



# Effects of ash dieback on leaf physiology and leaf morphology of *Fraxinus excelsior* L.

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## Abstract

**Key message** Ash dieback causes alterations in leaf physiology and morphology, particularly affecting the specific leaf area, which can be used to discriminate between different degrees of damage.

**Abstract** Since the introduction of the invasive fungal pathogen *Hymenoscyphus fraxineus* in Europe, the European common ash (*Fraxinus excelsior* L.) has been threatened by ash dieback. An infection leads, for example, to typical symptoms of dying shoots, but changes of leaf physiology and morphology are still largely unexplored. Therefore, five physiological and morphological traits, chlorophyll content, chlorophyll fluorescence, specific leaf area, leaf thickness, and fluctuating asymmetry, were investigated in four different study sites in southern Germany regarding possible changes due to ash dieback and their relationship to different degrees of damage. Both higher and lower levels of chlorophyll with increasing damage due to ash dieback were observed. Chlorophyll fluorescence and fluctuating asymmetry proved to be less suitable indicators of damage. Leaf thickness showed the tendency (however not significant) of an increase in more severely damaged trees. The specific leaf area was identified as a suitable indicator of the damage severity, with significant smaller values in less healthy trees. Therefore, ash dieback can also result in notable alterations in leaf physiology and morphology.

**Keywords** Chlorophyll content · Chlorophyll fluorescence · Specific leaf area · Fluctuating asymmetry · Leaf thickness

## Introduction

Ash dieback has become increasingly widespread in Europe since the first documentation of symptoms caused by the fungal pathogen *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz, Hosoyain (Baral et al. 2014) in the 1990s in Poland (Kowalski 2006; Timmermann et al. 2011) affecting the European common ash (*Fraxinus excelsior* L.) as well as the narrow-leaved ash (*Fraxinus angustifolia* Vahl) (Kirisits et al. 2010; Nielsen et al. 2017). Wind-dispersed ascospores of *Hymenoscyphus fraxineus* primarily infect leaves, further spreading into the woody shoots through the leaf petiole (Gross et al. 2014; Haňáčková et al. 2017; Nielsen et al. 2022). The infection leads to a thinning of ash crowns and

often ultimately to the death of the affected trees (Enderle et al. 2019). High mortality rates of up to 70 and 85% in woodland and ash plantations have been observed. Over the next 30 years, ash populations in Europe are expected to decline between 50 and 75% (Coker et al. 2019). However, *Fraxinus excelsior* L. plays an important role in Europe, from both an economic and ecological perspective (Enderle et al. 2019). Numerous species are dependent on ash trees and a decline in *Fraxinus excelsior* L. will have negative effects on other species (Hultberg et al. 2020; Agostinelli et al. 2021). Not all common ash trees react to an infection with *Hymenoscyphus fraxineus* in the same way, and differences in susceptibility to ash dieback have been documented in various studies across Europe (McKinney et al. 2011; Stener 2013; Lobo et al. 2015; Havrdová et al. 2016; Stocks et al. 2017; Wohlmuth et al. 2018; Adamčíková et al. 2023).

Plants often present physiological changes as a result of disease infestation (Berger et al. 2007; Kumari and Kumar 2015; Rodrigues et al. 2018). However, it is yet unknown how the disease progression of ash dieback is manifesting in leaf physiology and morphology of *Fraxinus excelsior* L. Plant stress arises from a deviation from an ideal and

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tolerable range of environmental conditions. This includes both biotic (biological origin such as fungal diseases induced by, e.g., *Hymenoscyphus fraxineus* or insects) and abiotic (physical or chemical attributes such as ambient temperature or nutrient availability) factors (Hopkins and Hüner 2009). When a plant experiences stress conditions exceeding its tolerance threshold, it is prone to undergo physiological and morphological alterations (Lamalakshmi Devi et al. 2017). Pathogens have been proven to negatively influence water transport, carbon flow processes, nutrient uptake, plant growth, and lead to a reduction of photosynthetic pigments and a reduced photosynthetic performance within the affected plant (Kumari and Kumar 2015). Photosynthesis, a vital part of a plant's physiology, can be heavily affected after an infection with fungal pathogens, where the degeneration of chloroplasts is coupled with a reduction in photosynthetic rates (Barón et al. 2012).

The loss of photosynthetic pigments leads to a reduced photochemical efficiency of photosystem II (PSII), which can be interpreted as an indicator for plant stress by measuring chlorophyll fluorescence. Chlorophyll fluorescence is sensitive to both biotic and abiotic stress and measures the reemitted light of PSII, assessing the impact of stressors on photosynthetic processes (Murchie and Lawson 2013; Banks 2017). Chlorophyll fluorescence has been found as a suitable indicator among others for waterlogging and salinity stress (Zeng et al. 2013), water stress, high-light stress, and temperature stress (Ibaraki and Murakami 2007) as well as leaf diseases (Duraes et al. 2001). As an important photosynthetic pigment, chlorophyll content can represent the photosynthetic energy efficiency available in the measured plant. There are various methods used to determine chlorophyll content; however, the utilization of SPAD values, representative of the chlorophyll content in the leaves, enables fast real-time measurements (Uddling et al. 2007; Li et al. 2020). SPAD values can also be influenced when a plant experiences stress, e.g., induced by diseases (Zhao et al. 2011; Khaled et al. 2018; Arafat et al. 2021).

Besides, the analysis of leaf morphology is also a suitable tool for identifying possible stress reactions. The specific leaf area (SLA), the ratio of leaf area to leaf dry mass, is commonly used to determine whether plants are experiencing stress (Garnier et al. 2001). It has been reported that SLA can change due to a number of environmental factors such as light and temperature (Awal et al. 2004), radiation (Liu et al. 2022), elevation (Hulshof et al. 2013), heavy metal load (Pleijel et al. 2021), water stress (Chaimala et al. 2023) or salt stress (Said et al. 2022). The thickness of leaves is also reported to be sensitive to stress associated with disease infection (Ahn et al. 2020; España-Guechá et al. 2020; McIntire 2023). Fluctuating asymmetry (FA) focusses on the symmetry of leaves and is characterized as a non-directional deviation from perfect symmetry in a bilateral trait, which

is sensitive to stress, mainly from environmental and/or genetic origin (Palmer and Strobeck 1986). Environmental stress, in the context of FA analysis, is a broad term including many factors such as water stress, nutrient limitation, pollutants, extreme climatic conditions, herbivory, and infection (Graham et al. 2010). Various studies focused on the importance of FA as an indicator for the severity of stress such as induced by electromagnetic fields (Freeman et al. 1999), elevation stress in mountain birch (*Betula pubescens*) (Hagen et al. 2008), industrial pollution effects for *Betula pendula* (Turmukhametova et al. 2021), pollution stress in *Robinia pseudoacacia* leaves (Klisarić et al. 2014), and climatic factors (Shadrina et al. 2023). However, FA also often proved to be an unsuitable indicator, e.g., for environmental stress in fragmented habitats in the case of *Quercus deserticola* (García-Jain et al. 2022) or for investigations on the influence of the heavy metals copper and nickel on cucumber (*Cucumis sativus*), sweet pepper (*Capsicum annuum*), and common bean (*Phaseolus vulgaris*) (Gavrikov et al. 2023). In the case of *Salix alba*, neither air pollution, shading, air temperature, humidity nor herbivory had an influence on leaf FA (Wuytack et al. 2011). The informative value of FA as an indicator for environmental stress is, therefore, varying, depending on the investigated environmental factor and the studied plants. For plant diseases, such as ash dieback, caused by a fungal pathogen, very few studies have yet been conducted addressing FA, none in the case of *Fraxinus excelsior* L. Hochwender and Fritz (1999) studied the impact of *Melampsora epitea*, a leaf rust pathogen on FA of *Salix* hybrids. While variations were observed between plants exposed to the pathogen and those protected against leaf rust, these differences did not reach statistical significance.

The leaves of *Fraxinus excelsior* L. are compound leaves and, therefore, add complexity to the analysis of FA, due to the presence of multiple leaflets. Compound leaves are less frequently studied with respect to FA. Various studies focused on different species, each with characteristic leaf shapes such as horse chestnut (*Aesculus hippocastanum*) (Velickovic 2008), soybean (*Glycine max*) (Freeman et al. 1999), *Rhus copallinum* (Freeman et al. 2004), honey locust (*Gleditsia triacanthos*) (Murphy and Lovett-Doust 2004) or parsley (*Petroselinum crispum*) (Rakutko et al. 2017), using different methods in determining FA. No exclusive studies of FA in *Fraxinus excelsior* L. compound leaves, focusing on the impact of ash dieback, have been conducted to date.

This study, therefore, investigates the effects of ash dieback on the physiology and morphology of *Fraxinus excelsior* L. leaves and assesses the suitability of various biomarkers and plant traits, such as chlorophyll content, chlorophyll fluorescence, specific leaf area, leaf thickness, and fluctuating asymmetry, as indicators for the damage severity caused by ash dieback.

## Materials and methods

### Study sites

This study was conducted at four different study sites, located in the south of Germany in the federal states of Bavaria and Baden-Wuerttemberg (Fig. 1).

The seed plantation, in close proximity to the city Emmendingen (48°6'38.50"N, 7°52'20.49"E, 209 m NHN), located in Baden-Wuerttemberg, was established in 1995 on an area of 2.7 ha. Ash trees were planted with a spacing of 10 m × 10 m. Originally, 228 trees were planted, but due to the extensive damage caused by ash dieback, only 86 ash trees remained on the plantation in the summer of 2023.

Another seed plantation (48° 46' 35.59" N, 9° 25' 31.00" E, 420 m NHN) is located near the city Schorndorf in Baden-Wuerttemberg. The plantation was established in 1992 in a 7 m × 7 m planting unit and has an area of 2 ha. In 2023, from initially 416 individuals, only 120 living ash trees remained on the plantation. Both ash seed plantations have already been the subject of ash dieback research in other studies (Enderle et al. 2015; Buchner et al. 2022; Eisen et al. 2022, 2023, 2024).

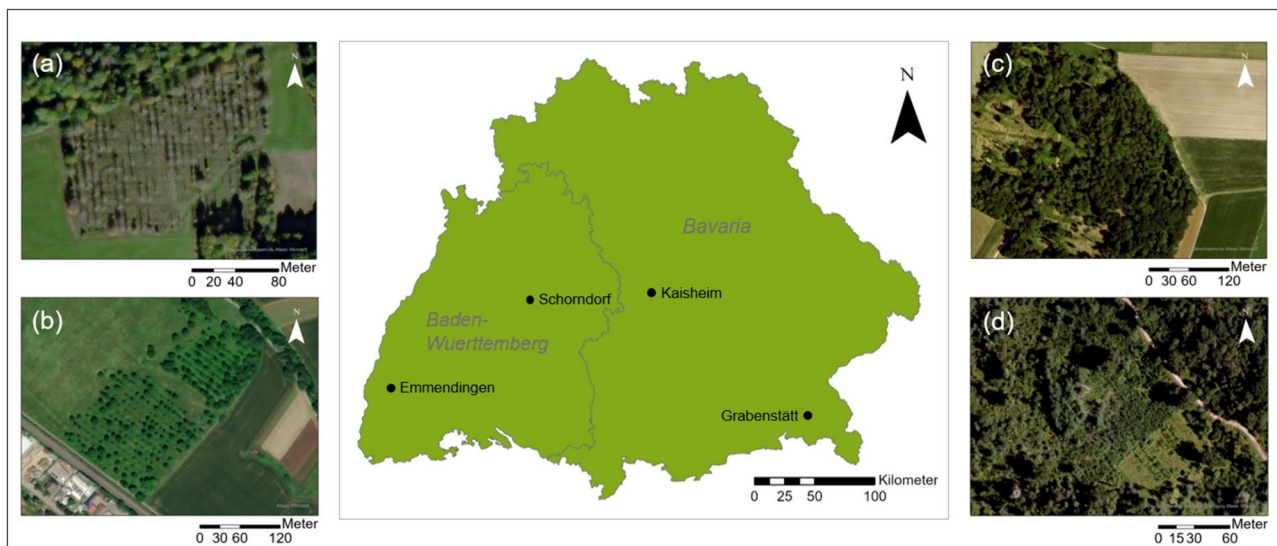
The third study site is a younger plantation densely stocked with ash clones (here referred as clone area) close to Grabenstätt near the lake Chiemsee in Bavaria (47° 50' 28.62" N, 12° 30' 41.87" E). In 2014, clones of 36 visually healthy trees from heavily infested stands with a total of 319 ramets were established in a randomized block design by the Bavarian Office of Forest Genetics (Fussi 2020). 213 ash

trees still existed in the summer of 2023, varying in height from less than 1 m to 8 m.

The mixed forest stand, Kaisheim (48° 48' 20.83" N, 10° 47' 33.34" E), is located about 1 km northeast of Sulzdorf in Bavaria. The approx. 80 years old and 30 m tall ash trees are approved as seed harvest stock ("Selected Propagation Material"). The stand is part of the intensive monitoring plots of the project FraxForFuture (Langer et al. 2022). Especially in the last 4 years, ash dieback has caused severe damage.

### Assessment of vitality

For each study site, 30 ash trees with various degrees of damage due to ash dieback were selected (exception: 33 in Kaisheim 2022) resulting in 123 studied trees in total. We assessed their vitality in the field, using the vitality scoring system developed by Peters et al. (2021). Vitality was assessed in the months July and August of 2022 and 2023 by taking the condition of the crown and overall leaf loss into consideration. The trees were divided into six categories, ranging from class 0 (no damage) to class five (dead). Only trees in class 0 can be classified as healthy, without typical ash dieback symptoms. Classes 1 and 2 are linked to mild damage symptoms (reduced foliage in class 1 and 2, few young dead shoots in class 2). Classes 3 and 4, with different degrees of advanced leaf loss, increased thinning of the crown, and multiple dead branches, represent severe damage due to ash dieback.



**Fig. 1** Location of the four study sites in the German federal states Baden-Wuerttemberg and Bavaria; **a** seed plantation Schorndorf, **b** seed plantation Emmendingen, **c** forest site Kaisheim, **d** clone area Grabenstätt (Map Source: ESRI Data and Maps)

## Chlorophyll content and chlorophyll fluorescence

Measurements of chlorophyll content and chlorophyll fluorescence were conducted in the field in the period of end of July until the beginning of August in 2022 and 2023. The chlorophyll content was determined using the SPAD-502Plus (Konica Minolta Optics, Inc., Japan). Recorded SPAD values correspond to the chlorophyll present in the leaf by measuring the transmittance of red and infrared radiation through the leaf (Uddling et al. 2007, Konica Minolta Optics, Inc. 2009). For each of the selected 30 trees per study site, measurements of 30 leaves were taken from various sun leaves throughout the tree crown. An average value was calculated to generate one SPAD value per tree. In addition, the standard deviation was assessed to infer on the variability of SPAD values from one tree.

Furthermore, chlorophyll fluorescence was investigated using the  $F_v/F_m$  meter, which is part of the Plant Stress Kit (Opti-Sciences, Inc., USA). The  $F_v/F_m$  meter uses dark adaptation clips for reliable measurements. The dark-adapted test is a measurement ratio indicative of the maximal potential quantum efficiency of PSII under the condition wherein all reaction centers are simultaneously in an open state (Murchie and Lawson 2013). Three clips were attached to each tree on three different leaves (Mevy et al. 2020), each time on the same position of the leaf, on the second leaflet from the top on the right side of the rachis. The leaves were then dark adapted for at least 40 min, until the chlorophyll fluorescence measurements were taken. Since the  $F_v/F_m$  ratio can differ between sun and shade leaves (Lichtenthaler and Babani 2004), attention was paid to exclusively select sun leaves. Healthy plants should exhibit  $F_v/F_m$  values in the range of 0.79 and 0.85 and lower values are indicative of plant stress (Maxwell and Johnson 2000). The standard deviation was calculated for each tree to infer on the variability of  $F_v/F_m$  measurements. Due to organizational and technical reasons, SPAD measurements for Kaisheim were only available in 2023, and no chlorophyll fluorescence measurements were possible in both years.

### Specific leaf area

For SLA, up to 20 fully expanded leaves from the light crown of the selected 123 trees were sampled and processed to calculate an average SLA value per tree for both 2022 and 2023. The standard deviation was assessed to infer on the variability of SLA values from the individual trees. Leaves with obvious damage caused by insects were avoided (Cornelissen et al. 2003). The sampled leaves were placed on moist tissue paper within sealed plastic bags and refrigerated. To prevent desiccation or decay of the leaves, the SLA analysis was performed in the laboratory in the days immediately following sampling. Since the rachis of

compound leaves is considered part of standardized SLA analysis (Cornelissen et al. 2003), the leaflets were not treated separately. Each sampled leaf was scanned in color with 300 dpi resolution using a scanner (CanoScan Lide 200, Canon Deutschland GmbH, Germany). Due to the size of the scanner and the sampling method in the field, the petiole of each leaf was cut below the last set of leaflets to ensure comparability. Subsequently, the leaves were dried for 48 h at 70 °C in an oven and the weight of each leaf was determined. The area of the leaves was then specified using the scanned image and the R package LeafArea (Katabuchi 2015) in R Studio (version 2021.09.0). This package implements the software ImageJ (version 1.53) and transforms the scanned color image into a black and white image. In the next steps, the area of the black leaf can be determined, and the SLA is calculated using the formula:

$$SLA = \frac{\text{Leaf area (cm}^2\text{)}}{\text{Leaf dry weight (g)}}$$

### Leaf thickness

Leaf thickness was measured on the same leaves analyzed for SLA, using a digital thickness gauge (Käfer Messuhrenfabrik GmbH & Co. KG, Germany). Every measurement was taken on the same position on the leaf, i.e., on the second leaflet from the top on the right side of the rachis. Close attention was paid to avoid the dense tissue of the leaflet midvein (White and Montes-R 2005). For each tree, we calculated the standard deviation and the average leaf thickness value out of the measurements of the 20 individual leaves.

### Statistical analysis

Leaf physiological and morphological data were tested for statistically significant differences between the vitality classes using a *t* test or Mann–Whitney *U* test depending on the condition of a normal distribution, which was tested using the Shapiro–Wilk test. Homogeneous variances were tested using the Levene’s test. For correlation analyses, we calculated the Spearman correlation coefficient and associated *p* values.

All statistical analyses were carried out using the software R Studio (version 2021.09.0).

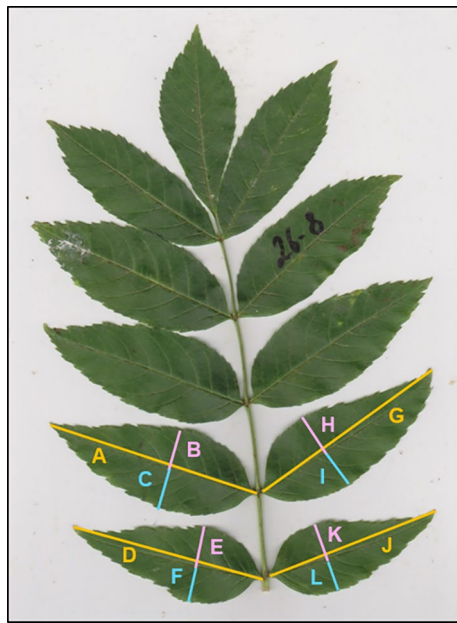
### Fluctuating asymmetry

The workflow for the determination of FA also builds on the images of the scanned leaves. For each tree, three scanned leaves were randomly selected (number of investigated leaves in total: 729). Since the leaves of *Fraxinus excelsior* L. are compound leaves, four leaflets per leaf were measured



and examined. These leaflets were the two youngest mature leaflets on both sides of the rachis. The software ImageJ (version 1.53) was used for the 12 measurements per leaf displayed in Fig. 2.

FA in general is calculated as the difference between the right (R) and left (L) side of the object under investigation (Palmer and Strobeck 1986; Graham 2021). The differences between these sides often are very small and the results of FA can be heavily affected by measurement error (Kozlov 2015; Kozlov et al. 2017); thus, the effect of measurement error has to be tested. A randomly chosen subset containing 5% of the 8,748 original individual lengths was measured



**Fig. 2** Measurements made on each leaf; for each leaflet, the right and left side was measured from the main vein at the widest point of the leaflet. The length of each leaflet was measured from the tip of the leaflet to the rachis of the leaf, independent of the main vein. A–L denote the different measured lengths

a second time by a different researcher. The two series of measurements were then statistically compared to each other using a *t* test. It is necessary to discriminate FA from other forms of asymmetry (Palmer and Strobeck 2003). Besides FA, there are two other forms of asymmetry whose possible presence must be tested: antisymmetry and directional asymmetry. FA exists when the variances between R and L adhere to a normal distribution centered around zero. Directional asymmetry occurs when these R – L differences exhibit a normal distribution, yet with a mean value significantly deviating from zero. Antisymmetry is identified by a platykurtic or bimodal distribution of R – L differences, converging around a mean value of zero, indicating that a left- or right-biased asymmetry exists (Palmer and Strobeck 1986; Palmer 1994; Klingenberg 2015; Maldonado-López et al. 2019). To determine the possible presence of directional asymmetry, a two-tailed *t* test against a mean of zero was applied (Mabrouk et al. 2020). Antisymmetry was evaluated using the Kolmogorov–Smirnov test, which tests for deviation from normality, a requirement for FA (Ambo-Rappe et al. 2008; Mabrouk et al. 2020).

Since larger leaves have had more time to develop more distinct FA than smaller leaves, the effect of size dependency was addressed. The traditional way of addressing size dependency is dividing R – L by  $(R + L)/2$  or  $R + L$ . Even if R and L are lognormally distributed, a log-transformation is a more elegant approach, since  $\log R - \log L$  is normally distributed. This method is especially suitable if the measurement error is small (Graham et al. 2003; Graham 2021). The transformation can be performed either using natural or base 10 logarithms (Palmer and Strobeck 2003). In this study, the absolute values of the right and left side were used for  $|\ln R - \ln L|$ , according to the trait calculations in Table 1.

While it is possible to simply averaging the deviations of multiple traits from symmetry after a log-transformation (Palmer and Strobeck 2003) or using a median leaflet as a representation of the entire leaf (Boeger et al. 2018), more precise methods exist. Leung et al. (2000) developed

**Table 1** Leaf traits and calculations for asymmetry and the trait level (leaf or leaflet level) based on  $|R - L|$ , measured parameters shown in Fig. 2

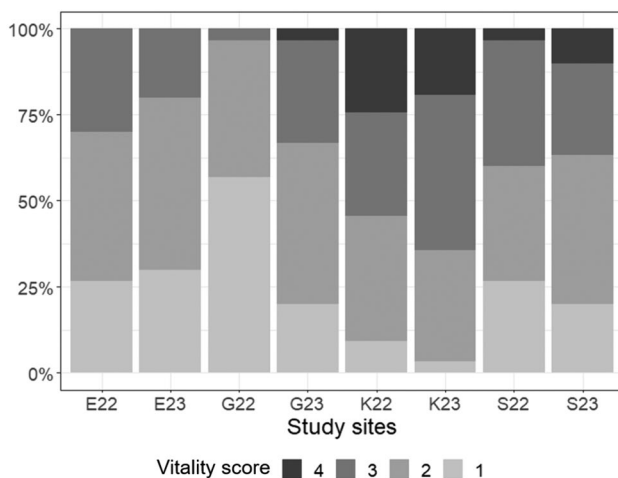
Trait level	Trait	Asymmetry
Asymmetry of the individual leaflets, ratio between the right and left side of the main vein for four individual leaflets	Lateral leaflet width (LLW)	B–C
		H–I
		E–F
		K–L
Asymmetry of the leaf, ratio between the right and left top side of the leaf of two pairs of leaflets	Lateral leaflet top (LLT)	E–K
		B–H
Asymmetry of the leaf, ratio between the right and left bottom side of the leaf of two pairs of leaflets	Lateral leaflet bottom (LLB)	F–L
		C–I
Asymmetry of the leaf, ratio between the right and left length of the leaflet of two pairs of leaflets	Lateral leaflet length (LLL)	A–G
		F–J

multiple Composite Fluctuating Asymmetry Indices, each taking into account multiple measured traits. In this study, however, individual traits might be dependent on each other and, thus, contain the same informative value. Therefore, a principal component analysis (PCA) was conducted to reduce the number of individual dependent values, resulting in a more compact dataset with fewer independent variables, while retaining the most significant variance present in the data. This approach highlights similarities and differences between leaf trait measurements by transforming the original variables into a new set of uncorrelated variables, known as principal components (PC) (Greenacre et al. 2022). The reduced dataset was then set in relation to the vitality assessment (mild or severe damage) of the individual ash trees. Two-factor ANOVA and MANOVA analyses were used to test whether trees with more severe damage due to ash dieback exhibit a significantly different FA compared to the trees with only mild damage. The application of the PCA and the further statistical analyses were carried out using the software R Studio (version 2021.09.0).

## Results

### Vitality assessment of ash trees

The vitality assessment showed a differentiated picture with regard to the distribution of the damage classes (Fig. 3), as the severity of the damage due to ash dieback varied for the four study sites. Especially the site Kaisheim was generally heavily affected with a large



**Fig. 3** Vitality scores of the selected ashes in the four study sites Emmendingen (E), Grabenstätt (G), Kaisheim (K), and Schorndorf (S) in 2022 and 2023. A vitality score of 1 and 2 accounts for mild damage due to ash dieback and vitality scores of 3 and 4 for severe damage. Note that the trees in Kaisheim were not all identical in 2022 and 2023 and 33 trees were selected in 2022

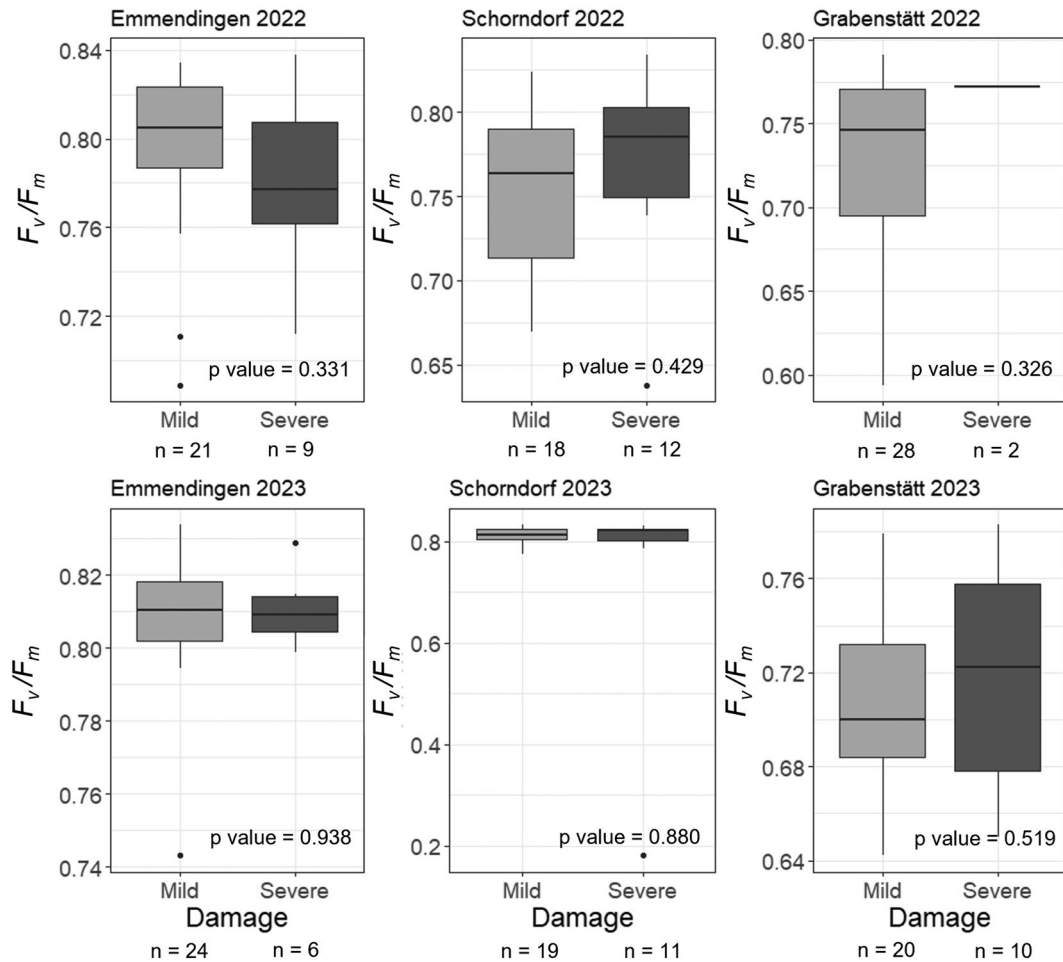
number of severely damaged trees. While for Emmendingen, Schorndorf, and Grabenstätt, the same trees were selected in both years, in Kaisheim, several trees had to be removed in 2023 due to the extensive damage of ash dieback and alternative trees were selected. A direct comparison of both years is, therefore, not possible for Kaisheim. Schorndorf and Grabenstätt showed a decline of trees sorted into class 1 from 2022 to 2023, indicating a decrease in only mildly affected trees in accordance with an increase of more severely affected trees. However, trees can also partially recover from one year to the next. In Emmendingen in 2023, less trees were grouped to class 3 than in the previous year. For all four study sites, no healthy trees (class 0) could be observed in both 2022 and 2023.

### Chlorophyll fluorescence and chlorophyll content of ash leaves

The results of the  $F_v/F_m$  meter measurements displayed in Fig. 4 present the distribution of the  $F_v/F_m$  values for each study site for mildly and severely damaged trees by ash dieback.  $F_v/F_m$  values  $< 0.79$  indicate stress; however, trees affected more severely by ash dieback demonstrated not always lower  $F_v/F_m$  values than mildly damaged trees. There were no significant differences in  $F_v/F_m$  values between the classes mild and severe damage for any of the study sites. The standard deviation of  $F_v/F_m$  values per tree ranged from a minimum of  $< 0.001$  to a maximum of 0.11 with a mean standard deviation for all measured trees of 0.02.

Only for Emmendingen 2022 and Schorndorf 2023, the percentage of stressed trees was higher for the more severely affected than mildly damaged ashes (Table 2). However, this was not the case for Emmendingen 2023, Schorndorf 2022, and for both years in Grabenstätt. For the study site Grabenstätt, the results differed greatly from the other two study sites with very high numbers of stressed trees, independent of their vitality status. Especially in 2023, all trees were linked to very low  $F_v/F_m$  values, and no unstressed trees could be detected.

For the two plantation sites Emmendingen and Schorndorf, SPAD values tended to be lower with severely affected trees. This, however, did not apply to the ash trees in Grabenstätt. Here, SPAD values in 2023 were slightly but non-significantly higher for severely damaged trees. Only for Emmendingen in 2023 ( $t$  test,  $p$  value = 0.005) and Schorndorf 2022 (Mann–Whitney  $U$  test,  $p$  value = 0.021) (Fig. 5), the differences for mildly and severely damaged ash trees were statistically significant. Overall SPAD values also differed between the different study sites: higher values were recorded for Emmendingen, Grabenstätt, and Kaisheim, whereas the trees displayed an on average lower chlorophyll content in Schorndorf. Note that the standard deviation of



**Fig. 4** Boxplots of  $F_v/F_m$  values differentiated for mildly and severely affected ash trees for three study sites in 2022 and 2023. Interquartile range (IQR) represented by height of boxes, median by bold horizontal lines, upper (lower) whiskers indicate minimum of maximum

(minimum) of metric and 1.5 times IQR, dots represent observations exceeding or falling below 1.5 times IQR. A different y-axis was applied for each plot

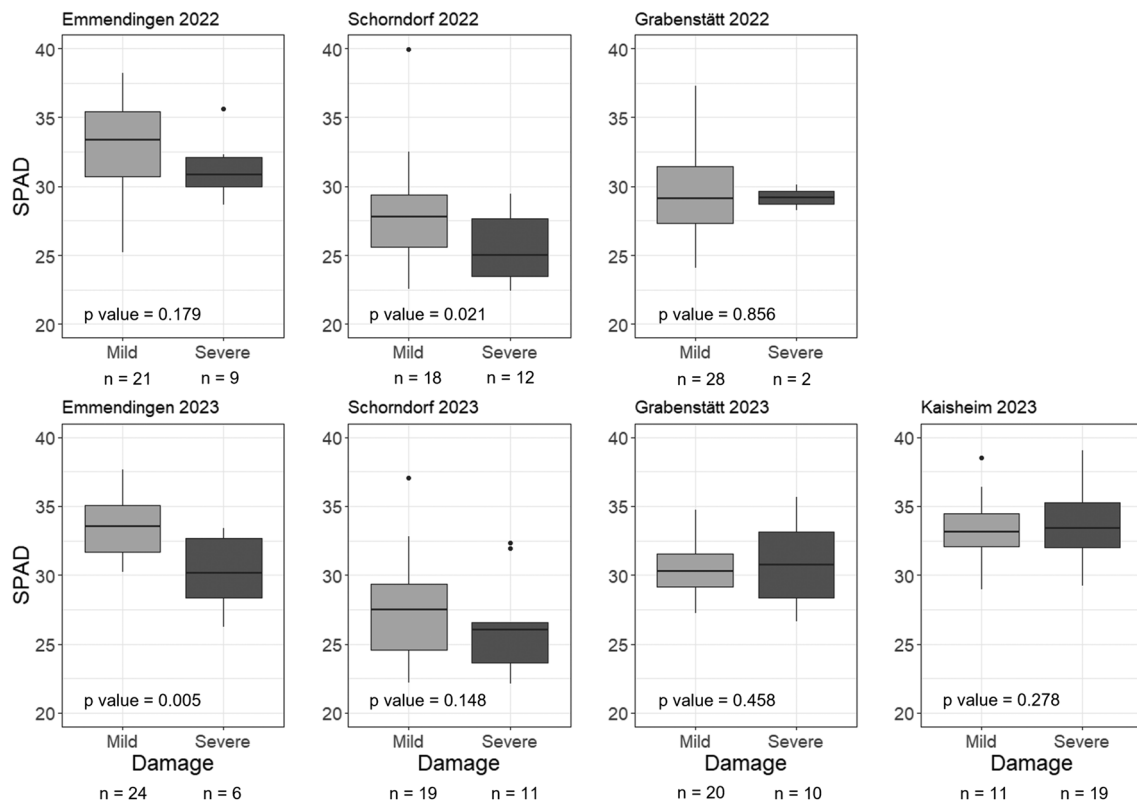
**Table 2** Percentage of stressed trees (low  $F_v/F_m$  values < 0.79 indicate stress, high  $F_v/F_m$  values from 0.79 to 0.85 indicate no stress) in mildly and severely damaged ash trees for the three study sites in 2022 and 2023

	Mild damage		Severe damage	
	Number of trees (n)	Percentage of stressed trees	Number of trees (n)	Percentage of stressed trees
Emmendingen 2022	21	28.6%	9	55.6%
Emmendingen 2023	24	20.8%	6	16.7%
Schorndorf 2022	18	66.7%	12	58.3%
Schorndorf 2023	19	10.5%	8	37.5%
Grabenstädt 2022	28	85.7%	2	100.0%
Grabenstädt 2023	20	100.0%	10	100.0%

the 30 SPAD measurements per tree ranged from a minimum of 0.90 to a maximum of 5.28, with a mean standard deviation for all measured trees of 2.27 pointing to a reasonable sample size.

**Specific leaf area**

The standard deviation of the SLA measurements ranged from a minimum of 3.0 to a maximum of 81.6, with a mean standard deviation for all measured trees of 15.5, demonstrating an overall suitable sample size, while also



**Fig. 5** Boxplots of chlorophyll content (SPAD) differentiated for mildly and severely affected ash trees for four study sites in 2022 and 2023. Interquartile range (IQR) represented by height of boxes,

representing the variability of the SLA of leaves within individual trees. In general, smaller SLA for severely affected ash trees were observed for all study sites for both investigated years. The difference in SLA was significant for Schorndorf in 2022 and for Schorndorf and Kaisheim in 2023, though Emmendingen and Kaisheim in 2022 were marginally significant (Fig. 6).

### Leaf thickness

Leaf thickness tended to be slightly higher in more severely damaged trees. However, the difference was only statistically significant for Schorndorf in 2022 ( $p$  value = 0.043) and Kaisheim 2023 ( $p$  value = 0.022). While the mean leaf thickness was similar across years, there were differences between the four areas. Leaf thickness was slightly higher in Schorndorf and Kaisheim, whereas ashes from Grabenstätt had overall thinner leaves (Fig. 7). The standard deviation of the 20 leaf thickness measurements per tree ranged from a minimum of 0.01 cm to a maximum of 0.12 cm, with a mean standard deviation for all measured trees of 0.04 cm; thus, many leaves are rather uniform in thickness, but some also exhibit a different pattern.

median by bold horizontal lines, upper (lower) whiskers indicate minimum of maximum (minimum) of metric and 1.5 times IQR, dots represent observations exceeding or falling below 1.5 times IQR

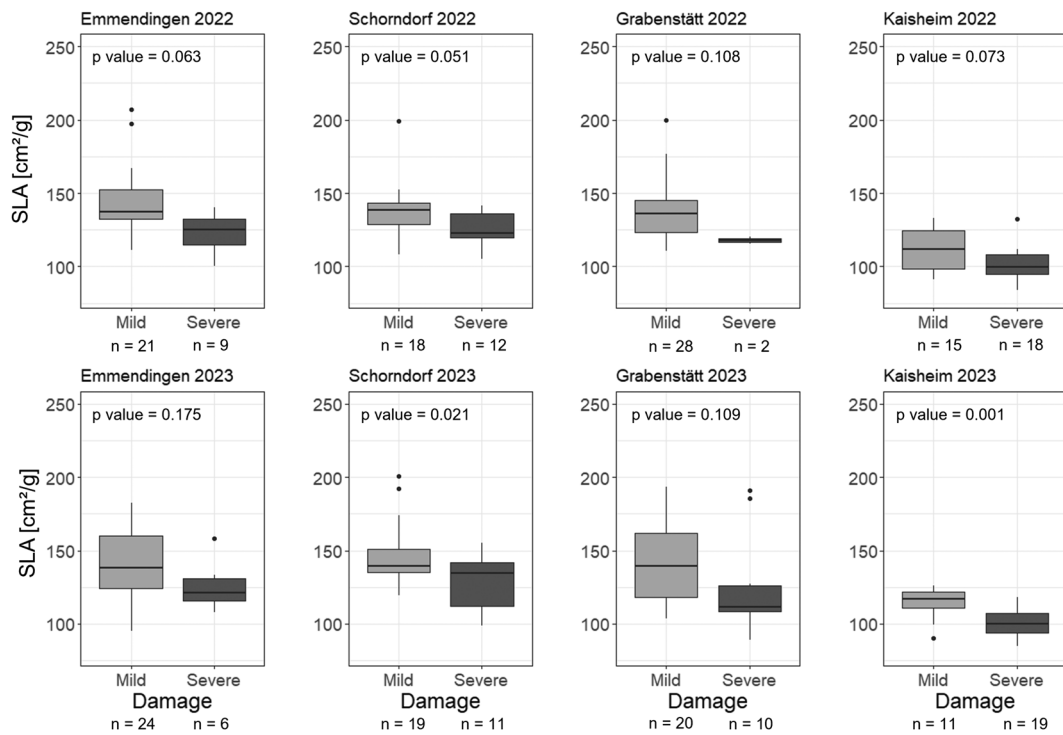
### Correlations of leaf traits

Leaf thickness correlated significantly (Table 3) with SLA, demonstrating that leaves with a lower SLA are related to thicker leaves than leaves with a high SLA. A significant negative correlation between SLA and chlorophyll content is shown in Table 3: trees with a smaller SLA were linked to a higher chlorophyll value. High  $F_v/F_m$  values, indicative of a non-stressed plant, corresponded to both thicker leaves and leaves with a high SLA. No significant correlations between chlorophyll content and leaf thickness or  $F_v/F_m$  were found.

### Fluctuating asymmetry

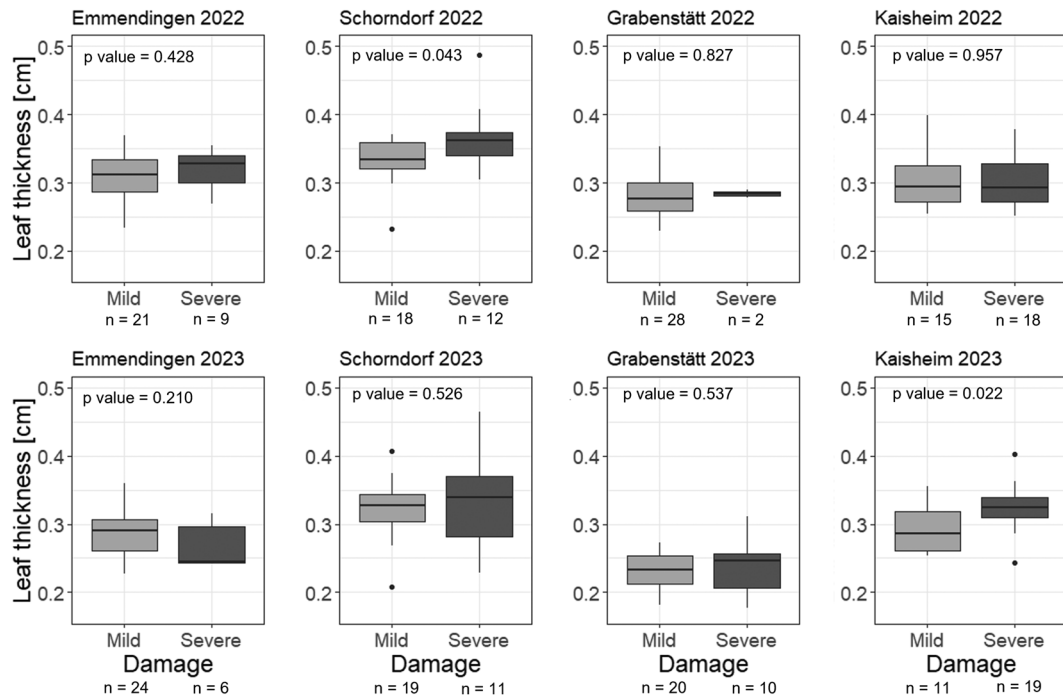
Related to the analysis of FA, the paired  $t$  test conducted to assess measurement error yielded a statistically highly significant result ( $p$  value < 0.001), and a strong correlation between the two sets of measurements was observed. Consequently, the measurement error was determined to be negligible. Given the significance of the Kolmogorov–Smirnov test for normal distribution ( $p$  value < 0.001) and the  $t$  test results indicating no significant deviation of recorded values from zero ( $p$  value = 0.745), both antisymmetry and directional asymmetry were rejected. Consequently, the





**Fig. 6** Boxplots of the impact of different degrees of damage due to ash dieback on SLA (specific leaf area) differentiated for mildly and severely affected ash trees for the four study sites in 2022 and 2023. IQR represented by height of boxes, median by bold horizontal

upper (lower) whiskers indicate minimum of maximum (minimum) of metric and 1.5 times IQR, dots represent observations exceeding or falling below 1.5 times IQR



**Fig. 7** Boxplots of leaf thickness for trees differentiated for mildly and severely affected ash trees for the four study sites in 2022 and 2023. IQR represented by height of boxes, median by bold horizontal

lines, upper (lower) whiskers indicate minimum of maximum (minimum) of metric and 1.5 times IQR, dots represent observations exceeding or falling below 1.5 times IQR

**Table 3** Spearman correlations between the investigated leaf traits (leaf thickness and SLA  $n=243$ , SPAD  $n=210$ ,  $F_{\sqrt{F_m}}$   $n=180$ )

	Leaf thickness	SLA	$F_{\sqrt{F_m}}$
SPAD	$r: -0.120$ $p$ value: 0.081	$r: -0.171$ $p$ value: 0.012*	$r: 0.079$ $p$ value: 0.291
$F_{\sqrt{F_m}}$	$r: 0.254$ $p$ value: <0.001*	$r: 0.216$ $p$ value: 0.003*	–
SLA	$r: -0.314$ $p$ value: <0.001*	–	–

\*Significant correlation with  $p < 0.05$

measurements were deemed to accurately represent FA. The PCA identified four principal components with eigenvalues  $> 1$ , each merging two to three traits (Table 4). Higher eigenvalues indicate a higher explanatory value of the respective PC. The traits included in PC 1 and PC 2, focusing on the FA of the entire leaf, therefore, exhibited a higher informative value than PC 3 and PC 4, which measured the FA of the individual leaflets. The higher the loading value, the better the observation is represented by the PC, as shown in Table 5. Our results show a clear distribution of the traits with none of them being loaded on more than one PC. In total, 67% of the variance of the dataset was retained by the application of the PCA (Table 4).

Each of the four principal components was set in relation to the four study sites and tested for significant difference between mildly and severely damaged ash trees regarding FA. However, only for Emmendingen and PC 4, the result

**Table 6**  $p$  values of two-way ANOVA analysis, testing the difference between mild and severely damaged trees for the four principal components (PC) and four study sites

	Emmendingen	Schorndorf	Grabenstätt	Kaisheim
PC 1	0.398	0.190	0.757	0.066
PC 2	0.338	0.737	0.975	0.485
PC 3	0.098	0.509	0.440	0.191
PC 4	0.023*	0.828	0.338	0.604

\*Significant difference with  $p < 0.05$

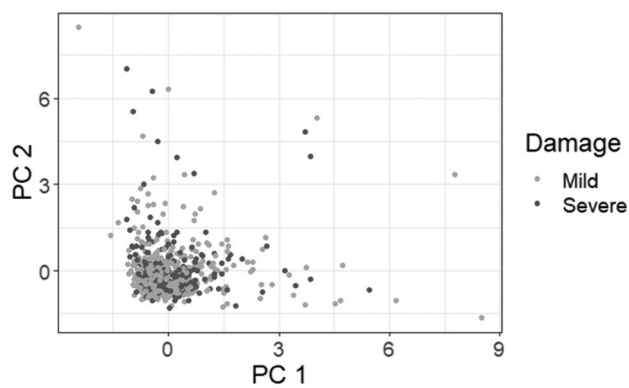
was significant (Table 6), demonstrating increased FA in more severely damaged trees. All other principal components showed no significant difference between disease severity for any of the study sites. A scatterplot of PC 1 and PC 2 for all study sites (Fig. 8) shows that leaves from both mildly and severely damaged trees are densely clustered near the origin [around (0,0)]. This suggests that the majority of leaves, regardless of damage severity, have similar PC scores. The lack of a clear separation between mild and severe damage along the two PCs implies that damage severity does not drastically alter the overall pattern of asymmetry, although individual leaves may still show differences. As there are some leaves demonstrating increased FA for both mildly and severely affected trees, changes in FA cannot be attributed to damage caused by ash dieback. A MANOVA analysis using the combined data of all four study sites revealed no significant difference between the four principal components and the damage severity ( $p$  value = 0.304).

**Table 4** Eigenvalue, proportion variance, and the respective included traits for the four PCs from the FA analysis of the sampled ash leaves

Factor	Eigenvalue	Proportion variance	Included traits
PC 1	2.03	0.21	First leaflet set from the bottom: LLL (D–J), LLB (F–L), LLT (E–K)
PC 2	2.05	0.20	Second leaflet set from the bottom: LLL (A–G), LLB (C–I), LLT (B–H)
PC 3	1.36	0.14	First leaflet set from the bottom: LLW (K–L), LLW (E–F)
PC 4	1.18	0.12	Second leaflet set from the bottom: LLW (B–C), LLW (H–I)

**Table 5** Loadings of the individual traits for the respective PCs from the FA analysis of the sampled ash leaves

Traits	Asymmetry	PC 1	PC 2	PC 3	PC 4
First leaflet set from the bottom: LLL	D–J	0.84	–	–	–
First leaflet set from the bottom: LLB	F–L	0.79	–	–	–
First leaflet set from the bottom: LLT	E–K	0.76	–	–	–
Second leaflet set from the bottom: LLL	A–G	–	0.83	–	–
Second leaflet set from the bottom: LLB	C–I	–	0.79	–	–
Second leaflet set from the bottom: LLT	B–H	–	0.75	–	–
First right leaflet from the bottom: LLW	K–L	–	–	0.81	–
First left leaflet from the bottom: LLW	E–F	–	–	0.75	–
Second left leaflet from the bottom: LLW	B–C	–	–	–	0.88
Second right leaflet from the bottom: LLW	H–I	–	–	–	0.58



**Fig. 8** Scatterplot displaying the values of FA of PC 1 and PC 2 of all four study sites, colored in the two damage classes “mildly affected” (light grey) and “severely affected” (dark grey)

## Discussion

Five different leaf physiology and morphology traits were tested within the scope of this study; however, only some were associated with significant differences between mildly and severely affected ash trees.

Using  $F_v/F_m$  values for the assessment of plant stress is a quick way of obtaining information; however, this method does not delineate the cause of stress. While for Emmendingen 2022 and Schorndorf in 2023, more stressed trees were observed in more severely damaged trees, this could neither be confirmed for the other year nor for the third study site Grabenstätt. For this study site, we recorded very high numbers of stressed trees, especially in 2023. It has to be noted that we observed visible symptoms presumably caused by drought stress during the measurements in early August. This highlights the difficulty of interpreting  $F_v/F_m$  values in the context of ash dieback. In general,  $F_v/F_m$  has been proven as a reliable indicator for diseases in multiple species. For *Plantago ovata*, affected by downy mildew disease, maximum quantum yield of PSII differed significantly between healthy and chlorotic leaves (Mandal et al. 2009). In the case of wheat (*Triticum aestivum*) affected by spot blotch, diseased plants presented reduced chlorophyll fluorescence variables, negatively correlated with severity (Rosyara et al. 2010). Chlorophyll fluorescence differences of white root rot in avocado stands, caused by the fungus *Rosellinia necatrix*, might even be suitable for identifying susceptible genotypes (Martínez-Ferri et al. 2016). For ash trees that were infested with emerald ash borer and treated with stem injections, chlorophyll fluorescence values improved, thus proving the general suitability of  $F_v/F_m$  as a measure of the photosynthetic capacity for this species (Hanavan and Heuss 2019).

Fungal pathogens can be differentiated in biotrophs (utilization of living host tissue) or necrotrophs (utilization of nutrients obtained from dead host tissue), or classified as

hemibiotrophic if they are able to switch from a biotrophic to a necrotrophic phase (Perfect and Green 2001). *Hymenoscyphus fraxineus* has been characterized as hemibiotroph, with an extended biotrophic phase in the stem tissue (Mansfield et al. 2019). Ajigboye et al. (2016) suggest that fungal pathogens, once switched to necrotrophic phase, are damaging the chloroplasts inside the leaves, thus changing the efficiency of PSII photochemistry. Therefore, we suggest that leaf infestation might not be detectable using  $F_v/F_m$  values as long as *Hymenoscyphus fraxineus* is in its biotrophic phase. Thus, we conclude that further studies should focus on  $F_v/F_m$  measurements while taking a larger number of leaves into account and measuring other environmental factors such as air temperature and edaphic conditions of the soil to assess the influence of other stressors. Extended time series are necessary to establish dependable relationships between  $F_v/F_m$  values and these stress factors.

The results of the analysis of chlorophyll content present two different dynamics. While there was a noticeable distinction between mildly and severely damaged trees at the two seed plantation sites, characterized by lower SPAD values for severely affected ashes, the clone area Grabenstätt exhibited the opposite pattern, with severely damaged trees showing higher SPAD values. Various studies in the context of plant diseases reported lower SPAD values for affected plants (Rosyara et al. 2010; Zhao et al. 2011; Yahya et al. 2020; Arafat et al. 2021). However, in the case of oil palm, affected by basal stem rot disease caused by *Ganoderma boninense* fungus, SPAD value differences were only recorded between healthy and diseased plants, without variation based on disease severity (Khaled et al. 2018). It has to be noted that it was not possible to include completely unaffected ash trees in this study due to the extended spread of ash dieback in Germany. The expected decrease of SPAD values associated with increased damage severity was only observed for the two ash seed plantations. Differences between the sites imply the presence of potential additional environmental factors influencing chlorophyll content.

A negative relationship between SPAD values and SLA was found, indicating a higher chlorophyll content in leaves with smaller SLA. This negative chlorophyll content–SLA relationship was also documented in six Amazonian tree species (Marenco et al. 2009), for groundnut (*Arachis hypogaea*) (Nageswara Rao et al. 2001; Nigam and Aruna 2008) and green gram (*Vigna radiata*) (Basu et al. 2019). This can be explained by the fact that elevated chlorophyll concentrations in a reduced leaf surface area facilitate enhanced absorption of solar radiation per unit leaf area by the plant (Basu et al. 2019) and, thus, counteract the possible negative effects of a reduced SLA.

In this study, SLA is significantly reduced in ash trees severely damaged by ash dieback. Plant diseases can have a high impact on SLA as demonstrated for American beech

(*Fagus grandifolia*) associated with a decreased SLA on leaves with severe symptoms caused by beech leaf disease. A clear difference in SLA between healthy trees and different degrees of damage was observed (McIntire 2023). A very similar result was reported for oil palms (*Elaeis guineensis*) affected by the plumero disorder (España-Guechá et al. 2020). Both studies are consistent with our findings of a decreased SLA for plants affected by varying degrees of a disease. However, an increased SLA was demonstrated for willow (*Salix* sp.) leaves damaged by leaf rust (*Melampsora epitea*) (Toome et al. 2010), illustrating species- and disease-specific reactions. SLA possibly demonstrates a high variability based on the position of the sampled leaves in the crown (Eriksson et al. 2005). Shade can have a crucial influence on leaves, with SLA increasing as a consequence of shadow (Wuytack et al. 2011). This was also documented for *Fraxinus excelsior* L., where SLA increased from top to bottom leaves (Petriřan et al. 2009) and shadowed leaves had a higher SLA (Legner et al. 2014). While in our study, close attention was paid to only collect leaves with exposure to direct sunlight, a possible variability in SLA due to the position of the leaf in the crown cannot be ruled out entirely. This fact may also be related to the variability of the measurements from different leaves of one tree: a relatively high standard deviation for some trees point to a possible high variability in SLA within individual trees. The SLA links the carbon and water cycle of a plant as it describes the distribution of leaf biomass in relation to leaf area, and thus changes in SLA also indicate changes in the latter (Pierce et al. 1994). It is yet unclear, if the reduction in SLA for severely damaged trees is a reaction to the infection with *Hymenoscyphus fraxineus* or leaves with a smaller SLA are more easily infected. However, the answer to this question would be highly interesting and further investigations are necessary.

Leaf thickness tends to be higher with advanced damage symptoms associated with a reduced SLA. The leaves of severely damaged ash trees, therefore, have a smaller area but exhibit an increased thickness. Changes in leaf thickness following stressors have been documented for various species. *Platanus acerifolia* growing under air-polluted conditions demonstrated higher leaf thickness than a non-polluted control group. The strengthened anatomic xeromorphic characteristics of the leaves were regarded as an adaptive strategy to air pollution (Dineva 2004). The cell wall of plants is dynamic and can change, when a disease occurs as a result of activated defense mechanisms (Zhao and Dixon 2014). Ahn et al. (2020) reported an increased thickness in the midribs of the leaves of Johnson grass as a response to the infection with *Colletotrichum sublineola*. Based on the presence of a thicker spongy mesophyll observed in highly susceptible clones, conclusions regarding the susceptibility of *Vitis vinifera* to downy mildew were possible; however,

the differences were not statistically significant (Alonso-Villaverde et al. 2011). Increases of the thickness of the epidermis and hypodermis cell layer of grape berries were positively correlated with resistance to the disease *Botrytis cinerea*; however, the thickness of the leaves was not investigated in this study (Gabler et al. 2003). Jarosz et al. (1982) found no significant correlation between the thickness of the leaf cuticle and resistance to an infection with powdery mildew *Erysiphe cichoracearum*, demonstrating the species-specific different dynamics. In the context of the evaluation of leaf thickness, leaf hydration may play an important role. McIntire (2023) concluded that the increased leaf thickness found in diseased leaves is caused by a higher water content in the leaves which leads to more hydrated leaves. White and Montes-R (2005) also emphasized the need to include leaf water content to minimize the effects of varying environmental conditions. The simple and fast measurements of leaf thickness applied in this study yielded promising results; however, reliable statements regarding the leaf thickness of trees under the influence of ash dieback cannot be concluded at this point. For future investigations on leaf thickness of *Fraxinus excelsior* L., it is recommended to take multiple measurements per leaf and additionally measure leaf water content. This study did not differentiate between the individual components in the leaves; however, this would be recommended for future studies, especially with regard to possible conclusions on susceptibility.

Fluctuating asymmetry is proved to be an unsuitable indicator for stress caused by ash dieback. Only for one principal component, one study site was linked to significant differences between FA values and the two different damage classes. This may indicate that stress caused by ash dieback does not lead to increased FA in the leaves. Many studies also reported difficulties in proving FA as a reliable indicator, e.g., to detect stress due to insect herbivory (García-Jain et al. 2022), heavy metal stress (Gavrikov et al. 2023), stress caused by air pollution (Ambo-Rappe et al. 2008), environmental and genetic stress in fragmented populations (Murphy and Lovett-Doust 2004) or stress caused by landfill leachate (Dimitriou et al. 2006). FA is a controversial concept as it is linked to uncertainties regarding sample size (Mogie and Cousins 2001), the lacking reproducibility of measurements (Kozlov 2015; Dodonov et al. 2024), and the high impact of measurement error (Kozlov et al. 2017; Dodonov et al. 2024).

Due to the widespread impact of ash dieback, it is now very rare to find completely healthy ash trees in Germany (Fuchs et al. 2024). Therefore, it was not possible to include healthy trees in this study and our results only highlight the differences between mildly and severely damaged trees. The observed tendencies of a reduction in photosynthetic pigments and the changes in leaf morphology in terms of SLA and leaf thickness are indicative of

the significant changes that *Hymenoscyphus fraxineus* can cause on *Fraxinus excelsior* L. leaves. A decline of photosynthetic activity suggests changes in energy conversion with possible additional implications further affecting the overall fitness of the infected trees (Berger et al. 2007). The noted effects may potentially lead to additional health disadvantages, exacerbating the overall well-being of the ash trees.

## Conclusion

The general health status of ash trees, infected by *Hymenoscyphus fraxineus*, can be easily recognized by the typically observed dieback of the shoots. In this study, we demonstrated that ash dieback has an influence on leaf physiology and morphology of *Fraxinus excelsior* L. Especially SLA enables a clear differentiation between the different degrees of damage by ash dieback. A reduced SLA of the leaves, in conjunction with alterations in leaf thickness and chlorophyll, may potentially contribute to additional adverse consequences, further impacting the overall health of the ash trees. Long-term data are needed for understanding the development of the observed physiological and morphological changes. Further studies on leaf physiology and morphology of *Fraxinus excelsior* L. affected by ash dieback are, therefore, highly recommended.

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**Data availability** The data supporting the findings of this study are not publicly available, as the project in which the study was conducted is still ongoing.

## Declarations

**Conflict of interest** The authors have no relevant financial or non-financial interests to disclose.

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