree of grouping is both attained by fewer solitary vessels and larger groups of vessel multiples, which must not inevitably be linked (cf. Fig. 2). The dependence of

Figure 4. Differences in **a** vessel grouping index (V_G), **b** vessel solitary fraction (V_S), and **c** mean group size of non-solitary vessels (V_M) in *Verbascum thapsus* growing at a dry (n = 6) and moist site (n = 8) on Big Island, Hawaii, USA (mean ± 1 se). Statistical significance is based on t-tests, * = P ≤ 0.05, ** = P ≤ 0.01.

vessel grouping on vessel size is an intriguing result: based on our limited data set, the increased hydraulic integration of the widest vessels in dry-site plants could enhance pathway redundancy, if some of these vessels cavitate while other (smaller) vessels of the same vessel group remain functional.
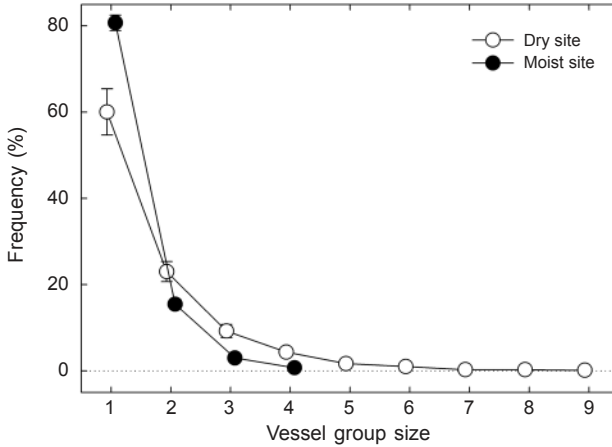

Figure 5. Relative frequency of different vessel group sizes (F_{DG}: including solitary vessels as groups of 1) in *Verbascum thapsus* growing at a dry and a moist site on Big Island, Hawaii, USA (mean ± 1 se). Vessel group sizes containing overall <25 vessels (when multiplying a specific group size by the number of such groups) are not shown. Symbols within vessel group sizes are jittered for better readability.
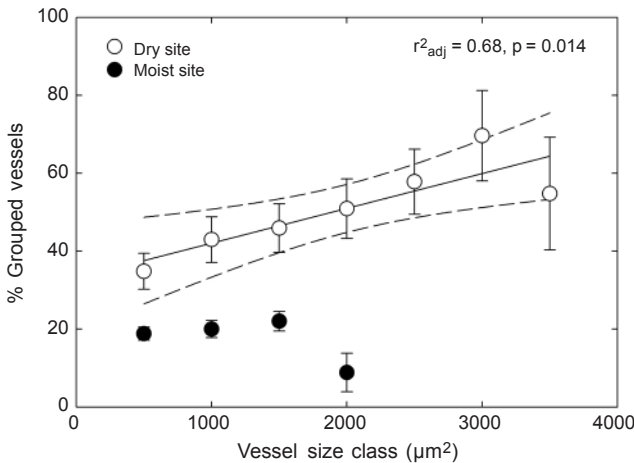

Figure 6. The percentage of grouped vessels per vessel size class (RG_{VA}) in *Verbascum thapsus* growing at a dry and a moist site on Big Island, Hawaii, USA (mean ± 1 se). Unlike at the moist site, grouping increased with vessel size in plants growing at the dry site. Only vessel size classes with ≥ 25 vessels included. Solid line – linear regression. Dashed line – 95 % confidence band.

Table 2. Comparison of vessel grouping parameters quantified with automatic ROXAS and manual inspection method for 3 individuals randomly chosen from the test dataset. See Table 1 for explanation of the acronyms of the vessel grouping parameters.

	Method	V_G	V_S	V_M	FD_G (number of vessels per group size)											
					1	2	3	4	5	6	7	8	9	10	11	12
Moist 1	ROXAS	1.096	0.837	2.155	1351	111	10	–	1	–	–	1	–	–	–	–
	Visual	1.096	0.851	2.191	1374	96	12	–	1	–	–	1	–	–	–	–
Moist 2	ROXAS	1.112	0.809	2.123	1121	109	46	–	–	–	–		–	–	–	–
	Visual	1.137	0.778	2.186	1192	128	27	1	–	–	–		–	–	–	–
Dry 1	ROXAS	1.434	0.504	2.563	2094	534	161	72	21	7	3	3	2	–	–	1
	Visual	1.412	0.526	2.602	2185	478	163	80	22	5	4	3	3	1	1	–

Accuracy of automatic detection of vessel grouping

The automatic results for V_G, V_S, and V_M differed from those obtained by manual inspection by 4 % and less. (Table 2). The differences in the frequency distribution of different group sizes (FD_G) were, with a few exceptions, very small as well. The accuracy of the percentage of grouped vessels per vessel size class (RG_{VA}) could not be checked by manual inspection, but should behave similarly, as it is also based on detected vessel groups. This demonstrates that differences of the ROXAS from the manual inspection approach are negligible. This accordance depends on correct vessel detection and recognition of vessel outlines (avoiding under- and overestimation of vessel lumen area) and a sensible distance threshold d' for the distinction of touching *vs.* non-touching vessels (cf. Materials and Methods). In addition, it was sometimes ambiguous by manual inspection, whether two closely positioned vessels were touching or not, which likely explains the few discrepancies to automatic results for FD_G.

Limitations of (automatic) vessel grouping analysis in cross sections

Two limitations require consideration when investigating vessel grouping in cross sections. First, when performing image analysis automatically or manually, a minimum cut-off vessel size has usually to be defined. This is because of limited image resolution, and because it is difficult to faithfully distinguish vessels from parenchyma cells below a certain size. If a very narrow vessel that is smaller than the threshold size connects to an otherwise solitary wider vessel, the latter will appear as solitary in the results (see Fig. 1b). As a result, vessel grouping will be underestimated. Yet, since this affects all samples, no bias should be expected, unless the narrowest vessels below the cut-off size are more frequent

in some of the samples. Furthermore, from a hydraulic efficiency point of view, the narrowest vessels contribute very little to overall conductivity (Fonti *et al*. 2010), although they may be important as a "safety net".

Second, if the cut runs through oblique reticulate or scalariform perforation plates between two joining vessels or vessel elements (cf. Fig. 1b), they may mistakenly appear as paired vessels from a cross-sectional perspective (Carlquist 2001). This situation may occur quite frequently when considering vessel element lengths of a few hundred microns. However, simple perforation plates between vessel elements seem to be more abundant in hardwood species than scalariform or reticulate perforation plates (Butter-field & Meylan 1982). Accordingly, vessel elements in *V. thapsus* are connected through simple perforation plates, while only intervessel pits are scalariform (Schweingruber 2005). Vessel grouping may also be systematically overestimated if the cutting plane runs exactly through the branching of two vessels. This will be rather rare, however, because vessel branching does not occur extensively along the plant axis (André 2005); vessels rather connect through some axial contact length, which is correctly considered as grouping in this case. Some of these limitations could potentially be removed by 3D visualization techniques such as high-resolution computed tomography (Brodersen *et al*. 2011, 2013); however, accessibility and current limitations with respect to image resolution and sample size may pose challenges for more widespread application.

Perspectives for vessel grouping analysis

The presented example allows to anticipate the promises of future studies of vessel grouping. We demonstrated the value of characterizing vessel groupings – especially grouped versus solitary vessels – that is not possible through previously approaches of whole-sample analysis such as point pattern analysis (Loepfe *et al*. 2007; Mencuccini *et al*. 2010; Martínez-Vilalta *et al*. 2012). Yet, we expect that for a full understanding of structure-function relationships of the xylem hydraulic system further grouping indices than those proposed here will be needed. The additional parameters suggested in this paper serve as examples for moving beyond the well-established vessel grouping index V_G – which is a mean value with limited functional information – and characterizing also variation of vessel grouping. We hope that this paper stimulates innovation towards the most useful quantification of spatial vessel arrangement. Particularly, quantification of vessel size-dependent grouping may prove to better connect structural with functional xylem properties under environmental variability. It is well possible, for example, that quantifying vessel size-dependent grouping should extend to the question of whether two grouped vessels are of similar or different size. In this sense, automated image analysis as performed by ROXAS opens the door for new types of data analyses.

CONCLUSIONS

The image analysis tool ROXAS accurately calculated established and novel aspects of vessel grouping patterns. Thanks to its efficiency entire cross-sectional samples can be analyzed, which likely provides sufficiently large numbers of vessel measurements to detect relatively small differences within species and even among annual rings of individuals. The large number of vessels analyzed by ROXAS therefore constitutes an

important methodological advancement. Other key advantages of automated quantification of vessel grouping lie within its objectivity and reproducibility.

Vessel grouping is a very poorly investigated functional trait of the xylem hydraulic system. Parameters used so far are rather simple and probably insufficient to cover the various functional meanings. Particularly unexplored parameters such as the frequency distribution of vessel group sizes, and the percentage of grouped (*vs.* solitary) vessels among different vessel size classes may prove to be of great ecological relevance and should therefore be considered in future studies. In addition, a better characterization of the structural context of vessels – such as the size of the vessels involved in grouping – may be necessary to understand whether vessel grouping is beneficial during water limitation. Thanks to its versatility, applications of ROXAS might also contribute to systematic studies that build on vessel grouping analyses (*e.g.* Lens *et al.* 2009), or to test hypotheses developed for woody clades with herbaceous species (*e.g.* Lens *et al.* 2013).

ACKNOWLEDGEMENTS

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