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Wood Anatomical Structure of *Rhododendron arboreum* Sm. in a Drought Manipulated Experiment under two Forest Types in Western Bhutan Himalayas

Dorji Dukpa[1], Om Katel[2], and Yeshey Khandu[2]

Abstract

Formation of woody structure in plant is a dynamic process and is affected by environmental conditions such as moisture availability. This experiment set up tested the hypothesis that drought leads to changes in wood anatomical characteristics, accompanied by decreased vessel size that is compensated by increased vessel density in *Rhododendron arboreum* Sm. The study was carried out in a cool temperate broadleaved forest dominated by oak species (*Quercus lanata* Sm., *Quercus griffithii* Hook. f. and Thomson ex Miq. and in a cool temperate conifer forest dominated by hemlock – *Tsuga dumosa* (D. Don) Eichler. *R. arboreum* wood samples were collected from four homogeneous replicated plots of 725 m^2 (two control and two roofed plots) established in 2014 at each forest type. The wood samples were sliced to 20 µm thickness using microtome, dehydrated using different concentration of ethanol before embedding them onto a glass slide. Micro section image was captured using digital camera and analysed using the software ImageJ32. Drought did not impact wood anatomical characteristics and did not lead to a decrease in vessel size. However, drought led to increased vessel density in both the forest types. At the same time, potential seepage of lateral interflow and leakage from roof may have impacted results and therefore further investigations applying deeper trenching against interflow and good roofing over an extended period are recommended to confirm this present finding.

Keywords: Control plot, roofed plot, vessel density, vessel size, wood structure

Introduction

Wood formation in plant is a dynamic process that depends on environmental conditions such as moisture and nutrient availability (Dünisch and Bauch, 1994; Puech *et al*., 2000; Wind *et al*., 2004). Among these environmental factors which are expected to vary with global climate change is the soil water scarcity (IPPC, 2007). Soil water scarcity has been recognised as the major factor affecting plant growth, development, and productivity (ibid). Drought is an important stress factor and can lead to physiological and structural response to maintain balanced water relations (Maherali and DeLucia, 2000; Arend and Fromm, 2007; McDowell *et al.*, 2008). Drought stress occurs when soil water content drops below a threshold inducing restrictions to growth and transpiration (Breda *et al*., 2006). Drought affects trees' morphological and functional features (Orshan, 1989; Floret *et al*., 1990) and also wood anatomy and its spe-

[1] Ugyen Wangchuck Institute for Conservation and Environmental Research, Lame Goempa, Bumthang
[1] Corresponding author: dendro1972@gmail.com
[2] College of Natural Resources, RUB, Lobesa

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cific properties (Bouriaud *et al.,* 2005).

Tree ring width and vessel features store a wide range of environmental information (Campelo *et al*., 2010). Altering the required amount of water results in changes in tree rings (Fonti, 2012). The pattern of this ring formation is prominent in spring and summer and such rings are called annual rings (Edlin, 1975). Annual rings provide specific information on varying environmental conditions that the tree was subjected to (Downes *et al*., 2002). The intra-annual climatic information is difficult to extract from tree ring width, but other anatomical features can be used to generate signals with high temporal resolution (Wimmer, 2002). An increase in wood density has been associated with decrease soil water supply, e.g. in *Eucalyptus globulus* Labill. (ibid).

Wood anatomical structures are widely used for assessing the drought stress in forest stands. Characterization of such anatomical structures is based on cell characteristics (wall thickness) affected by environmental factors during the process of growing (Eckstein, 2004; Fonti *et al*., 2009). Arend and Fromm (2007) reported that drought led to decrease in vessel size, which was compensated by increasing the number of newly formed vessel cells in *Populus* sp. A study carried out by Noshiro and Suzuki (1995) showed that wood anatomical variation for 26 different *Rhododendron* species in Nepal is homogeneous and at the same time, intra-specific variation is less pronounced than inter-specific variation. However, Merev and Yavuz (2000) showed that wood characteristics vary with large altitudinal differences, because the intra-specific variation of wood anatomical characteristics tends to be large for species with a large altitudinal distribution. For example, five Turkish *Rhododendron* L. species, *R. luteum* Sweet., *R. ungernii* Trautv., *R. caucasicum* Pallas., and *R. ponticum* L. growing from sea level to 2230 m showed that wood anatomical characters were significantly correlated with altitude.

In Bhutan, where ecosystem services derived from forests are the mainstay of the national economy (NEC, 2011), an understanding of anticipated changes in forest composition are mandatory. *Rhododendron* species are a very important understory component and represent an ecological filter that determines regeneration success of over story species and thus has a close direct impact on forest dynamics (Gratzer *et al.,* 2004). *R. arboreum* is a dominant understory species in both, mixed conifer as well as cool temperate broadleaf forests (ibid). As such, the species is well suited to explore differential responses between two major forest types. Knowledge on the resilience of this species by characterizing wood anatomical acclimation potential to drought will therefore provide essential information on changes to be expected with global environmental change at the ecosystem level in some of the most important forest types of Bhutan. This study tested, in an experimental setup, the hypothesis that drought leads to changes in wood anatomical characteristics accompanied by decreased vessel size that is compensated by increased vessel density in *R. arboreum* growing in cool temperate conifer and cool temperate broadleaf forests.

Materials and Method

Study site

This study was carried out in the forests of two districts, namely; Thimphu and Wangdue. The two sites comprised of conifer and broadleaved forests (Figure 1). The drought experiment study was started in March 2014 having cool temperate conifer zone and cool temperate broadleaved respectively. The forest types comprised of varied temperature and soil moisture, which are further constrained by altitude and precipitation (Wangda and Ohsawa, 2006).

The first study site was in Tashigang Goempa under Thimphu district in the cool temperate conifer zone dominated by *Tsuga dumosa* D. Don. followed by *Quercus semecarpifolia* Smith*, R. arboreum, Picea spinulosa* Griff.*,* and *Acer campbelli* Hiern. which occur in greater densities. The site was located at 3280 m with mean summer temperature (JJA months) of 13.7 °C and the mean winter temperature (DJF

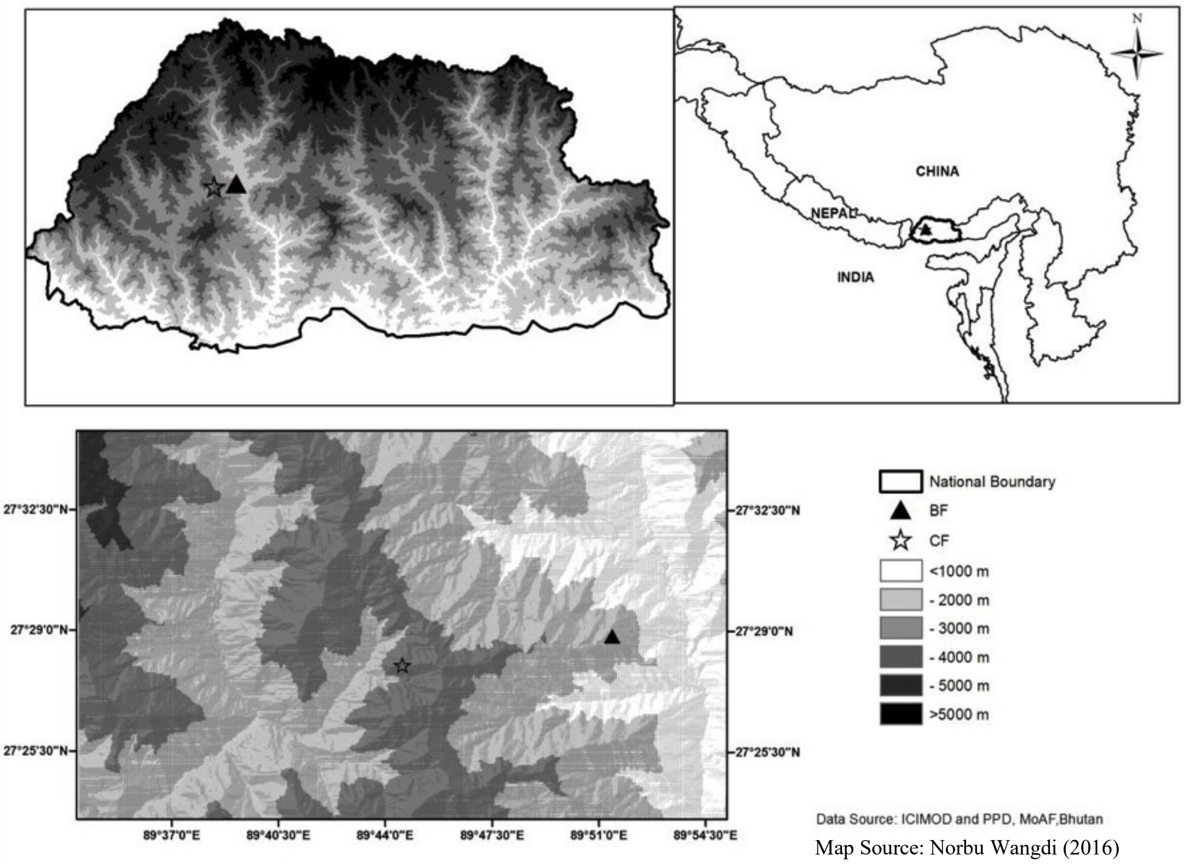


Figure 1: Study area

months) of 2.5 °C for the June 2014-June 2015 period. The total precipitation measured on site (July 2014-June 2015) was 1175 mm (Wangdi, 2016). The soil type at this site was described as Cambisol with clay loam texture (Om, 2016).

The second study site was in Pangsho Goempa under Wangdue district in the cool temperate broadleaved zone dominated by *Quercus lanata* Smith. and *Quercus griffithii* Hook. f. and Thomson ex Miq. followed by *R. arboreum*, *Symplocus* spp., and *Lyonia ovalifolia* Wall. The study site was located at 2480 m with mean summer temperature (JJA months) of 17.4 °C and mean winter temperature (DJF months) of 6.3 °C June for 2014-June 2015 period. The total precipitation measured on site (July 2014-June 2015) was 1027 mm (Wangdi, 2016). The soil was characterized by Luvisol soil type with silty clay texture (Om, 2016).

Site selection

Each study site was selected based on same tree composition, tree density, vegetation cover, gradient, and minimum number of dead trees in order to avoid bias because wood structure, vessel size, and density differ are influenced by the above criteria. Sites having compacted soils, steep slopes, shallow ground water, understory bamboo growth, and nearby existing footpaths were excluded to minimize disturbance impact.

Plot size and layout

In each site, four plots (two drought manipulated (roofed) and two control plots) were established, which summed up to eight plots for two locations to have equal representative from each site. Plot size of 25 m (along contour) x 29 m (towards slope) length was established, which was a standard forest plot size (Gratzer, 2014, pers. comm.). The above plots were laid out to compare wood anatomical characteristics, vessel size, and vessel density (number) between roofed and control plots. The site selection and laying out was a combined collaboration with

Department of Forest and Park Services.

In the roofed plots, rainfall exclusion was carried out using transparent plastic sheeting mounted on a bamboo frame. The height of the roof was set at eight feet. A deep trench (1 m) covered with plastic sheet was constructed just above the plot boundary and lateral sides of the plot to prevent water flow into the plot and to prevent trees to absorb moisture. The experimental plots were roofed from April to end of August for the year 2014 and April to late October for the year 2015. During the remaining months plastic roofs were removed. These roofed plots were considered as drought induced plots.

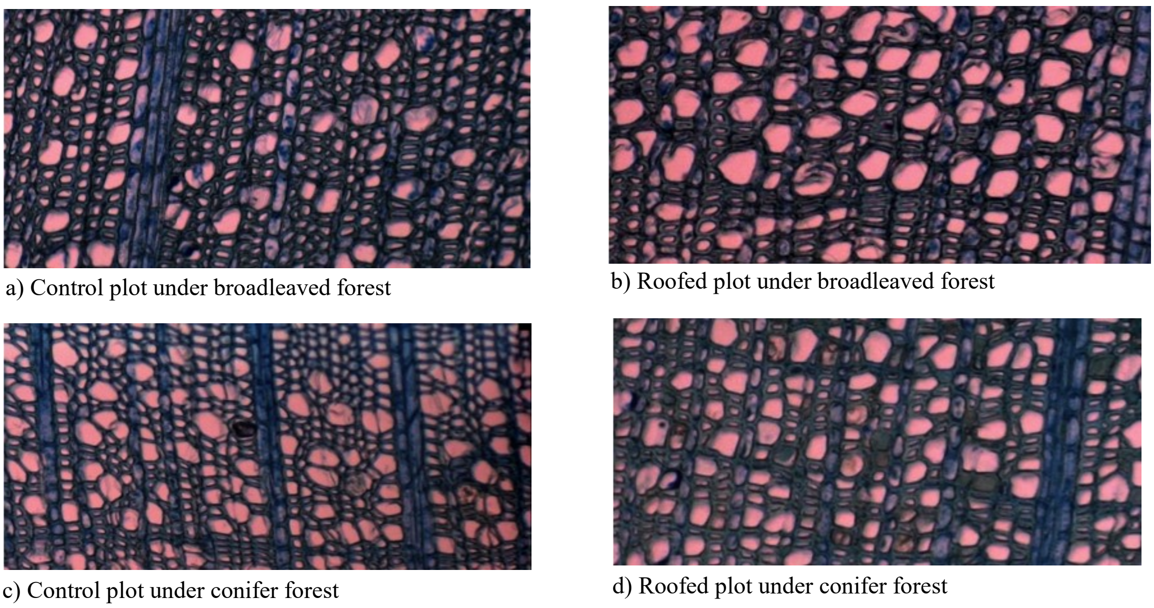
For the control plots (no plastic roof), only red painted wooden pecks on four corners of the each plot were erected. These plots were kept in a natural condition.

Wood sample collection

To test the hypothesis that drought leads to changes in wood anatomical characteristics, accompanied by decreased vessel size that is compensated by increased vessel density in *R. arboreum*, two wood samples (1 x 1 x 3 cm cube under bark) from each *R. arboreum* tree were taken using a homemade chisel (Grabner, 2014, pers. comm.). Sampled trees had a mean diameter at breast height (dbh) of 14 cm and heights ranging from 4.1 to 8.5 m. The sample was collected facing the contour line at breast height (1.3 m) from the ground to avoid reaction wood and missing rings. A total of 22 wood samples were collected from broadleaved forest (6 trees from each treatment) and conifer forest (6 trees from roofed and 4 trees from control plots, respectively). The wood samples were immersed in a mixture of 96% alcohol and glycerin in a volume proportion of 1:1 inside a labeled zip-lock plastic bag to avoid vessel shrinkage during transportation to the laboratory.

Laboratory work

Micro slides of 20 µm thickness were obtained from each wood sample. The sliced wood section was stained in an Astrablue solution and then dehydrated using 30%, 60%, 96%, and 100% ethanol consecutively before placing them onto a glass slide and embedding them in Apparel. The images of the micro sections were captured with a Zeiss Axiocam HRc digital camera attached to a binocular microscope (Zeiss Axioplan 2). The captured images were stitched and analyzed using the software ImageJ32. The laboratory process was carried out to compare the wood structure, vessel size, and density of each forest type and treatment.



a) Control plot under broadleaved forest

b) Roofed plot under broadleaved forest

c) Control plot under conifer forest

d) Roofed plot under conifer forest

Figure 2: Wood anatomical structure

Statistical test

We tested whether the drought manipulation had a significant effect on vessel size and vessel density over the time period of two years using Two-way Repeated Measures ANOVA. We conducted this test because this study had two treatments located at two different places/ elevations and the data were collected over two consecutive years (2014 and 2015). The data were normally distributed.

Results and Discussion

Wood anatomical characteristics

The experiment result showed that irrespective of forest type and drought treatment over a two-year period, *R. arboreum* trees have diffuse porous wood with distinct rings or indistinct boundaries and solitary vessels with an angular outline (Figure 2). Similarly, irrespective of treatment and forest type, *R. arbo-*

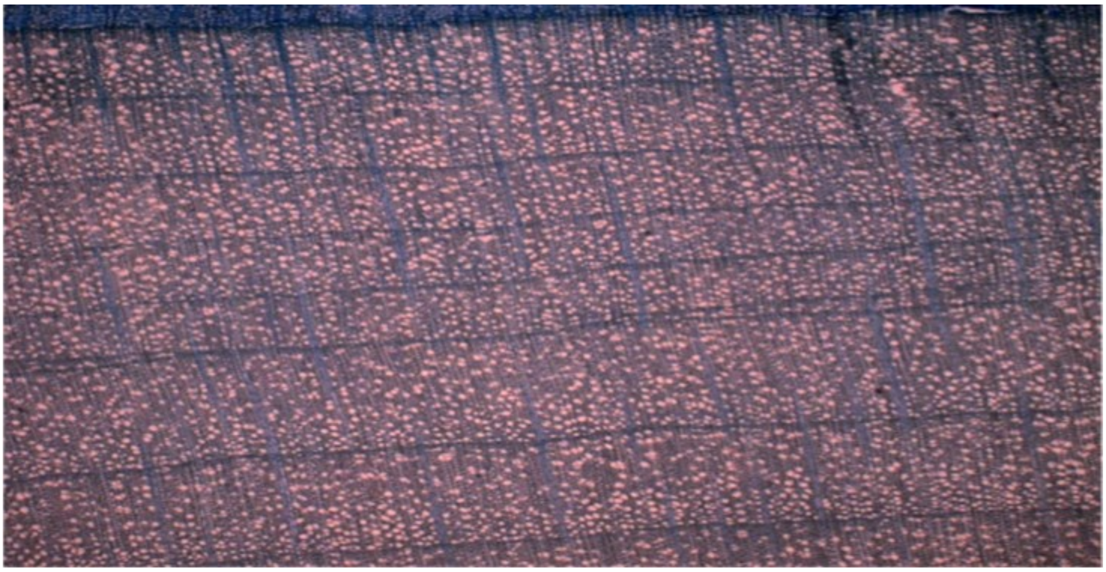


Figure 3. Discontinuous rings

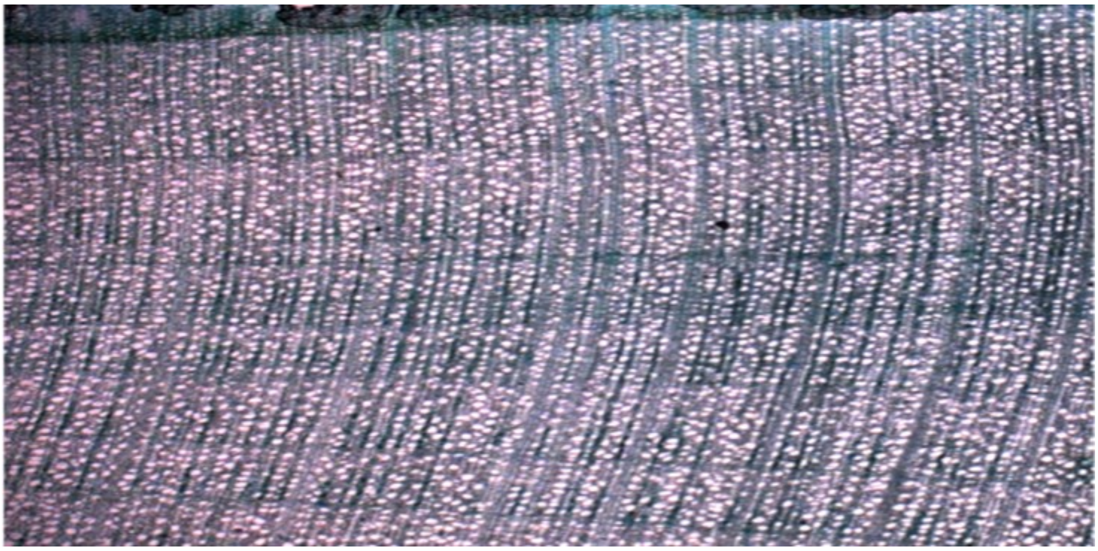


Figure 4. Narrow annual rings

reum frequently showed discontinuous rings (Figure 3) and on extreme sites the annual rings were narrow (Figure 4). In general, wood anatomical characteristics were homogenous (diffuse porous, solitary vessels with an angular outline and a few discontinuous rings). In a similar study in Nepal, Noshiro and Suzuki (1995) found that wood anatomical variation in 26 different *Rhododendron* species was homogeneous and the intra-specific variation was less pronounced than the inter-specific variation. However, Merev and Yavuz (2000) found that if there is a wide difference in altitude variation then wood characteristics changes because intra -specific variation of wood anatomical characters tends to be larger for species with a large altitudinal range. In the present study, the altitude variation was not large between the two sites (3280 m for the cool temperate conifer forest versus 2480 m for the cool temperate broadleaved forest) when compared to the entire altitudinal range of *R. arboreum* that ranges from 1430-3460 m (Noshiro and Suzuki, 1995). Besides this species, other *Rhododendron* species such as *R. ponticum* also show smaller wood anatomical variation over a larger altitudinal range (Merev and Yavuz, 2000).

Vessel size and density under broadleaved forest

Time (year 2014-2015) had no significant effect on vessel size ($F_{1,1} = .01$, $p = .93$) and vessel density ($F_{1,1} = .15$, $p = .74$) (Table 1 and 2). Between treatments, vessel size ($F_{1,1} = 15.34$, $p = .05$) was significantly different with no effects on vessel density ($F_{1,1} = 4.49$, $p = .17$). The interaction of time and treatment showed a significant impact on vessel density ($F_{1,1} = 16.04$, $p = .05$), but not on vessel size ($F_{1,1} = 10.77$, $p = .08$). These proved the hypothesis that drought induced by roofing led to smaller vessel size, which is compensated by increased vessel density over time. As per the study by Wangdi (2016) on the same sites on drought stress tolerance and climate change adaptation potentials of the concerned forest ecosystems, roofing had a significant effect on the soil moisture content ($p < .05$) and the soils were significantly drier than control plots from the onset of the rainy season onwards irrespective of the year. Gimbel *et al.* (2015) also reported a reduction in soil moisture and soil water potential at all depths in forest precipitation reduction experiment.

There is a trade-off between wood density and hydraulic conductivity for some angiosperms species (Stratton *et al.,* 2000). This trade-off may be the low hydraulic conductivity allowing cell wall materials to take up more wood volume (increased density) with small/ less conduit (Searson *et al.,* 2004). A similar response was reported that increased vessel number in drought treated poplar might be interpreted as a compensatory response to the loss of hydraulic conductivity resulting from the reduction in vessel size (Arend, 2007). Drought induces vessel size, which is compensated by increasing the number of newly formed vessel cells in *Populus nigra* L. (Breda *et al*., 2006). Vessel features, fiber length, and vessel diameter decrease as pore density increases with increasing drought or decreasing temperature (Baas, 1982) as shown for *Rhododendron* (Noshiro and Suzuki, 1995), *Alnus nepalensis* (Noshiro *et al.,* 1994), *Syringa oblata* var. *giraldii* (Zhang *et al.,* 1988), *llex* sp. (Baas, 1973), *Symplocos* sp. Jacquin. (Oever, 1981), and the Oleaceae family (Baas *et al.,* 1988). The formation of smaller vessels in oak species in response to drought is consistent with dendroecological studies (Villar-Salvador *et al.,* 1997; Garcia-Gonzalez and Eckstein, 2003; Corcuera *et al*., 2004; Eilmann *et al.,* 2006) and appears to be an adaptive response because smaller vessels are thought to be less susceptible to drought induced xylem embolism (Sperry and Saliendra, 1994; Lo Gullo *et al*., 1995; Hacke and Sauter, 1996; Sperry and Tyree, 1998).

The other potential explanation for the increase in vessel density is potential damages to the xylem tissue. Vessels may disrupt due to sap tension that exceed a threshold value (Chaves *et al*., 2003). This occurs when there is decline in soil water content and stomata close, leading to limits in water fluxes in the plants (ibid). Davis *et al.* (1999) reported that tracheid-bearing conifer and diffuse-porous angiosperms with mean diameter vessels < 30 µm show no freezing-induced cavitation under modest water stress (xylem pressure = -0.5 MPa), whereas species with mean diameter vessels > 40 µm are cavitated under the same conditions and species with intermediate mean diameter vessels 30–40 µm show partial cavitation by freezing. Such effect is possible in case of Tashigang Goempa study site. However, *R. arboreum* had less than 30 µm mean vessel diameter so the collapse of vessels was not observed. However, Chhetri and Lepcha (2015) revealed rapid disruption of physiological and biochemical activities in *R. arboreum*. This is due to drought induced polyethylene glycol-6000 formation, equivalent to -0.2 and -0.5 MPa osmotic stress level, which increases the production of protein, soluble carbohydrates, and proline leading to membrane deterioration. The cellular water deficit can change concentration of solutes, membrane structure, disruption of water potential, and denaturation of proteins (Choudhuri and Choudhur, 1993). Increasing drought intensity causes permanent disruptions of steady state water transfer in xylem, which is detrimental to tree survival (Breda *et al.*, 2006).

Table 1: *R. arboreum's vessel size under broadleaved forest*

	Vessel size (um^2)				
	Mean	***SE***	**Mean Square**	***F***	***p***
Year					
2014	466.2	144	37.72	0.009	0.93
2015	470.4	164			
Treatment					
Control	619.1	116	274,954.22	15.34	0.05
Roofed	316.4	190			
Year*Treatment					
2014*Control	641.7	106	6,606.57	10.77	0.08
2014*Roofed	291.1	184			
2015*Control	597.4	131			
2015*Roofed	341.6	197			

Table 2: *R. arboreum's vessel density under broadleaved forest*

	Vessel density (mm^2)				
	Mean	***SE***	**Mean Square**	***F***	***p***
Year					
2014	372.69	60.82	179.79	0.15	0.74
2015	364.94	44.47			
Treatment					
Control	226.97	26.96	241,444.93	4.49	0.17
Roofed	510.66	117.09			
Year*Treatment					
2014*Control	223.19	20.38	702.71	16.04	0.05
2014*Roofed	522.18	128.3			
2015*Control	230.75	34.22			
2015*Roofed	499.14	106.02			

Drought induced by roofing in this study might have led to smaller vessel size, which was compensated by increased vessel density over time. However, this study could not confirm if cavitation have occurred in the vessel, which was not visible under the microscopic.

Vessel size and density under conifer forest

Time (year 2014-15) had no significant effect on vessel size ($F_{1,1}$ = 111.58, p = .06) and vessel density ($F_{1,1}$ = 0.15, p = .73) (Table 3 and 4). Between treatments, there was no effect on vessel size ($F_{1,1}$ = 1.50, p = .43) and vessel density

Table 3: *R. arboreum's vessel size under conifer forest*

	Vessel size (um^2)				
	Mean	***SE***	**Mean Square**	***F***	***p***
Year					
2014	339.03	25.10	2,548.23	111.58	0.06
2015	374.73	21.72			
Treatment					
Control	328.99	0.60	6,220.42	1.45	0.44
Roofed	384.77	46.22			
Year*Treatment					
2014*Control	305.41	6.67	263.13	0.41	0.64
2014*Roofed	372.65	56.87			
2015*Control	352.58	7.87			
2015*Roofed	396.88	35.57			

Table 4: *R. arboreum's vessel density under conifer forest*

	Vessel density (mm^2)				
	Mean	***SE***	**Mean Square**	***F***	***p***
Year					
2014	372.69	60.82	179.79	0.15	0.74
2015	364.94	44.47			
Treatment					
Control	226.97	26.96	2,41,444.92	4.49	0.17
Roofed	510.66	117.18			
Year*Treatment					
2014*Control	223.19	20.38	702.71	16.04	0.05
2014*Roofed	522.18	128.30			
2015*Control	230.75	34.21			
2015*Roofed	499.14	106.02			

($F_{1,1}$ = 4.50, p = .16). The interaction of time and treatment showed significant impact on vessel density ($F_{1,1}$ = 16.04, p = .05) but not on vessel size ($F_{1,1}$ = .41, p = .64). The study could not confirm the hypothesis that drought leads to decrease in vessel size that is compensated by increase vessel density in *R. arboreum* growing in cool temperate conifer forest since the result showed that drought induced by roofing led to increase in vessel density but not the vessel sizes. The reason could be due to 20% reduction of volumetric soil water content. As observed in the broadleaved forest (Wangdi, 2016), it is possible that drought induced in the conifer forest might also require a minimum of 31% reduction in soil water content to observe decrease in vessel size with density increase. An increase in wood density is associated with decreased soil water supply in *Eucalyptus globulus* Labill. (Wimmer *et al.,* 2002), *E. nitens* (Deane and Maiden), and Maiden trees (Beadle *et al.,* 2001). This could be the reason as to why *R. arboreum* growing under the conifer forest might be performing similar to *Eucalyptus globulus, E. nitens,* and Maiden tree. This trend may be associated with site factors such as temperature or nutrient supply (Macfarlane and Adams, 1998; Catchpoole *et al.*, 2000). Other possible explanation could be that the drought affects physiological responses in forest tree types as per site and species-specific trends (Carnicer *et al.,* 2011).

In both the forest types, drought led to increased vessel density. However, in broadleaved forest vessel size reduced with no effect on cool temperate conifer forest. The difference could be due to higher annual evapotranspiration (*E*) of coniferous forests (Tsukamoto, 1998; Kuraji, 2003). However, *E* of broadleaved forest is similar to the *E* of young coniferous forests and higher than *E* of old coniferous forests (Komatsu *et al.,* 2007). Even though the present study did not assess the total vessel area, it is likely that the increased density of smaller vessels did not lead to substantial changes in total vessel area in the broadleaved forest. On the other hand, increased density of constant sized vessels in conifer forests may lead to an increase in the total vessel area, necessitated by higher *E* under drought conditions in the conifer forest.

Also, drought produced both positive and negative physiological responses in forest tree types with wide variety of site dependent and species-specific trends (Carnicer *et al.,* 2011). Davis *et al*. (1999) reported that tracheid-bearing conifer and diffuse-porous angiosperms with intermediate mean diameters vessels 30–40 µm show partial cavitations by freezing and 44 µm at or above freeze–thaw cycle at -0.5 MPa. The current study did not observe any vessel collapse under the microscopic because mean diameter vessels was less than 30 µm.

Conclusion

The formation of woody structures is a dynamic process, and is affected by environmental condition such as water availability. As a result of two years of drought induced in *R. arboreum* grown in two forest types, the number of vessels increased while the vessel size and wood structure remained constant irrespective of forest type. This result indicated that there is a role of drought in changing vessel density. Drought seems to increase transpiration potential of *R. arboreum* by increasing the overall vessel cross sectional area. This potentially has positive implication for increasing the resilience of the species against the impacts of climate change, in particular drought. Potential water seepage from lateral interflow and leakage from roof may have impacted results and therefore, further investigations applying deeper trenching against interflow over an extended period are recommended to confirm the present findings.

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