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Strong increase of photosynthetic pigments and leaf size in a pruned *Ginkgo biloba* tree

H.K. LICHTENTHALER[*,+], E. ABUSLIMA[**,***], and P. NICK[**]

Botanical Institute, Molecular Biology and Biochemistry of Plants, Karlsruhe Institute of Technology (KIT), D-76133 Karlsruhe, Germany[*]
Botanical Institute, Molecular Cell Biology, Karlsruhe Institute of Technology (KIT), D-76133 Karlsruhe, Germany[**]
Department of Botany, Faculty of Science, Suez Canal University, 41522 Ismailia, Egypt[***]

Abstract

A 50-year-old solitary, sun-exposed ginkgo tree had strongly been pruned in the fall of 2021. Very few buds for the formation of new leaves, twigs, and branches were left over. In spring 2022, these few remaining buds responded with the formation of a different leaf type. These leaves were 2.7 times larger and also thicker than in the years before. In addition, the mean content of total chlorophylls [Chl (*a*+*b*)] per leaf area unit of dark-green leaves was 1.45, those of green leaves two times higher as compared to the years before pruning and the two other ginkgo trees which had been investigated in parallel. A comparable increase was also found for the level of total carotenoids (*x*+*c*). The mean content for Chl (*a*+*b*) were 1,118 mg m^{-2} for dark-green and 898 mg m^{-2} for green leaves as compared to 435 to 770 mg m^{-2} in leaves of other trees. The higher values for Chl (*a*+*b*) and total carotenoid content showed up also on a fresh and dry mass basis. Thus, with the formation of a new, larger leaf type by changes in morphology (leaf size and thickness) and the increase of photosynthetic pigments, the pruned ginkgo tree was able to compensate for the much lower number of leaves and photosynthetic units.

Keywords: chlorophyll *a*/*b* ratio; chlorophyll levels; leaf size; leaf thickness; ratio of chlorophylls to carotenoids; total carotenoid content.

Introduction

Ginkgo trees (*Ginkgo biloba* L.), which exhibit fan-shaped bilobated leaves, are native to East China and have long been cultivated in China and Japan, and since about 300 years also in Europe as ornamental park trees. This species is a living fossil, the last representative of the gymnosperm class of the Ginkgophyta, which, according to paleontological records, was widespread about 270 million years ago. It differs from the conifers by a flagellate spermatozoid that is released by the bursting pollen tube around 50 µm before reaching the egg cell (Fujii 1899) and thus represents a missing link between the Pteridophytes and the Spermatophytes. The photosynthetic plasticity of ginkgo trees appears to be one major point to explain their survival across the Triassic–Jurassic mass

Highlights

- Strong pruning of a ginkgo tree led to a change in leaf morphology and pigment content
- The new leaves were much thicker and up to 5 times larger than before pruning
- They contained around 1.4 times more Chl (*a*+*b*) and carotenoids per leaf area unit

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[+]Corresponding author
e-mail: hartmut.lichtenthaler@kit.edu

Abbreviations: *c* – carotenes; Chl – chlorophyll; Chl (*a*+*b*) – total chlorophyll content; Chl *a*/*b* – ratio of Chl *a* to Chl *b*; DM – dry mass; FM – fresh mass; *x* – leaf xanthophylls; (*x*+*c*) – total carotenoids.

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extinction boundary (Yiotis *et al*. 2017). In recent years ginkgo trees are often planted as trees in gardens and also as alley trees, *e.g*., in towns such as New York City. Their great advantage, as compared to many other deciduous trees, is that ginkgo trees are not only fairly resistant to fungi and insects but also to air pollutants (Kim *et al.* 1997). Moreover, in contrast to many trees, such as oak, poplar, aspen, *Platanus*, or *Robinia* that emit the volatile semi-terpene isoprene during hot and dry summer months (*e.g*., Zeidler and Lichtenthaler 1998, Sharkey *et al*. 2008), strongly promoting ozone formation, ginkgo trees do not evolve isoprene. They accumulate instead nonvolatile hexacyclic terpenes, termed ginkgolides, which are deposited in the leaves. These particular properties of ginkgo trees render them well-suited as urban trees.

In a residential area of Karlsruhe-Durlach, a 50-year-old ginkgo tree growing in the garden next to a house became too large (height of 11 m) and had to be very strongly recut in the fall of 2021. In spring 2022, this drastically pruned and shortened ginkgo tree developed a qualitatively different type of leaves, on the relatively low number of stem and branch buds, which were left over after pruning. These leaves were considerably larger, thicker, and much greener than that in the years before, yet they retained their bi-lobed and multi-lobed shape. The morphological characteristics of this unusual leaf-type found on the pruned ginkgo tree, along with its higher content of photosynthetic pigments (chlorophylls and carotenoids) are compared to the smaller leaves from earlier years and to those from two other non-pruned ginkgo trees. In addition, we also checked whether these new leaves can be understood in terms of a light adaptation response, known to produce smaller but thicker sun leaves rather than thinner but larger shade leaves (*see e.g*., Lichtenthaler and Babani 2004, Sarijeva *et al*. 2007). Yet, this seems not to be the case.

Materials and methods

Plant materials: The leaves used in the experiments were taken from three single standing light-exposed ginkgo trees (*Ginkgo biloba* L.) growing in the Karlsruhe area.

Tree 1: This 50-year-old male tree grows in a private garden on the hill slope 'Kennental' in Karlsruhe-Durlach. Since all rainwater from the neighboring gardens is running down over the stand and roots of this ginkgo tree, it developed into a strong and thick tree. Its stem diameter amounted in spring 2022 already to 67 cm at a height of 1 m above ground. This tree, getting too large for this garden stand, was very strongly recut and pruned in the fall of 2021. By cutting off most twigs and smaller branches and also shortening thicker branches, the tree was considerably shortened to a height of only 6 m, thus losing *ca*. 90% of its tree crown. As a consequence, the number of buds for forming new leaves, twigs, and branches was reduced to far below 10% as compared to the situation prior to pruning. In spring 2022, this pruned tree responded with the formation of much larger and also thicker leaves than usual, thus compensating for their much lower number. All new leaves, formed first in spring 2022, were fully dark green, whereas those in the years before were of a lighter green. Among the very first leaves being formed at each stem-bud in spring 2022, there was one particularly very large dark-green leaf besides several smaller dark-green ones. Such a very large and thick leaf had not been seen on the stem buds in the years before pruning of ginkgo tree 1. Also, on the branch buds of the still-existing few branches, the newly formed leaves were fully dark-green and clearly larger than prior to pruning. This solitary ginkgo tree 1 is sun-exposed and, due to the strong pruning of the tree, all new leaves were developed at full exposure to daylight. For this reason, one could have expected that the newly formed leaves would show the typical sun-type leaves (*e.g*., Sarijeva *et al.* 2007), yet this was not really the case as is reported below in the result section. All leaves of this pruned ginkgo tree 1 showed very clearly the typical bilobated morphology with a distinct central division into two deep lobes. Since most of the leaves also exhibited further, somewhat smaller lobes of a secondary order, the leaves of this ginkgo tree 1 display a multi-lobed leaf morphology, as had been the case in the years before pruning.

Tree 2: This old and fully healthy solitary female ginkgo tree was planted about 260 to 270 years ago in the former garden of the Karlsruhe Palace and is today part of the Campus of the Karlsruhe Institute of Technology (KIT). Its stand is in the half shade, and this tree has developed a very large tree crown. Most of its leaves are not bilobate, no matter whether the leaves are formed on short or on long shoots. Only the most recently formed few leaves on the outer, light-exposed ends of long shoots and branches showed the bilobate leaf structure and are also somewhat smaller. Most leaves of this tree 2 were dark green, others showed normal green coloration. The smaller leaves at the outer ends of shoots were light green. This tree 2 exhibited a stem diameter of 138 cm at a height of 1 m above ground in 2022. It possesses a broad crown and is around 18 m high.

Tree 3: This 41-year-old very slender male ginkgo tree grows on a ginkgo alley in Karlsruhe-Durlach near the pruned garden ginkgo tree 1. Standing on the highest point of the alley it receives only a little rainwater. The stem diameter of this road ginkgo was 21 cm at 1 m above ground and the height of this tree was about 10 m. It is fully sun-exposed, exhibits a fully open, narrow crown, and its leaves are almost exclusively exposed to the sun, such that its leaf morphology shows all characteristics of typical sun leaves. The bilobate leaf structure is found in about one-third of its leaves but is not strongly expressed.

Leaf thickness: The thickness of leaves was determined using a classical nonius measuring tool (micrometer caliper). For each leaf type of the three ginkgo trees usually two or up to five leaves were stapled and measured together. The individual values indicated in the tables are based on at least 15 measurements.

Leaf area: The leaf contours (perimeters) were marked on a special millimeter–centimeter paper and the total leaf area was determined by counting the cm^2 and mm^2 of the respective leaf contour. For all three ginkgo trees, the area of 100 leaves was determined. This had also been done the same way for 50 leaves of the non-pruned ginkgo tree 1 in 2005.

Water content and dry mass: Ginkgo leaves with known leaf area were weighed before and after drying the leaves for *ca*. 1 up to 2 h in a drying oven at 80°C. The percentage water content (% of FM), as well as the specific leaf area SLA [expressed as $cm^2\ g^{-1}$(DM)] and the specific leaf mass SLM [expressed as $mg\ cm^{-2}$(leaf area)], were determined from these data.

Photosynthetic leaf pigments: Chlorophylls *a* and *b* and total carotenoids (x+c), *i.e.*, xanthophylls (x) and carotenes (c) were determined in a two-wavelength spectrophotometer using the specific extinction coefficients and equations by Lichtenthaler (1987) as well as Lichtenthaler and Babani (2022). In brief, 3–6 punched leaf discs (7-mm diameter), which were obtained using a rim-sharpened laboratory cork driller, were ground in 100% acetone (5–10 ml) in a mortar and a pestle by adding some quartz sand. The acetone needs to be a high-quality acetone (*Carl Roth GmbH*, Karlsruhe, Germany, article no. 7328.1). In addition, a tiny amount of magnesium oxide or carbonate (MgO or $MgCO_3$) was added in order to block pheophytin formation. The resulting pigment extract was centrifuged for 10 to 15 min, *e.g.*, at 2,000 rpm (*ca*. 400 to 500 × *g*) in a *Universal 320R* centrifuge (*Andreas Hettich GmbH & Co*, Tuttlingen, Germany). Part of the fully clear supernatant was transferred into a spectrophotometer cuvette (path length of 1 cm) paying careful attention not to stir up any parts of the sediment. Using a *V-750* UV-visible spectrophotometer (*Jasco Deutschland GmbH*, Pfungstadt, Germany) the optical density (absorption A) was measured at 661.6, 644.8, and 470 nm. The following equations were applied to determine the pigment concentration C in µg per ml of extract solution from the absorbance readings (A). By considering the total pigment extract solution and the number of extracted leaf discs, the obtained pigment values per ml were then converted to the pigment amounts per cm^2(leaf area).

Chlorophyll *a*: $C_a = 11.24\ A_{661.6} - 2.04\ A_{644.8}$

Chlorophyll *b*: $C_b = 20.13\ A_{644.8} - 4.19\ A_{661.6}$

Total carotenoids (x+c): $C_{(x+c)} = (1000\ A_{470} - 1.90\ C_a - 63.14\ C_b)/214$

The absorbance readings in the spectrophotometer in the red absorption maximum at 661.6 nm need to be performed in the absorbance range of 0.3 to 1.0 in order to obtain the correct Chl *a* and Chl *b* values of green extract solutions. In more diluted light green solutions with an absorbance at 661.6 nm of below 0.3, the Chl *b* values become too high and those of Chl *a* too low.

For more details and examples of the pigment values of several plants *see* Lichtenthaler and Buschmann (2001a,b). Practical instruction is also found in the link: https://www.botanik.kit.edu/molbio/download/1A_Chlorophylls_Carotenoid_determin-HKLi_2010.pdf.

The whole procedure of pigment extraction, the centrifugation step, and the absorbance readings were performed within 30 min in order to obtain reliable Chl *a* values. Longer resting times of the extracts have to be avoided because the high and narrow absorption peak at 661.6 nm for Chl *a* steadily decreases and broadens with increasing time after extraction due to the successive formation of allomeric Chl *a* forms. This would result in values underestimating Chl *a*, and values overestimating Chl *b*, resulting in too low values for the ratio Chl *a*/*b*.

Statistical analysis: The differences in pigment contents and pigment ratios between leaves of the three ginkgo trees in 2022 as well as those of sun and shade leaves of the non-pruned ginkgo tree 1 in 2005 were checked for significance using the *Student*'s *t*-test. The differences were assessed *via* the one-way analysis of variance (*ANOVA*). Differences with $P<0.05$ are significant and those with $P<0.01$ are highly significant.

Results

Leaf characteristics

Leaf size: The dark-green, green, and light leaves of the pruned ginkgo tree 1 that had newly been formed in spring 2022 were on average 2.3 times larger (mean leaf size of 54.1 cm^2) as compared to the years before pruning (mean leaf size of 23.5 cm^2). These leaves of the pruned ginkgo were also 2.7 times larger than those of the two other ginkgo trees 2 and 3 analyzed here. This is also clearly seen by comparing the leaf images of pruned ginkgo tree 1 (Figs. 1, 2) with the normal-sized leaves of two other ginkgo trees (Figs. 3, 4). The mean leaf size of ginkgo trees 2 and 3 amounted to only 20.8 and 21.2 cm^2 (Table 1). These differences were highly significant.

Fig. 1. Dark-green leaves of a male ginkgo tree (tree 1) in summer 2022 which had very strongly been recut and pruned in the fall of 2021. One very large dark-green leaf was formed as first in spring 2022 at each of the few buds on thin stems. These leaves had an unusual large leaf size of 128 cm^2 (*left*) and 91 cm^2 (*right*). The three smaller light-green leaves shown were formed in June at the end of newly formed branches (shoots) of the same tree. (The razor blade shown serves as a measure, it has a length of 2 cm.)

In fact, the leaf-size distribution of ginkgo trees 2 and 3 were very similar (Fig. 5), whereas the leaf-size distribution of the pruned ginkgo was quite different and ranged from 14 cm^2 up to a leaf size of 128 cm^2 (Fig. 6). As in other deciduous trees, we found for the ginkgo trees 1 and 2 that leaves originating at the inner part of the crown were larger in area, which is a typical half-shade or shade response. In contrast, those leaves formed on long shoots and branches reaching out of the crown were smaller, which is a typical sun-type response. However, in ginkgo tree 3 with its fully open crown, where all leaves received full sunlight for several hours per day, this effect was barely noticeable.

Expression of the bilobate leaf structure: The three ginkgo trees showed distinct differences in the degree of their bilobate leaf structure. Pruned ginkgo tree 1: Except for the few very large and some medium size leaves being formed in springtime at stem-buds, the dark-green, green, and light-green leaves of the pruned ginkgo tree 1 exhibited full expression of the bilobate leaf character with clear primary and also secondary lobes. Most leaves exhibited this multi-lobed leaf structure (Figs. 1, 2), which had been seen also in the years before pruning, yet then with smaller leaves. Ginkgo tree 2: The bilobate leaf structure was not seen for the majority of dark-green or green leaves (Fig. 3), whereas in the small light-green leaves, formed towards the outer end of twigs and longer shoots, the bilobate character was clearly manifested. Ginkgo tree 3: The bilobate character was seen in about one-third of the leaves, however only as a small indentation (Fig. 4). Thus, the expression of the bilobate morphology of leaves seems to vary widely among the investigated trees planted in parks and urban street alleys. After checking also other ginkgo trees in the Karlsruhe area, we can state that the strongly bilobate and even multi-lobed character seen in the pruned ginkgo tree 1, before and after pruning, appears to be a less common trait.

Fig. 2. Dark-green relatively large ginkgo leaves in summer 2022 being formed in the spring on the buds of one of the few left-over branches of the strongly pruned male ginkgo tree (tree 1). (The razor blade shown serves as scale, it has a length of 2 cm.)

Fig. 3. Dark-green normal size ginkgo leaves in August 2022 from an old female tree at the KIT campus (tree 2). The largest leaf (*down right*) had only a leaf area of 46 cm^2. (The razor blade shown serves as scale, it has a length of 2 cm.)

Fig. 4. Green normal-size sun leaves in August 2022 from a younger male ginkgo tree (tree 3). (The razor blade shown serves as scale, it has a length of 2 cm.)

Petiole length: In addition, also the petioles were considerably longer in leaves from the pruned ginkgo tree 1, with a mean of 6.4 cm and a range from 2.3 to 11.6 cm as compared to ginkgo tree 2 (mean 4.5 cm) and ginkgo tree 3 (mean 5.3 cm) (Table 1). These differences in petiole length were significant. We also observed that ginkgo tree 3 lacked the very short leaf petioles (< 2.3 cm) found in trees 1 and 2; but produced petioles that were longer than 4.1 cm and ranged up to 7.4 cm.

Leaf thickness: Concerning leaf thickness, we could differentiate the pruned ginkgo tree 1 with its exceptionally large leaves between three leaf types: (*1*) dark-green leaves formed on the stem-buds and also on the branches representing the major part of leaves, (*2*) green leaves

Table 1. Leaf characteristics of the three ginkgo trees investigated: leaf thickness (in µm), petiole length (in cm), and leaf area (in cm^2). Tree 1 is the pruned male garden tree in Durlach with much larger leaves; tree 2 is the old female tree on the KIT Campus and tree 3 is the male alley tree in Durlach. Mean values with standard deviation. These are based on 15 determinations (leaf thickness), on 100 leaves (leaf area), and 50 leaf petioles. The much higher values in all three parameters of the pruned tree 1 in leaf thickness, leaf area and leaf petiole length are printed here in bold face. The differences of the values of tree 1 to those of the two other ginkgo trees 2 and 3 are highly significant ($P<0.01$).

Leaf area [cm^2]	Mean	Range
Ginkgo tree 1		
Dark-green leaves	**54.1** ± 28.6	14–126
Light-green leaves	26.9 ± 11.0	11–44
Ginkgo tree 2		
Dark-green leaves	20.8 ± 8.6	6–35
Ginkgo tree 3		
Green sun leaves	21.2 ± 6.1	7–36
Petiole length [cm]		
Ginkgo tree 1		
Dark-green leaves	**6.4** ± 2.1	2.3–11.6
Ginkgo tree 2		
Dark-green leaves	4.5 ± 1.9	1.8–9.1
Ginkgo tree 3		
Green sun leaves	5.3 ± 0.9	4.1–7.4
Leaf thickness [µm]		
Ginkgo tree 1		
Dark-green leaves (few)	**507** ± 7	484–510
Dark-green leaves (mass)	**446** ± 23	430–465
Green leaves	**308** ± 10	300–320
Light-green leaves	251 ± 11	230–265
Ginkgo tree 2		
Dark-green leaves	271 ± 18	247–320
Green leaves	243 ± 9	231–252
Ginkgo tree 3		
Green sun leaves	318 ± 36	290–347

formed on the newly developed branches and shoots, as well as (*3*) somewhat smaller lighter green leaves being formed as latest leaves (at the end of June 2022) at the outer end of long shoots and branches. These three leaf types we also found in ginkgo tree 2. A special particularity among the dark-green leaves of the pruned ginkgo tree 1 was, that the very first few leaves in early spring were particularly thick (mean thickness 507 µm), whereas the majority of dark-green leaves formed thereafter had a mean thickness of 446 µm. The green leaves and light-green leaves of this pruned ginkgo tree 1 being formed later had a lower mean thickness of only 308 and 251 µm, respectively, as shown in Table 1. In contrast to the pruned ginkgo tree 1, all leaves of ginkgo tree 2 were significantly thinner, with a mean thickness of 271 µm for its dark-green leaves (the major part of leaves) and a mean of 243 µm for the low amount of green to light-green leaves. The leaves of ginkgo tree 3 (predominantly green sun leaves) were significantly thicker than those of tree 2, but in the same range (mean 318 µm) as the green leaves of the pruned tree 1 which were also sun-exposed leaves (mean 308 µm) (Table 1).

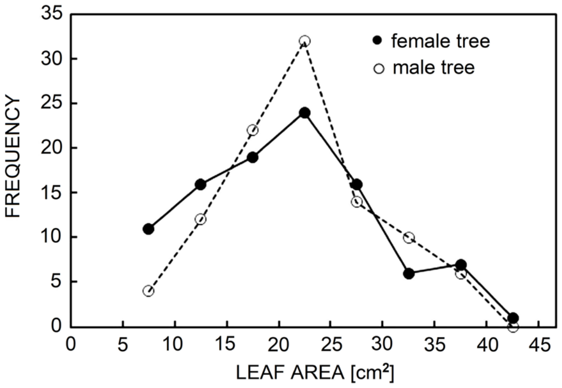

Fig. 5. Leaf-area size distribution of 100 leaves each of two ginkgo trees (tree 2 and 3). The points shown indicate the number of leaves in the range of 5–10, 10–15, and 15–20 cm^2, *etc*., up to 40–45 cm^2.

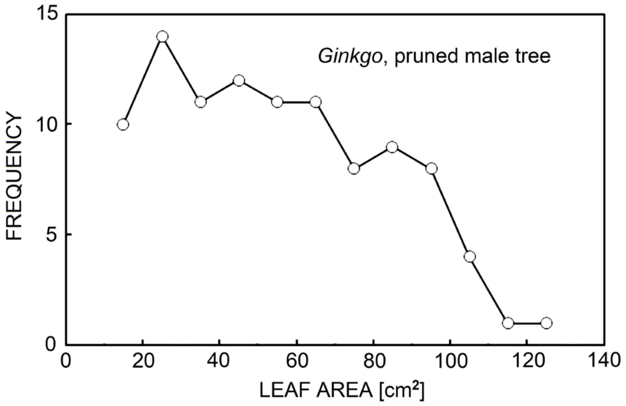

Fig. 6. Leaf-area size distribution of 100 leaves of ginkgo tree 1 which had very strongly been pruned in the fall of 2021 and showed in 2022 extra-large leaves. The points shown represent the number of leaves in the range of 10–20, 20–30, 30–40 and 40–50 cm^2, *etc*.

Photosynthetic pigments

Chlorophylls: The leaves of the pruned ginkgo tree 1 had not only larger and thicker leaves as compared to the two other ginkgo trees but also accumulated significantly higher chlorophyll (*a*+*b*) and carotenoids (*x*+*c*) contents per leaf area unit (mg per m^2). In fact, the dark-green leaves of ginkgo tree 1 altogether contained 1,118 mg Chl (*a*+*b*) per

m^2 leaf area, which is 45% more chlorophyll as compared to the dark-green leaves of ginkgo tree 2. In green and light-green leaves of ginkgo tree 1 the Chl (*a*+*b*) content per leaf area units was even 1.95 times and 2.38 times higher, respectively, as compared to the corresponding leaves of ginkgo tree 2 (Table 2). In addition, the pruned ginkgo tree 1 had even in its green leaves 56% more Chl (*a*+*b*) as compared to the green leaves of ginkgo tree 3 (Table 2).

We also expressed the differing Chl (*a*+*b*) content between both ginkgo trees 1 and 2 on the basis of dry mass (DM in g) and fresh mass (FM in g) as shown in Table 3. Also, in these two reference systems, the Chl (*a*+*b*) contents of the pruned ginkgo tree 1 were significantly higher as compared to the corresponding leaves of ginkgo tree 2. On a dry-mass basis, the Chl (*a*+*b*) contents in dark-green, green, and light-green leaves of tree 1 were 1.55, 1.54, and 2.72 times higher than in the corresponding leaves of ginkgo tree 2. On a fresh mass basis, the Chl (*a*+*b*) contents of the corresponding leaves were 1.21, 1.24, and 2.37 times higher than that of ginkgo tree 2.

Carotenoids: Also, the total carotenoid (*x*+*c*) contents of dark-green, green, and light-green leaves of the pruned ginkgo tree 1 were considerably higher as compared to the corresponding leaves of ginkgo tree 2. In fact, the (*x*+*c*) values [mg m^{-2}(leaf area)] were 1.3 (dark-green leaves), 1.73 (green leaves), and 1.75 times (light-green leaves) higher as in the corresponding leaves of ginkgo tree 2 (Table 2). The (*x*+*c*) content of green leaves of ginkgo tree 1 was also 1.33 times higher than that of ginkgo tree 3. Similarly, higher values for the (*x*+*c*) contents of ginkgo tree 1 as compared to ginkgo trees 2 and 3 were also found when the (*x*+*c*) content was expressed on a DM and an FM basis (data not shown).

The ratio of Chl *a*/*b*: In contrast, the values for the mass ratio of Chl *a*/*b* of ginkgo trees 1 and 2, taken between mid to end of July 2022, were in a similar range from 2.59 to 2.75 for the three leaf types dark-green, green, and light-green and more or less independent of the total chlorophyll content of the leaves. For ginkgo tree 2, this result could be expected due to its stand in the half-shade, but not for the sun-exposed pruned ginkgo tree 1. The typical differentiation into higher Chl (*a*+*b*) contents with higher Chl *a*/*b* values for the sun as compared to shade leaves, as shown for the same, then non-pruned ginkgo tree 1 in 2005 (Table 2) was not observed in 2022. In fact, by the middle to end of July, when the pigment values were taken, the pruned ginkgo tree 1 had formed numerous new leaves and only some of these were in the half-shade. Yet, despite the very high Chl (*a*+*b*) contents, otherwise known for sun-exposed leaves, the typically expected higher values of the Chl *a*/*b* ratio, were missing. In contrast, the sun-exposed leaves of ginkgo trees 1 and 3 (both taken already in 2005) showed the higher Chl *a*/*b* ratio values characteristic for sun leaves of 3.13 and 2.96, respectively (Table 2).

Ratio Chl (*a*+*b*)/(*x*+*c*): The values of the mass ratio of total chlorophylls to total carotenoids Chl (*a*+*b*)/(*x*+*c*) were distinctly higher (values from 4.41 to 5.05) in the leaves of

Table 2. Differences in the contents of Chl (*a*+*b*) and total carotenoids (*x*+*c*) per leaf area unit (mg m^{-2}) and in the pigment ratios Chl *a*/*b* and chlorophylls (*a*+*b*) to carotenoids, (*a*+*b*)/(*x*+*c*), between the dark-green, green and light-green leaves of a pruned ginkgo (tree 1) and a non-pruned ginkgo (tree 2). For comparison the values of green sun leaves of a third ginkgo tree (tree 3) are added as well as the values of ginkgo tree 1 obtained in 2005 long before pruning (*see* Sarijeva *et al.* 2007). Mean values of 4 to 8 determinations per leaf-type. For a better comparison of the differences, the contents of Chl (*a*+*b*) and total carotenoids (*x*+*c*) of the three trees the highest values of each tree are shown as bold numbers. The higher chlorophyll (*a*+*b*) and carotenoid (*x*+*c*) contents in the pruned ginkgo tree 1 as compared to the values in corresponding leaf types of ginkgo tree 2 and tree 3 are significant ($P<0.05$). 1) of stem buds; 2) green to dark-green leaves of branch buds and new shoots; 3) the last leaves formed on the outer end of the newly formed shoots and branches.

Tree	Chl (*a*+*b*)	(*x*+*c*)	Chl *a*/*b*	(*a*+*b*)/(*x*+*c*)
Ginkgo tree 1				
Dark-green leaves1)	**1,118** ± 104	**222** ± 20	2.69 ± 0.09	5.04 ± 0.07
Green leaves2)	898 ± 25	178 ± 9	2.53 ± 0.16	5.05 ± 0.10
Light-green leaves3)	626 ± 47	142 ± 12	2.71 ± 0.19	4.41 ± 0.47
Ginkgo tree 2				
Dark-green leaves	**769** ± 73	**171** ± 18	2.75 ± 0.16	4.50 ± 0.14
Green leaves	471 ± 55	103 ± 10	2.73 ± 0.15	4.57 ± 0.18
Light-green leaves	263 ± 19	81 ± 8	2.59 ± 0.19	3.25 ± 0.19
Ginkgo tree 3 (values of 2005)				
Green sun leaves	**574** ± 36	**134** ± 12	2.96 ± 0.13	4.22 ± 0.27
Green leaves of *Ginkgo* tree 1 in 2005				
Sun leaves	**494** ± 31	**119** ± 7	3.13 ± 0.08	4.15 ± 0.15
Shade leaves	322 ± 11	58 ± 5	2.73 ± 0.09	5.56 ± 0.31

Table 3. Differences in the percentage water content (% H_2O of fresh mass), specific leaf area (SLA) and specific leaf mass (SLM) as well as the total Chl (*a*+*b*) contents per leaf area [mg m^{-2}], per dry mass [mg g^{-1}(DM)] and per fresh mass [mg g^{-1}(FM)] of two ginkgo trees: the pruned ginkgo tree 1 and the non-pruned ginkgo tree 2. For a better comparison the Chl (*a*+*b*) contents per leaf area unit are printed in bold face.

Parameter	Dark-green	Green	Light-green
Ginkgo tree 1			
H_2O [% of FW]	72.8 ± 2.9	71.2 ± 2.3	67.7 ± 3.2
SLA [cm^2 g^{-1}(DM)]	107.6 ± 8.5	116.8 ± 7.1	107.9 ± 17.3
SLM [mg cm^{-2}]	9.5 ± 0.7	8.8 ± 0.7	9.5 ± 1.6
Chl (*a*+*b*) [mg m^{-2}]	**1,118** ± 104	**898** ± 25	**626** ± 47
Chl (*a*+*b*) [mg g^{-1}(DM)]	11.9 ± 0.6	10.5 ± 0.4	6.8 ± 1.5
Chl (*a*+*b*) [mg g^{-1}(FM)	3.23 ± 0.37	2.91 ± 0.09	2.16 ± 0.27
Ginkgo tree 2			
H_2O [% of FW]	65.1 ± 1.2	64.5 ± 2.6	63.5 ± 2.6
SLA [cm^2 g^{-1}(DM)]	100.4 ± 7.8	142.3 ± 6.8	95.0 ± 2.6
SLM [mg cm^{-2}]	10.1 ± 0.8	7.3 ± 0.3	10.5 ± 0.3
Chl (*a*+*b*) [mg m^{-2}]	**769** ± 73	**472** ± 55	**263** ± 19
Chl (*a*+*b*) [mg g^{-1}(DM)]	7.7 ± 0.6	6.8 ± 0.5	2.5 ± 0.3
Chl (*a*+*b*) [mg g^{-1}(FM)	2.67 ± 0.16	2.34 ± 0.13	0.91 ± 0.06

the pruned ginkgo tree 1 as compared to the corresponding leaves of ginkgo tree 2 (range 3.25 to 4.57) as shown in Table 2. In both trees the light-green leaves, being formed latest at the end of June at the outer parts of sun-exposed shoots and branches, had lower values for the ratio Chl (*a*+*b*)/(*x*+*c*) lower values of 4.41 and 3.25, respectively.

Additional tree parameters

Further tree parameters are listed for the pruned ginkgo tree 1 and ginkgo tree 2 in Table 3. These are the water content of leaves as a percentage of the leaf fresh mass, (H_2O, % of FM), the specific leaf area SLA, *i.e.*, cm^2 of leaf area per 1 g of dry mass, and the specific leaf mass SLM expressed as mg of leaf mass per cm^2 of leaf area.

H_2O (% of FM): The pruned ginkgo tree 1 had a somewhat higher water percentage of 72.8 and 71.2% for its dark-green and green leaves, respectively, as compared to its fully sun-exposed light-green leaves with only 67.7%. In the case of ginkgo tree 2, the water percentages were very similar ranging from 63.5 to 65.1% for the three leaf types. These somewhat lower values of tree 2 are due to its particular somewhat dryer stand because of some pavement slabs surrounding the tree, which thus receives less rainwater as compared to tree 1.

SLA and SLM: The values of the specific leaf area were found to vary for the dark-green, green, and light-green leaves of both ginkgo trees 1 and 2 in the range of 95 to 142 as is usually also found for other trees. Yet, specific differences between the leaves of the two trees could not be detected. The same is true for the specific leaf mass SLM which varied in the range of 7.3 to 10.5 (Table 3).

Discussion

The results of this investigation showed that ginkgo tree 1, strongly pruned in the fall of 2021 and, thus, forming only very few stem or branch buds for forming new leaves, responded in the following spring of 2022 with the formation of much larger leaves. These leaves were also thicker and contained significantly higher amounts of photosynthetic pigments per leaf area unit and also on a fresh mass and dry mass basis as compared to regular years before pruning as well as in comparison to the two other non-pruned ginkgo trees (trees 2 and 3). Such high contents of Chl (*a*+*b*) per leaf area unit as those found in the pruned ginkgo tree 1 (1,118 mg m^{-2} in the first formed dark-green leaves and 898 mg m^{-2} in later formed dark-green leaves) had, to the best of our knowledge, not been reported for any other tree so far. Sun-exposed leaves (sun leaves) of all trees tested so far are well-known to be thicker and smaller than shade leaves and exhibit higher Chl (*a*+*b*) contents on a leaf area unit as compared to shade leaves (*see e.g.*, the reviews of Lichtenthaler *et al.* 1981, 2013; Lichtenthaler and Babani 2004). This light-adaptation response of leaves was also found for ginkgo tree 1 in the non-pruned stage (Lichtenthaler 2007, Sarijeva *et al.* 2007, Lichtenthaler *et al.* 2013). In 2005, its sun leaves had a mean Chl (*a*+*b*) content of 494 mg per m^2, and its thinner shade leaves were only 322 mg per m^2 (*see* Table 2). For sun leaves of beech (*Fagus sylvatica* L.), mean Chl (*a*+*b*) contents of 521 mg m^{-2} were reported, and for sun leaves of pedunculate oak (*Quercus robur* L.), a mean of 435 mg m^{-2} leaf area (*e.g.*, Lichtenthaler *et al.* 2013), while the values of the thinner, yet larger shade leaves were much lower.

There is one report (Leigh *et al.* 2011) describing that leaf size in ginkgo trees can differ between short and long shoots, whereby leaves on short shoots are larger (mean leaf area of 28 cm^2) while those on long shoots are significantly smaller (mean leaf area of 11 cm^2). These smaller leaves on long shoots, which are reaching out further of the tree crown, had also a much higher stomata frequency as compared to the short-shoot leaves which are found predominantly in the shade and half-shade. An elevated stomata frequency is, however, a very typical trait of sun leaves of trees contrasting with shade leaves exhibiting a much lower stomata frequency (*e.g.*, Salisbury 1928, Osborn and Taylor 1990, Idris *et al.* 2019). The same holds true for sun-exposed leaves of herbaceous plants (*e.g.*, Tichá 1982) when compared to low-light leaves. Thus, the leaf-size differences described by Leigh *et al.* (2011) for ginkgo trees seem to reflect characteristic features of sun and shade leaves, although these authors did not mention (and were probably not aware of) the typical differences between shade and sun leaves as investigated and reviewed in detail by Lichtenthaler's group (*e.g.*, Lichtenthaler *et al.* 1981, Lichtenthaler and Babani 2004).

However, the unusually large and pigment-rich leaves in the pruned ginkgo tree 1 cannot be explained in terms of sun and shade leaves, for different reasons. Here, all leaves were thicker and much larger, while sun leaves are thick as well, but also small. Moreover, these leaves were also significantly larger than the usual relatively thin shade leaves in the normal years of this tree. This particularly applies to the first, extremely large and thick leaves directly emerging from the few remaining buds of the strongly pruned ginkgo tree 1. Since they are particularly thick, but also particularly large, they do not fall into the typical adaptative response to either a sun or shade differentiation during leaf formation. Instead, the extremely reduced number of buds caused by the strong pruning has apparently initiated a compensatory developmental response, where leaf surface size and chlorophyll content per leaf area unit were stimulated to such a degree that the photosynthetic light-absorbing leaf area and photosynthetic capacity of the pruned tree with its initially only a few leaves were at least partially restored. In this respect, one needs to reconsider that the bud opening in spring and the formation of the first leaves are promoted by the sucrose being formed from starch in the tree stem and being transported to the buds. Yet the further development and formation of new shoots and leaves are strongly dependent on the photosynthetic sugars and metabolites produced in the chloroplasts of the first leaves.

This tendency of forming larger and thicker leaves with more Chl (a+b) and total carotenoids per leaf area unit and a higher photosynthetic capacity continued when many further leaves were formed on the newly developing branches and shoots. Only towards the end of June 2022, when the formation of new branches and shoots ended, the last leaves being formed on the outer end of these shoots were again smaller, yet still larger than those formed in normal years or in the corresponding leaves of ginkgo tree 2. Through this spontaneous increase in the photosynthetic capacity of not only a few first but also the following leaves formed in the new branches and shoots of the pruned ginkgo tree 1, enough photosynthetic assimilates were produced to sustain the quick continuous formation of new shoots and branches with many additional leaves contributing to photosynthesis. In fact, already in mid-July of 2022, the pruned ginkgo tree had fully been covered again by many new branches, shoots, and leaves. These then formed a new, rather bushy tree crown, so that the consequences of the severe pruning in the fall of 2021 could no longer be recognized when looking at the tree from some distance. A further particular response of the pruned ginkgo tree 1 was that the autumnal leaf discoloration with a breakdown of the chlorophylls started four weeks later than in the two other non-pruned ginkgo trees 2 and 3.

The impact of heavy pruning on development and photosynthesis had also been investigated in roadside linden trees, *Tilia cordata* (Suchocka *et al.* 2021). These authors found that the pruned linden trees also developed a much larger leaf area as compared to the unpruned control trees and that the autumnal discoloration of the leaves also started later. In addition, they reported that after four years the tree crowns had mostly been rebuilt, but not entirely. In some cases, the severe pruning even evoked a dieback of the tree. In contrast, the strongly pruned ginkgo tree 1 rebuilt a full tree crown with a very large number of new shoots and branches already in the first year after pruning and with very high numbers of new buds at each shoot. In addition, now in spring 2023, the second year after pruning, all of these new numerous buds formed in summer 2022 – on shoots, thick branches, and on the main stem of ginkgo tree 1 – were opening and developing new leaves. Yet, such extraordinarily large single leaves on each of the few remaining buds, as in spring 2022, did not show up on the certainly more than 2,000 buds of the pruned ginkgo tree 1 in spring 2023. In fact, as compared to leaves formed in 2022 (mean leaf size of 54.1 cm^2, range: 11 to 128 cm^2), the new leaves of 2023 were much smaller again with a regular mean leaf size of 26.4 cm^2 (range: 9 to 49 cm^2) as determined on 12 May 2023. In addition, also the mean thickness of the new leaves was much lower in 2023 (231 ± 22 µm) as compared to 2022 (with 507 µm for the first bud leaves and 446 µm for the mass of leaves).

The physiological mechanism behind this compensatory response of forming much larger and thicker leaves with a higher chlorophyll content in the pruned ginkgo tree 1 in 2022 warrants further elucidation, but might be linked with the fact that, during early spring, when the remaining buds compete as sinks for resources such as sugars and amino acids, but also for stimulating signals, such as auxins and cytokinins, this mutual competition is mitigated, since the individual bud can recruit more of these limiting factors. Auxins are produced in the aerial plant parts and particularly in the main shoot top (King 1975) and are also known to suppress the growth of side shoots known as apical dominance. After removal of the tree top by strong pruning the apical dominance is blocked. Cytokinins, in turn, being formed in the roots, are promoting cell division and are counterparts of auxin (Letham 1968, Skene 1975) In fact, pruning has an essential impact on the auxin–cytokinin pathway as shown by measuring phytohormone contents in pruned and non-pruned apple trees (Grochowska *et al.* 1984). These authors found that vigorous shoot growth following heavy pruning was accompanied by an increase of cytokinin concentration in the apple tree tissues in early spring which activated cell division and cell expansion. Moreover, sugar demand, not auxin, seems to be the initial regulator of apical dominance (Mason *et al.* 2014), and axillary buds are released when the shoot tip is removed which leads to an enhanced bud outgrowth. Transferred to the severely pruned ginkgo tree 1, this means that in spring high amounts of sugar should be mobilized from starch in the tree stem and, together with the cytokinins formed in the large root system of the tree, would saturate the outgrowth of the few buds left over after pruning.

In several reports and investigations on sun and shade leaves of trees, it had been concluded that the induction of the very first leaf primordia being formed in spring depends on the light conditions (either shade or full sun) prevailing at the bud formation site in the preceding summer (Nordhausen 1903, Hansen 1959, Eschrich *et al.* 1989). With respect to the sun and shade leaves of the beech, we could confirm this dependence on the light conditions at the bud formation site (Lichtenthaler, unpublished). Concerning the pruned ginkgo tree, those low numbers of buds being left over after pruning had all been formed in the fall of 2021 in the shade. Hence, one could have expected that these buds would form in 2022 thin and somewhat larger leaves as compared to the usual smaller and thicker sun leaves of this tree on the buds formed in full sun. Instead, we observed leaves of a new type – with respect to area these leaves were much larger than usual shade leaves, but, unlike shade leaves, these leaves were also much thicker with higher Chl (*a*+*b*) and carotenoid concentrations, which is a typical trait of sun leaves. However, for sun leaves higher values for the ratio of Chl *a*/*b* (range 2.8–3.1) would be expected, which was not found. Instead, lower mean Chl *a*/*b* ratio values of 2.59 to 2.75 were seen as they are usually found in shade or half-shade leaves (Table 2). Yet, one has to consider in this respect that sun chloroplasts are usually only formed in the palisade parenchyma cells of the upper sun-exposed part of a leaf, whereas chloroplasts in the lower leaf part (*e.g.*, the spongy parenchyma) are half-shade or shade chloroplasts with lower values for the ratio of Chl *a*/*b*. Based on this observation, one may conclude that the major proportion of chloroplasts in the very thick leaves of the pruned ginkgo with their extremely high Chl (*a*+*b*) content per leaf area unit displayed characteristics of half-shade or shade chloroplasts with their medium or low values for the ratio Chl *a*/*b*. Hence, sun chloroplasts may certainly have been formed in the upper leaf layer of these sun-exposed leaves of the pruned ginkgo tree 1, but due to their relatively low proportion in the very thick leaves with their very high Chl (*a*+*b*) content, the higher Chl *a*/*b* ratio values of sun chloroplasts did not show up when as here punched leaf disks of the whole leaf were extracted.

Thus, the compensatory response of ginkgo tree 1 with thicker and much larger leaves cannot be understood in terms of the wide-spread shade *vs.* sun leaf differentiation response. In this context, it is of interest in this respect, that about 10 or 12 years ago the same ginkgo tree 1 had lost during springtime about 80% of its very young still developing first leaves and twig buds during a very late frost period with extremely low temperatures lasting for about 2 to 3 d. Thereafter, no new leaves or new twigs were formed in these frostbitten places. However, also then, those leaf buds, that had not yet opened, formed thereafter much larger and greener leaves than usual. Yet, two years later the leaves had again their normal size. This demonstrates that this ginkgo tree and possibly all ginkgo trees can adjust size and pigment content to compensate for reduced numbers of photosynthetic organs, no matter, whether the reduction was caused by late spring frosts or extreme pruning of the tree.

To respond *via* the spontaneous formation of much larger and thicker leaves with higher chlorophyll and carotenoid contents, after a strong pruning as shown here for the 50-year-old large ginkgo tree 1, appears to be a very special response of adult ginkgo trees with a large root system. Similar compensatory responses, albeit at much lower amplitude, have been observed for very young (two years in age) ginkgo trees. Here, pruning promoted leaf area growth and increased chlorophyll (*a*+*b*) content of leaves to some extent, particularly in the third year after pruning (Cao *et al.* 2022), but the effect was much weaker than in the current case, which might be linked with the fact that the larger root system of adult trees can provide a larger source for cytokinins as compared to a very young tree. Also, in young peach trees (one year in age), severe pruning removing 60% of the shoots of the plants, induced shoot formation, such that already 2.5 months after pruning the plants had restored the initial root/shoot ratio (Mediene *et al.* 2002). Likewise, pruned fig plants exhibited a faster leaf expansion as compared to non-pruned branches (González-Rodríguez and Peters 2010), but this effect remained transient, such that at the end of the season the values had almost converged. Thus, the very strong increase in leaf area, leaf thickness, and chlorophyll content seen in the strongly pruned ginkgo tree, seems to reflect a response that appears to be specific for *Ginkgo biloba*.

In the same garden in Durlach, besides ginkgo tree 1, also four other trees at the age of 40 to 45 years had been pruned considerably, including an American tulip tree (*Liriodendron tulipifera* L.), a beech (*Fagus sylvatica* L.), a maple (*Acer negundo* L.), and a poplar (*Populus nigra* L.). Yet these trees did not respond in the following year by the formation of considerably larger and thicker leaves with higher chlorophyll and carotenoid contents per leaf area unit. In addition, this did also not happen in pruned *ca*. 30-year-old apple and cherry trees in the neighbouring gardens. These observations support the view that the compensatory response is specific to ginkgo trees. This adds to the other specificities of this living fossil ginkgo, such as the particular accumulation of terpenoid ginkgolides instead of emitting the volatile isoprene as in most other deciduous trees (*e.g.*, Zeidler and Lichtenthaler 1998, Sharkey *et al*. 2008). However, also linden trees do have a similar response with larger leaves to severe pruning as mentioned above (Suchocka *et al*. 2021). Whether *Ginkgo biloba* and *Tilia cordata* have retained here an old ancestral trait that was lost during the evolution of most angiosperm trees, or whether they acquired this trait exclusively, is not known. In this context, it would be rewarding to test further angiosperm trees and also, whether conifers or ferns show comparable compensatory responses to severe reduction in shoots and photosynthetic organs.

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