Functional adjustments of xylem anatomy to climatic variability: insights from long-term Ilex aquifolium tree-ring series

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The present study assessed the effects of climatic conditions on radial growth and functional anatomical traits, including ring width, vessel size, vessel frequency and derived variables, i.e., potential hydraulic conductivity and xylem vulnerability to cavitation in Ilex aquifolium L. trees using long-term tree-ring time series obtained at two climatically contrasting sites, one mesic site in Switzerland (CH) and one drought-prone site in Italy (ITA). Relationships were explored by examining different xylem traits, and point pattern analysis was applied to investigate vessel clustering. We also used generalized additive models and bootstrap correlation functions to describe temperature and precipitation effects. Results indicated modified radial growth and xylem anatomy in trees over the last century; in particular, vessel frequency increased markedly at both sites in recent years, and all xylem traits examined, with the exception of xylem cavitation vulnerability, were higher at the CH mesic compared with the ITA drought site. A significant vessel clustering was observed at the ITA site, which could contribute to an enhanced tolerance to drought-induced embolism. Flat and negative relationships between vessel size and ring width were observed, suggesting carbon was not allocated to radial growth under conditions which favored stem water conduction. Finally, in most cases results indicated that climatic conditions influenced functional anatomical traits more substantially than tree radial growth, suggesting a crucial role of functional xylem anatomy in plant acclimation to future climatic conditions.

Keywords: European holly, precipitation, ring width, temperature, vessel characteristics, wood anatomy.

Introduction

The geographic distribution, growth and survival of tree species are strongly controlled by climatic factors (Kozlowski and Pallardy 1997), and for these reasons climate change can be regarded as an important selective force on trees and forests (IPCC 2014). Among climatic variables, precipitation and air temperature exert a primary influence on plant communities. In some regions, particularly the Mediterranean, a substantial drought due to changes in both precipitation and temperature patterns is expected to impact trees and forests (Giorgi and Lionello 2008, Raftoyannis et al. 2014). Recent evidence showed the potential for drought to cause extensive forest die back, and that warm and dry summers negatively affected trees growing at southern sites (e.g., Anderegg et al. 2012a, Galván et al. 2014). During drought, vulnerability of xylem conduits to cavitation and embolism results in a critical plant condition, which might jeopardize the water conducting system, heavily limit plant performance and eventually compromise plant survival (Tyree and Sperry 1989). Nardini et al. (2013) documented these damage consequences to the plant hydraulic system in Mediterranean tree species exposed to severe and prolonged summer droughts.

Xylem vulnerability to cavitation and run-away embolism shows substantial variability among tree species (e.g., Maherali et al. 2004). However, major physiological and structural adjustments are also possible at the plant scale, and therefore plant phenotypic plasticity can be regarded as an important...
process trees adopt to cope with drought stress. Effective tolerance to reduced water availability might arise, for example, by modified carbon storage and allocation patterns, which results in modifications to plant hydraulic architecture, achieving a favorable trade-off between water transport efficiency and safety against xylem embolism (Tyree and Ewers 1991, Magnani et al. 2002, Anderegg et al. 2012b, Mencuccini 2014).

Another important phenotypic response is related to changes in xylem structure. The Hagen–Poiseuille model predicts that wider vessels contribute to increased hydraulic conductance and facilitate higher stomatal conductance with more photosynthetic carbon gain; however, these vessels are expected to be at greater risk of drought-induced embolism (Brodribb and Holbrook 2003, Mencuccini 2003, Sperry et al. 2006). Structural xylem changes might compensate for cavitation safety risks, for instance by clustering vessels and modifying vessel number and distribution (Hacke et al. 2006, Loepfe et al. 2007). Indeed, substantial environmental effects have been evidenced by changes in wood anatomical structure and xylem vulnerability, and climatic factors, atmospheric CO$_2$ concentration and nutrient availability exert a major role, among the others effects (e.g., Fonti et al. 2010).

Tree annual rings provide ecological information related to climate change, and studies have shown tree-ring time series to be important archival data to explore past environmental conditions and woody species functional acclimatisation to changes in climatic variables over time (e.g., Hughes 2002, Fonti and Garcia-Gonzalez 2004, Fonti et al. 2010, Sanders et al. 2014). However, to date, scant data are available on the associated long-term response of tree growth, xylem structure and hydraulic conductivity to environmental conditions, particularly in the Mediterranean (Cherubini et al. 2003). A notable challenge in dendroecological studies is to evaluate whether data on seasonal climatic influences derived from anatomical traits is redundant or novel, and enhances data obtained from tree-ring analyses (Fonti et al. 2010).

In the present study, we tested the hypothesis that functional xylem anatomy, more than xylem growth, might be affected by climatic conditions. In addition, xylem changes depend on climatic regimes, reflecting a trade-off between plant growth and hydraulic safety. We obtained tree-ring cores from $I.\ aquifolium$ L. (European holly) at climatically contrasting sites in Southern Europe, and combined quantitative wood anatomy with tree-ring analyses. $I.\ aquifolium$ was chosen as a suitable model species due to its ecological amplitude, wide geographic distribution and the primary role in spreading in a temperate deciduous forest (Peterken and Lloyd 1967, Berger and Walther 2006). Furthermore, $I.\ aquifolium$ has origins in humid Tertiary climates, and therefore the species might also be vulnerable to more intense and prolonged drought periods forecasted for the Mediterranean region.

Materials and methods

Study sites and climatic data

Our research was performed at two sites with divergent climatic conditions. One site was located at San Giorgio in southern Switzerland (CH) (45°54′N, 8°57′E, 800 m above sea level, a.s.l.); the climate (mean annual temperature: 7.7 °C, annual precipitation: 1600 mm) is defined by dry mild winters and damp warm summers; the presence of summer rains (650 mm between June and September) makes a strong difference from Mediterranean climate, while prolonged drought frequently occurs in winter (Uttinger 1946). Forest species composition is dominated by deciduous trees. Castanea sativa Mill. is the dominant tree species up to 900 m a.s.l. (Conedera et al. 2004) and Quercus petraea Liebl., Quercus pubescens Willd., Tilia spp., Fraxinus spp. and Ostrya carpinifolia Scop. are subordinate species. Previous studies (Walther et al. 2001, Berger and Walther 2006) reported the CH site is one of the best examples of evergreen species, largely $I.\ aquifolium$, spread into temperate deciduous forest.

The other site (ITA) was chosen at Monte Pollino in Southern Italy (39°56′N, 16°14′E, 1000 m a.s.l.). High summer temperatures and inconsistent precipitation, with a minimum in summer, characterizes the climate (mean annual temperature: 9.5 °C, annual precipitation: 1077 mm); snow occurs, and typically persists from November to May; dew and fog, common in winter and spring, play an important ecological role. Fagus sylvatica L. is the dominant forest tree species, and Abies alba Mill., Acer pseudoplatanus L., Tilia spp. and Taxus baccata L. are associated subordinate community members. $I.\ aquifolium$ occurs primarily in the $F.\ sylvatica$ forest understory (Todaro et al. 2007, Rita et al. 2014).

We obtained climate data from meteorological stations close to our study sites for analyses. Data from the Lugano meteorological station (46°00′N, 8°57′E, 335 m a.s.l.) was applied to CH (Swiss Weather Service, MeteoSwiss); data from the Castrovillari (39°83′N, 16°19′E, 343 m a.s.l.) and San Lorenzo Bellizzi (39°88′N, 16°32′E, 851 m a.s.l.) meteorological stations (Servizio Idrografico e Mareografico Italiano, SIMI) were collected for ITA. Temperatures were corrected for altitude by applying a coefficient of $-0.007$ °C·m$^{-1}$ (International Civil Aviation Organization 2002).

Long-term series of mean annual temperature ($T_m$) and total annual precipitation ($P$) for the two study sites are shown in Figure 1. Strong oscillations in $T_m$ were evident at CH over the last 60 years with a steep rise during the last decade, whereas at ITA, $T_m$ exhibited a gradual and regular rise since 1980. Clear trends in $P$ were not discernible at CH over the last 60 years, whereas $P$ decreased at ITA from 1970 to 1990, and then increased again over the last 20 years (Figure 1). A significant low correlation was found between $T_m$ and $P$ in both sites.

Tree sampling and tree-ring series

In summer 2012, a forest stand was selected at each study site where no sign of manipulation or disturbance (e.g., logging,
transverse sections. Histological preparations were obtained using the following method described by Schweingruber and Poschlod (2005): sections were placed on a slide and stained with 2% astrablue and 1% safranin solutions, which resulted in un lignified cells appearing blue and lignified cells appearing red; sections were subsequently dehydrated using a series of ethanol solutions of increasing concentrations, washed with xylol and embedded in Canada balsam.

Annual ring images were captured with a CCD digital camera (Olympus ColorView III, Soft Imaging System, Germany) mounted on a reflected light microscope (Olympus BX41, Japan). Sequential images were subsequently stitched using the Microsoft Image Composite Editor (ICE 1.3.5, Microsoft Corp., Redmond, Washington) (see Figure S1 available as Supplementary Data at Tree Physiology Online), and analyzed with the image-analysis software ImageJ v.1.40 (National Institutes of Health, Bethesda, MD, USA, available for download from http://rsb.info.nih.gov/ij/index.html). In particular, this software led to identification of xylem cells as objects. First, to make easier the identification of the features in an image, the contrast was enhanced; then, through an appropriate image conversion process (i.e., 24-bit color into 8-bit grayscale) the objects contour was produced in a threshold binary image (mask) in which only the particles of interest were kept, in our case the vessels pore lumen (see Figure S2 available as Supplementary Data at Tree Physiology Online); before any measuring, the image was calibrated from a scale bar of known length in the image. In each ring, a rectangular ($W\times l$, where $W$ is ring width and $l = 2\, \text{mm}$) surface was defined, and a semi-automated particle analysis was applied to determine the Cartesian coordinates of each vessel, vessel number ($N$) and vessel lumen area ($A$); careful visual inspection was also performed to verify all vessel elements and non-vascular elements were included.

Vessel frequency ($f_v$) was determined as the ratio between the number of vessels and the area analyzed, and vessel diameter ($D_v$) was calculated as follows:

$$D_v = \sqrt{\frac{4\, A}{\pi}}$$

Vessels are not exactly circular; therefore $D_v$ of ~1000 vessels was compared with the calculated elliptical transverse section diameter ($D_e$), following Lewis and Booze (1995):

$$D_e = \sqrt{\frac{2\alpha^2 \beta^2}{\alpha^2 + \beta^2}}$$

where $\alpha$ and $\beta$ are major and minor perpendicular lumen diameters. The difference between $D_v$ and $D_e$ did not exceed 1%, thus $D_e$ was used in subsequent calculations to determine the hydraulically weighted mean diameter ($D_h$) for each ring, according to Tyree and Zimmermann (2002):

$$D_h = \left(\frac{1}{n} \sum_{i=1}^{n} D_i^4\right)^{1/4}$$
Potential hydraulic conductivity ($K_s$) was estimated according to the modified Hagen–Poiseuille equation reported by Tyree and Ewers (1991, their Eq. (4)).

\[
K_s = \frac{\pi \rho D}{128 \eta S} \sum_{i} D_i^4
\]

where $\rho$ and $\eta$ are the density and dynamic viscosity of water at 20 °C, $S_i$ is ring surface, $n$ is the number of vessels per unit of surface and $D_i$ is the vessel diameter defined in Eq. (1).

Finally, xylem vulnerability to cavitation ($V_x$) was estimated according to Carlquist (1977) as follows:

\[
V_x = \frac{D_i}{\delta_i}
\]  

### Data analysis

For all variables, data from different sampled trees were averaged to obtain mean values for each annual ring. Successively, to remove non-climatic signals, data were standardized by applying a 32-year cubic spline function with a 50% frequency response (Cook and Kairiukstis 1990) using the library ‘dplR’ (Bunn 2008) in the R statistical suite (R Development Core Team 2013). The observed values were divided by the spline function estimates to obtain indices, and autoregressive modeling was performed to remove the first-order autocorrelation (AC1). Standard dendrochronological statistics were subsequently computed to evaluate the quality of chronologies between tree mean (with area $A$) follows the Poisson distribution with mean $= \lambda A/A$. Given $n$ events in $A$, event positions follow an independent sample from the uniform distribution on $A$. First, a test of complete spatial randomness (CSR) was obtained by testing for departure from a Poisson null model. Then, Ripley’s $K$-function was used to study the spatial pattern; $K(r)$ is a function of the mean number of neighbors over all vessels within a distance $r$ (circle radius) of a typical point of the process; results are the deviation from expectation at each vessel under CSR; a weighting factor was used to reduce edge effects (Haase 1995, 2001). The estimated $K(r)$ is typically compared with the Poisson $K$ function: $K(r) > \pi r^2$ suggests clustering, while $K(r) < \pi r^2$ suggests a regular pattern. Results are interpreted more easily by calculating the common $L$-function $L(r)$ by normalizing the $K$-function, and obtaining a zero benchmark.

\[
L(r) = \frac{K(r)}{\pi r - \pi r^2}
\]

Values of $L(d) > 0$ indicate a clumped vessel pattern, while $L(r) < 0$ indicates a random vessel pattern. Significance of the point pattern was tested using simultaneous Monte Carlo simulations to estimate 95% confidence limits (Haase 1995).

Xylem trait and climatic variable relationships were assessed for 1925–2011 and 1934–2012 at CH and ITA, respectively. First, generalized additive models (GAMs) were applied to examine tree-ring feature response curves to the climate predictor variables ($T_m$ and $P$). Generalized additive models are non-linear and non-parametric regression techniques, which do not require a priori functional relationship specifications between dependent and independent variables. Model strength is the production of a link function that defines a relationship between the response variable mean and a smoothed function of each explanatory variable (Hastie and Tibshirani 1990). A random intercept term was incorporated in the model to account for variation among trees. The smoothing parameter estimate in the semi-parametric regression was achieved by employing restricted maximum likelihood as an alternative to a generalized cross validation (GCV) score. The Akaike information criterion (AIC), which penalized the model’s goodness-of-fit by the number of parameters was also calculated. Generalized additive model analysis was performed using the library ‘mgcv’ (Wood and Wood 2014) in the R statistical suite.

Successively, bootstrapped response and correlation functions were calculated using the package ‘bootRes’ (Zang and Biondi 2013) in the R statistical suite to assess the significance of the change in mean annual temperature ($T_m$) and annual precipitation ($P$) on standardized tree-ring width and xylem traits. The 95% confidence level based on 1000 bootstrap resamplings was calculated to assess the statistical significance of the regression coefficients.

### Results

Xylem traits exhibited substantial variation between the two sites (Figure 2). CH showed higher values than ITA for ring width ($W$), average vessel size ($A_v$), vessel frequency ($f_v$), and more notably, potential hydraulic conductivity ($K_s$), whereas a difference between sites was not detected for xylem vulnerability to cavitation ($V_x$).

Xylem traits displayed evident temporal fluctuations at both sites (Figure 3). Sinusoidal variation was discernible in $W$ at ITA over the last century, with a decreasing trend since 1980. Over the same period, the same decreasing trend was evident at CH, where in the previous period, a more stable pattern was apparent; $A_v$ remained rather stable across years at CH, whereas oscillations and an increase was apparent at ITA in recent years; $f_v$ displayed a steep increase at both sites since 1980, more marked at CH; the same pattern was displayed in $K_s$ but a less steep rise was observed since 1980; $V_x$ variation was essentially the inverse to $f_v$ at both sites, as expected from Eq. (5).
Functional adjustments of xylem anatomy to climatic variability

We evaluated relationships between ring width and xylem traits, where measured anatomical traits were independent of ring width (Figure 4). \( A_{av} \) displayed a negative relationship with \( W_r \) at ITA, whereas a flat relationship was observed at CH; notable negative relationships were evident at both sites for \( f_v \), which were, as expected, in the opposite direction to the positive relationships observed between \( W_r \) and \( V_x \).

Point pattern analysis results indicated that vessel distribution differed between CH and ITA sites (Figure 5). In particular, a significant departure from CSR was detected at ITA, where clustering was evident from the middle-to-late portions of the ring width. However, departure from CSR was not observed at the CH site.

The relationships between climatic variables and xylem traits at CH and ITA were explored using GAMs. All GAMs analyses conducted for both sites revealed significant results for \( T_m \) and \( P \) smoothed terms (Figure 6). In general, relationships with climatic variables were weak or not observed at CH, while relationships with climate were more evident at ITA. At CH, in particular, GAMs showed a slightly negative relationship for temperature with vulnerability to cavitation (\( V_x \)), and a slightly positive relationship with vessel frequency (\( f_v \)) and potential hydraulic conductivity (\( K_s \)). At ITA, curvilinear relationships for temperature were observed; in particular, positive relationships were displayed for temperature with \( A_{av} \), \( f_v \) and \( K_s \) up to 10 °C maximum, followed by a decrease, whereas \( V_x \) exhibited a decrease until reaching 10 °C, followed by an increase. The GAMs did not show evident relationships between precipitation and xylem traits at CH. However at ITA, negative relationships between \( P \) and \( W_r \), \( P \) and \( V_x \) and positive relationships between \( P \) and \( A_{av} \) and \( f_v \) and \( K_s \) were detected (Figure 6). It is noteworthy that at both sites, GAMs did not detect relationships between any climatic variables with ring width (\( W_r \)).

Relationships between tree-ring traits and climatic variables (annual and seasonal averages) were further explored by bootstrap correlation functions (Figure 7). Tree-ring width (\( W_r \)) did not reveal a significant climate signal at both the study sites. A clear interpretation emerged from results between sites following assessment of relationships based on annual climatic averages. CH xylem traits were primarily affected by temperature; and \( K_s \) and \( V_x \) exhibited respective positive and negative relationships with temperature. Precipitation had the greatest effects at ITA; \( A_{av} \) showed a positive relationship with temperature and precipitation, \( K_s \) and \( V_x \) exhibited positive relationships with precipitation, and \( V_x \) was negatively associated with precipitation. Similar results for relationships between xylem traits and climatic variable averages on a seasonal basis were found.

**Discussion**

**Xylem trait relationships and vessel clustering**

Xylem traits, including ring width (\( W_r \)), average vessel size (\( A_{av} \)), vessel frequency (\( f_v \)) and potential hydraulic conductivity (\( K_s \)), excluding vulnerability to cavitation (\( V_x \)), exhibited higher values at the mesic CH site compared with the drought-prone ITA site (Figure 2). This result is congruent with the established pattern of xylem trait variability within tree species derived from climatically contrasting regions and precipitation gradients.
relationships between vessel size (and frequency) and ring width were absent (at CH) or negative (at ITA), therefore a positive association did not emerge between positive relationships between vessel size, hydraulic conductivity and growth were reported (e.g., Carlquist 2001, Hacke and Sperry 2001, Zanne et al. 2010), suggesting higher conductivity allowed for increased gas exchange rates, resulting in higher growth rates (Brodribb and Holbrook 2003, Mencuccini 2003). Flat and negative relationships between vessel size and ring width, consistent with our results, suggested radial growth was not the destination for carbon allocation under conditions favoring stem water conduction and carbon uptake.

The spatial arrangement of conduits might notably affect functional xylem attributes. Significant grouping observed at ITA supported vessel clustering (Figure 5), and this might improve hydraulic safety in habitats suffering from drought conditions. Indeed, research showed that water bypassing of air-filled conduits through alternative pathways was easier in clustered vessels, and that vessels grouping may improve xylem hydraulic

Figure 3. Average chronologies of tree-ring width (W), average vessel size (Aav), vessel frequency (fv), potential hydraulic conductivity (Ks) and xylem vulnerability (Vx). Filled and empty circles are CH and ITA sites, respectively. The continuous lines correspond to a locally weighted polynomial regression; dotted lines indicate the 95% confidence interval.

**Climatic variable relationships**

Long-term tree-ring series have often been used to investigate the response functions of tree growth to climate. In most cases, ring width was used as the main response variable to climatic variables. However, in the last decade, research efforts have been devoted, as we do here, to establishing a relationship between climatic conditions and anatomical wood characteristics. The objectives of these studies were to annually reconstruct the functional xylem modifications to temporally changing environmental conditions (e.g., Fonti et al. 2010). Overall, relationships between climatic variables and anatomical xylem traits or derived variables (vessel size $A_{v}$, vessel frequency $f_{v}$, potential hydraulic conductivity $K_{s}$ and vulnerability to cavitation $V_{x}$) revealed site-specific functional adjustments. Notably, in most cases climatic conditions influenced anatomical traits more than tree radial growth (Figure 6).

The relationship between xylem traits and temperature was supported at the mesic (no water limitations) CH site, whereas a stronger precipitation signal was emphasized at the drought-prone ITA site (Figures 6 and 7). To date, rather clear relationships between functional anatomical traits and temperature have been reported for conifer species growing in temperature-limited environments. Under certain conditions, for example, climate warming favors wider tracheid cell lumen, wider rings and enhanced hydraulic conductivity (Fonti et al. 2010, Bryukhanova and Fonti 2013). However, Gea-Izquierdo et al. (2013) reported...
less evident relationships between xylem anatomy and temperature for deciduous tree species growing in temperate environments. Interactive climatic effects were frequently associated with drought, and tree acclimation to climatic variability might result from the coordination of several complementary traits (Gea-Izquierdo et al. 2013).

A hydraulic conductivity, $K_s$, increase with temperature at CH was associated with a rise in vessel frequency ($f_v$), whereas
vessel size ($A_{av}$) remained unchanged. This resulted in an increased lumen fraction (i.e., the ring surface proportion covered by vessels), accompanied by a decrease in cavitation vulnerability ($V_x$). Tyree and Zimmermann (2002) reported that smaller vessels were less efficient in water transport, however at lower cavitation risk. Overall at CH the combined effect of rising temperature on vessel frequency and size seems to be associated with enhanced xylem conductivity and reduced risk of drought-induced embolism. This might suggest potential to acclimate to future increasing temperatures and water stress in *I. aquifolium* at mesic sites. Berger and Veste (2007) showed a positive temperature response in this species for photosynthetic rates suggesting a potential climate-warming advantage in the species. The positive association between temperature and hydraulic functionality might also indicate the possibility for enhanced performance and opportunistic spread of the species in forest gaps, and geographic range expansion under climate-warming scenarios (Bañuelos et al. 2003, Walther et al. 2005, Skou et al. 2012, Stokes et al. 2014). Under Mediterranean drought-prone conditions, a marked precipitation effect was expected on tree growth and xylem properties, resulting in acclimation of hydraulic architecture to changing water availability (see Cherubini et al. 2003 for review). Rather surprisingly, GAMs revealed a negative relationship between ring width and annual precipitation at ITA. This might also suggest a preferential carbon allocation to tree height growth rather than stem radial growth under favorable water balance conditions, resulting in trees growing to approach the upper canopy layer and more advantageous light conditions. Valladares et al. (2005) provided experimental evidence showing *I. aquifolium* should not be considered an obligate understory species under relatively mild Mediterranean conditions.
Increased precipitation was associated with formation of an increased number of vessels with larger diameter, consistent with other deciduous tree species subjected to improved water balance conditions (Abrantes et al. 2013 in Quercus ilex; Rossi et al. 2013 in Olea europaea). However, this does not appear to be a general pattern. For example vessel frequency increased in response to prolonged drought in the ring-porous Quercus canariensis (Gea-Izquierdo et al. 2012). As previously stated, vessel frequency and spatial arrangement, such as vessel clustering, could play an integral role in the balance between hydraulic conductivity safety and prevention of xylem embolism.

At ITA, the curvilinear response of xylem traits to rising temperature was difficult to interpret. Previous physiological work on I. aquifolium (Aranda et al. 2008) suggested the species might lack the plasticity necessary to tolerate the harsh climatic conditions in the Mediterranean region, and our results seem to confirm these findings.

The following conclusions were drawn from this study: (i) compared with the drought-prone site, the mesic climatic conditions favored radial growth and xylem traits associated with hydraulic efficiency in I. aquifolium; at both studied sites, trees showed the potential to modify radial growth and xylem structure over the last century; (ii) potential anatomical modifications due to effects of climate warming and models projecting more severe drought periods might arise as a consequence of the positive relationships between temperature and vessel frequency (which increased markedly at both sites in recent years) and vessel clustering, quite evident at ITA, which might increase resilience to drought-induced embolism in a drier habitat; and (iii) in most cases, climatic conditions showed stronger influence on anatomical traits than tree radial growth, and therefore radial growth could not represent the preferential carbon destination under conditions favoring water uptake and conduction.

Conflict of interest
None declared.

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Supplementary data
Supplementary data for this article are available at Tree Physiology Online.

Conflict of interest
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