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# Uncovering the secrets of monoculture yield decline: trade-offs between leaf and root chemical and physical defence traits in a grassland experiment

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Plant monocultures growing for extended periods face severe losses of productivity. This phenomenon, known as 'yield decline', is often caused by the accumulation of above- and below-ground plant antagonists. The effectiveness of plant defences against antagonists might help explain differences in yield decline among species. Using a trait-based approach, we studied the role of 20 physical and chemical defence traits of leaves and fine roots on yield decline of 4- and 18-year-old monocultures of 27 grassland species. We hypothesized that yield decline is lower for species with high defences, that root defences are better predictors of yield decline than leaf defences, and that in roots, physical defences better predict yield decline than chemical defences, while the reverse is true for leaves. We additionally hypothesized that the relationship between defences and yield decline increases with time and that species increasing the expression of defence traits after long-term monoculture growth would suffer less yield decline. We summarized leaf and fine root defence traits using principal component analyses and analysed the relationship between the most informative components along with their temporal changes and monoculture yield decline. The significant predictors of yield decline were traits related to the so-called collaboration gradient of the root economics space (specific root length and root diameter) as well as their temporal changes and traits related to the leaf physical vs chemical defence tradeoff (leaf dry matter, silicon and cellulose content, toughness and phytochemical diversity). We were unable to unequivocally identify the mechanisms relating the effect of those traits to yield decline as they could mediate plant responses to several stressors such as antagonist

accumulation, nutrient depletion or drought. Further studies are needed to differentiate between these alternative mechanisms and to gain a comprehensive understanding of the drivers of yield decline in relation to root and leaf defence traits.

Keywords: antagonists, collaboration gradient, functional traits, mutualists, performance change, trait plasticity

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## Introduction

Most crop monocultures growing in the same field for extended periods of time face severe losses of productivity (Bennett et al. 2012, Zhao et al. 2020). In agricultural settings, this phenomenon is known as 'yield decline' (Bennett et al. 2012). Recently, it has also been observed for non-crop species in several grassland diversity experiments (Guerrero-Ramírez et al. 2017), including the Jena Experiment (Meyer et al. 2016, Dietrich et al. 2020). In these biodiversity experiments, monoculture yield decline is one of the processes promoting an increased positive biodiversity ecosystem functioning relationship through time (Eisenhauer et al. 2012, Meyer et al. 2016, Marquard et al. 2013). One of the major drivers of yield decline is the accumulation of below- and aboveground plant antagonists through time (Bennett et al. 2012, Benitez et al. 2021), which has been well documented in agricultural (Bennett et al. 2012) and experimental plant–soil-feedback studies (Mills and Bever 1998, Maron et al. 2011, Schnitzer et al. 2011, Latz et al. 2012, Kulmatiski et al. 2012, van der Putten et al. 2013, Cortois et al. 2016, Wang et al. 2019). These antagonists include pathogenic fungi, bacteria and protists (Petermann et al. 2008, Hilton et al. 2013, 2021, Xu et al. 2015, Neupane et al. 2021, Li et al. 2022) as well as herbivores, such as plant-feeding nematodes (Jones et al. 2013, Grabau and Chen 2016, Wilschut et al. 2019) and arthropods (Brust and King 1994, Spencer et al. 2014). To counteract the effects of aboveground and belowground antagonists, plants evolved a series of defence strategies (reviewed by Hanley et al. 2007, Raguso et al. 2015, Moore and Johnson 2017). The differences in type and strength of plant defence strategies in leaves and fine roots could be one mechanism to explain the differences in yield decline observed between plant species (Fig. 1a). In plant ecology, defence strategies are generally divided into physical and chemical defences and often characterised using plant functional traits, such as tissue toughness or the presence of toxic compounds (Poorter et al. 2004). Using a trait-based approach, we aim to study the importance of physical and chemical defence traits in leaves and fine roots for yield decline of 4- and 18-year-old monocultures of 27 grassland plant species.

In grassland systems, the effect of belowground antagonists on plant fitness often exceeds aboveground effects (Stanton 1988, Rasmann and Agrawal 2008). Root herbivores, such as root-feeding nematodes and insect chewers are among the most abundant and effective antagonists (Andersen 1987, Ingham and Detling 1990, Zvereva and Kozlov 2012, Johnson et al. 2016b, van den Hoogen et al. 2019) and are often the cause of yield decline for several crop species (Bennett et al. 2012) along with root fungal and bacterial pathogens (Petermann et al. 2008, Hilton et al. 2013, 2021, Xu et al. 2015, Neupane et al. 2021, Li et al. 2022). The importance of root antagonists in long-term monocultures is further amplified by their lower mobility compared to aboveground antagonists, which move more easily between hosts in the canopy (Brown and Gange 1990). In addition, aboveground antagonists are more subject to biotic and abiotic fluctuation (i.e. seasonal changes) that could promote more regular and severe die-off, than their belowground counterparts. In the short term, the reduced mobility of root antagonists decreases the probability of a belowground attack compared to an aboveground one. However, in long-term monocultures, once a suitable host is found, root antagonists likely enforce a stronger and more persistent pressure on the plant compared to aboveground antagonists (Johnson et al. 2016a). Thus, in long-term monocultures, the probability and severity of a belowground attack increases over time, while it may not increases as much aboveground. According to optimal defence theory, allocation to defence depends on the value of the plant tissue, the benefit from defence, and the probability of attack (Stamp 2003). A higher probability and severity of belowground attack should therefore support a higher allocation of resources to root defences to counteract the accumulation of root antagonists. Thus, belowground defences are likely more important than aboveground ones to buffer yield decline promoted by the accumulation of antagonists (Fig. 1c-1). However, the benefit of defence depends on the efficiency of protection against the most important antagonists. Physical defences, such as tissue toughness, are known to be a major defence, especially against chewing root or shoot herbivores (Hanley et al. 2007, Caldwell et al. 2016, Johnson et al. 2016b, Hervé and Erb 2019, Freschet et al. 2021b). Plant physical defences are strongly associated with compounds such as cellulose, lignin or silica (Moore and Johnson 2016). In addition to increasing the mechanical strength of a tissue, these compounds also reduce tissue palatability for herbivores (Cooke et al. 2016, Moore and Johnson 2016). Another strategy to counteract negative effects of belowground antagonists, is to collaborate with mutualists. Along the recently defined root economics space (Bergmann et al. 2020), this is captured by the collaboration gradient. This gradient is defined as a trade-off between specific root length and root diameter, and is positively related to the presence of mycorrhizal fungi. In grassland species, the colonisation of roots by arbuscular mycorrhizae (AMF) is an effective defence against several groups of root antagonists such as insects, nematodes, bacteria and fungi (Dehne 1982, Whipps 2004, Rasmann et al. 2011) due to competition for space and resources, by promoting plant tolerance and by
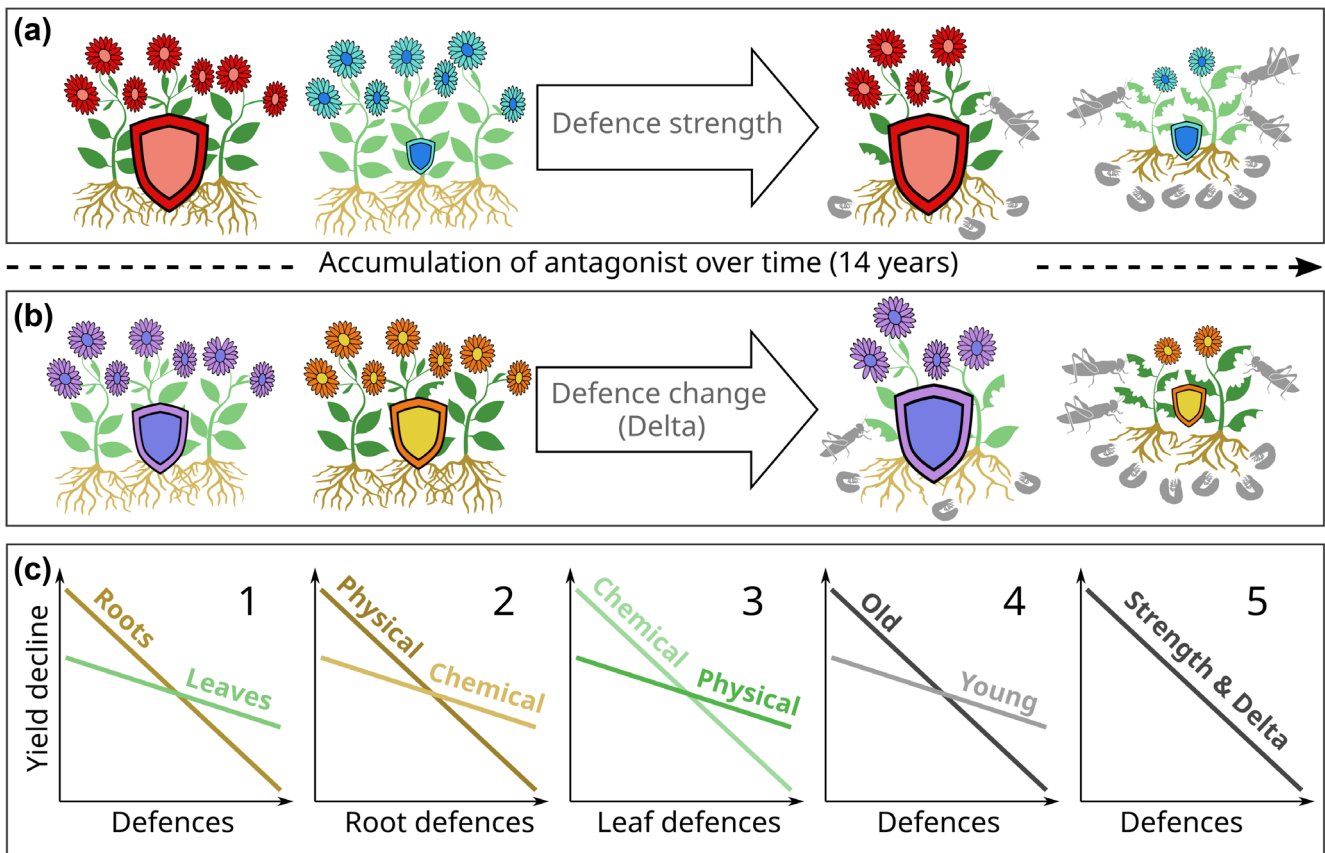
Figure 1. Graphical illustration of antagonist accumulation in response to defence strength (a) and defence temporal changes (delta defences; b) and our five hypotheses (c; from 1 to 5). Flower colour represents species, plant size represents biomass and the size of the shield represents the defence of each species. The number of aboveground and belowground antagonists indicates the overall pressure of antagonists. Plants on the left side are young monocultures (four years) while plants on the right side are old monocultures (18 years) of the same species. (a) Species with higher defence traits have lower yield decline than species with lower defence. (b) Species with a higher increase in defence after 14 years in monoculture, calculated as the difference between defence traits in old and young monoculture, have lower yield decline than species with a decrease in defences after 14 years in monoculture. For details on the hypotheses 1–5 see the main text.

inducing plant defences (reviewed by Frew et al. 2022). In contrast to physical or anatomical defences, chemical defence allows for more diverse and flexible strategies which may be more effective against highly diverse and mobile antagonists with higher temporal and spatial turnover. Given the lower mobility and fluctuation of belowground antagonists, species that invest in belowground physical defence strategies and in collaboration with mutualists, such as AMF, rather than chemical defences, should be able to counteract the accumulation of major belowground antagonists and reduce yield decline in long-term monocultures (Fig. 1c-2).

Aboveground, yield decline has primarily been linked to invertebrate herbivores and leaf spot diseases caused by fungi or protists (Fernandez et al. 1998, Bailey et al. 2001, Jalli et al. 2021). Antagonist accumulation over time is mostly associated with soil- or litter-bound larval or dormant stages (Reavey and Gaston 1991, Johnson et al. 2006, Judelson 2008, Jain et al. 2019). However, during their aboveground life stages, antagonists are more susceptible to environmental fluctuations that could cause antagonist die-off, potentially promoting a quicker turn-over rather than an intense accumulation

and persistence compared to belowground antagonists. In addition, the high mobility of aboveground antagonists increases the likelihood of finding a suitable plant host or changing the host in shorter intervals (Johnson et al. 2016a). Furthermore, aboveground insect herbivores are expected to have a higher species richness and feeding guild diversity than their belowground counterparts (Rasmann and Agrawal 2008). As a consequence, aboveground plant canopies face a more diverse antagonist community than plant roots, and attacks aboveground may be more frequent, but potentially less severe (Rasmann and Agrawal 2008, Zvereva and Kozlov 2012). This calls for a more diverse and flexible defence strategy aboveground. Plants harbour an extremely diverse arsenal of defensive phytochemicals (Wetzel and Whitehead 2020). These can act either directly as toxins or indirectly through the attraction of natural enemies (Raguso et al. 2015), potentially providing a quick and effective defence against the multitude of aboveground antagonists. Whitehead et al. (2021) found that the number of apple antagonist species, including insects and fungi, that are negatively affected by a mixture of phenolics in bio-assays increases with the structural diversity

and richness of phenolics in the mixture. This suggests that phytochemical diversity is an important dimension of plant defence when plants are facing a quick turn-over of multiple antagonist species. Leaf physical defences, effective protection against leaf chewers (Hanley et al. 2007, Loranger et al. 2012, Muiruri et al. 2019), may be less effective to cope with the high diversity and quick turn-over of aboveground antagonists. Thus, unlike belowground where physical defences are effective against major belowground antagonists, aboveground a strategy relying on the diversity of defensive phytochemicals, which are more effective against a high diversity and turnover of aboveground antagonists, seems more promising than physical defences to forestall yield decline in long-term monocultures (Fig. 1c-3).

Plant functional traits provide a quantitative framework that might help to understand the relationship between plant fitness and the environment by quantifying plant morphological, physical, and phenological characteristics (Violle et al. 2007). Plant defence traits are those traits that promote plant fitness in the presence of antagonists relative to when antagonists are absent (Didiano et al. 2014). The type and intensity of defence can vary substantially across species (Moles et al. 2013). Species investing in a high expression of specific defence traits, which are well-suited against dominant antagonists may be able to maintain a high yield in monocultures over time (Fig. 1a). However, plant defence traits show high phenotypic plasticity in response to current selective pressure byantagonists, even within short time frames (i.e. one growing season) (Poorter et al. 2019, Ojha et al. 2022). Given more time, strong selection by antagonists can result in altered plant defence trait expression through microevolution

(Didiano et al. 2014). Indeed, plant species growing either in monoculture or mixture for eleven years showed genetic and epigenetic trait divergence in the Jena Experiment (van Moorsel et al. 2018, 2019). Thus, if the accumulation of antagonists over time is a major selective pressure in monocultures, and an increased level of defence promotes fitness by slowing down the accumulation of antagonists or their negative effect, the relationship between plant defences and yield decline should become stronger with monoculture age (Fig. 1c-4). In addition, plants growing in long-term monocultures should express higher levels of defence traits compared to young monocultures (delta defence, Fig. 1b). Overall, plant species with either a high expression of specific defence traits or species able to increase their defence in response to the accumulation of antagonists over time, should show lower levels of yield decline in long-term monocultures (Fig. 1c-5).

In this study, we measured 20 physical and chemical defence traits (summarised in Table 1) of 27 grassland plant species growing in monocultures for 4 (young monocultures) and 18 years (old monocultures) in the Jena Experiment. For each species, we estimated total above- and below-ground physical and chemical defences by summarizing the 20 individual defence traits with principal component analyses. We used the scores of the most informative principal components as a proxy of species defence strength in old and young monocultures and calculated the delta between old and young monocultures as a measure of temporal change of defence. We estimated yield decline for each species, in the old and young monocultures, using aboveground biomass temporal trends over 18 and 4 years, respectively. We then

Table 1. List of leaf and fine root defence traits selected in this study, their directional effect and role on defence and related references. The symbols '+' and '-' in the column 'Direction' indicate that defences levels are respectively increased or decreased, with higher value of the respective trait. Physical and chemical defences are reported in sperate sections. Abbreviations: LMA=leaf mass per area, N=nitrogen, DMC=dry matter content, Si=silicon content, RD=root diameter and SRL=specific root length, PI=protease inhibitor. *With the term 'palatability' we refer to the nutritional quality of the plant tissue.

| Tissue | Trait | Direction | Mechanisms | References |
|---|---|---|---|---|
| **Physical defences** | | | | |
| Leaf | water repellency | + | surface barrier: reduced attachment and mobility of antagonists | Gorb and Gorb 2017, Hanley et al. 2007 |
| Leaf | hair density | + | | |
| Leaf | hair length | + | | |
| Leaf | LMA | + | palatability* and mechanical strength | Hanley et al. 2007, Johnson et al. 2010, Schuldt et al. 2012, Loranger et al. 2012, Caldwell et al. 2016, Hartley and DeGabriel 2016, Moore and Johnson 2016 |
| Leaf/root | DMC | + | | |
| Leaf/root | N | − | | |
| Leaf/root | cellulose | + | | |
| Leaf/root | Si | + | | |
| Leaf/root | toughness | + | | |
| Root | SRL | − | protection through AMF | Cortois et al. 2016, Johnson et al. 2016b, Frew et al. 2022 |
| Root | RD | + | | |
| **Chemical defences** | | | | |
| Leaf /root | PI (trypsin) | + | toxicity | Johnson et al. 2016b, Moore and Johnson 2016, Whitehead et al. 2021 |
| Leaf/root | features richness | + | | |

used defence strength in the old and young monocultures and delta defences to explain different levels of yield decline across species. We tested the following hypotheses:

1. Fine root defences are a stronger predictor of monoculture yield decline than leaf defences (Fig. 1c-1).
2. Fine root physical defences and mutualistic collaboration are stronger predictors of monoculture yield decline than fine root chemical defences (Fig. 1c-2).
3. Leaf chemical defences are a stronger predictor of monoculture yield decline than leaf physical defences (Fig. 1c-3).
4. The relationship between plant defences and yield decline is stronger in old compared to young monocultures (Fig. 1c-4).
5. Defence strength and temporal changes (difference in defence between old and young monocultures) of fine roots and leaves are both important predictors of yield decline (Fig. 1c-5).

## Material and methods

### Study site and experimental design

The monocultures sampled in this study are part of a large grassland biodiversity experiment, the Jena Experiment. The experiment is located along the Saale River's floodplain near Jena (Thuringia, Germany, 50°57'N, 11°37'E, altitude 130 m a.s.l.). The regional mean annual air temperature is 9.9°C, and annual precipitation is 660 mm (1980–2010) (Hoffmann et al. 2014). In 1960, the experimental site was converted from grassland to a highly-fertilized arable field until the start of the experiment. Sixty species of the Arrhenatherion mesophilic grassland type (Ellenberg 1988) belonging to four functional groups were selected for the experiment. The classification of functional groups was based on above- and belowground functional traits and differentiates grasses (16 species), legumes (12 species), small herbs (12 species), and tall herbs (20 species) (Roscher et al. 2004). For each of the sixty species, two monocultures were established randomly within the four blocks of the experiment in 3.5 × 3.5 m plots. Monocultures were sown in May 2002 using 1000 viable seeds per m². In November 2002, species with no or spare cover were re-sown (Roscher et al. 2004, Heisse et al. 2007). After that, no additional sowing was done. In 2008, one of the two monoculture replicates was abandoned, and in 2009 the plots were reduced to 1 × 1 m. We hereafter refer to these monocultures as 'old monocultures'.

In 2016, additional monoculture plots of 1 m² for all sixty species, hereafter called 'young monocultures', were established randomly within the four blocks of the experiment in soil not previously conditioned by the target species. To reproduce the original soil conditions at the start of the Jena Experiment, the top 30 cm of the soil were removed and replaced with soil from an adjacent field (north of the site). A 30 cm deep plastic sheet barrier was placed around the

plots to avoid contamination of the new soil from the area outside the plot. The young soil had been under the same management regime as the experimental site prior to the start of the Jena experiment. Laboratory analysis of the young soil confirmed that the soil structure, carbon content, and nutrient content closely resemble conditions of the soil in 2002 (Vogel et al. 2019). Seeds from the same supplier as in 2002, were sown in the young soil using the same approach used for the old monocultures in 2002.

Both, old and young monocultures, were maintained by weeding of non-target species two to three times per year in spring, summer, and autumn (Weisser et al. 2017). Plots were mowed in June and September every year, and the biomass removed to simulate the common hay meadow management of the region.

At the time of sampling for this study, in May 2020, the old monocultures were 18 years old while the young monocultures were 4 years old and thus 14 years younger than the old monocultures. After four years, plant soil-feedback effects and monoculture yield decline in the young monocultures could already be in place, however the short duration of this effect may not have promoted as strong responses in plant functional traits as in the 18-year-old monocultures. The comparison between old and young monocultures allows us to investigate the effect of time on plant–soil feedback effects and yield decline. The advantage of this experimental design is that our analysis is independent of different climatic conditions between years.

### Yield decline

We estimated yield decline in 1) the old and 2) the young monocultures over the full period, 18 and 4 years, respectively, as well as in 3) the last 14 years of the old monocultures. This last measure of yield decline is used to test the effect of temporal changes in defences on yield decline (hypothesis 5) while taking into account that the young monocultures were already four years old at the time of sampling. To estimate yield decline in the old monocultures (1 and 3), we used the annual aboveground biomass in the period from 2003 to 2020. Within this period, aboveground biomass was measured twice a year: end of May and end of August. From 2003–2009 two biomass samples were harvested using a 0.2 × 0.5 m frame in a random position within the central part of each plot (excluding 0.5 m margin). From 2010–2020, one biomass sample was collected with the same frame and only if no individual of the target species was present within the frame the harvest area was doubled. Plants were cut at 3 cm above the ground, and the harvested material was dried at 70°C for 48 h before weighing. The annual aboveground biomass was calculated as the sum of the biomass of the two harvests per year extrapolated to 1 m². To estimate yield decline in the young monocultures we used the annual aboveground biomass in the period from 2017 to 2020 collected as described above.

We estimated yield decline with species-specific linear regressions using scaled plant annual aboveground biomass as response variable and the natural logarithm (ln) of year

since the start of the experiment as explanatory variable. Aboveground biomass scaling was done separately for the old and young monocultures by dividing the annual aboveground biomass of each species by the mean annual aboveground biomass of that species over the full period in each monoculture type. The 'scaled annual aboveground biomass' accounted for differences in plant biomass across species. Without the scaling, linear regression slopes would be primarily influenced by species mean biomass. With the scaling, the slope is expressed as unit distance to the mean of species biomass, which allows for comparison across species. We used the natural logarithm of year since the start of each experiment to account for a stronger biomass decline in the first years of each experiment. Scatterplots of linear regressions of the old (full and selected period) and young monocultures for the sampled species are shown in the Supporting information. The slopes of those regressions were multiplied by '−1' and are hereafter called yield decline: scaled aboveground biomass ~ − Yield decline × ln(year) + $b$. This was done to transform negative slope values into positive numbers so that high values indicate species with high yield decline (more negative slopes), simplifying the interpretation of the results. Monocultures of all sixty species of the Jena Experiment, except *Ajuga reptans*, showed yield decline after 18 years. Due to extinction or low cover of some old or young monocultures, only twenty-seven full species pairs with viable old and young monocultures out of the sixty species of the Jena Experiment could be included in this study. The distribution of yield declines for the sampled species in the old monocultures does not represent the yield decline distribution for all the 60 species (data not shown): the extinction of several species led to a strongly skewed yield decline for all sixty species, with the majority of the species undergoing stronger yield decline than the sampled species. Thus, our sample represents a conservative estimate of potential effects of yield decline. Among the sampled species, the extent of yield decline varies substantially and is independent of plant functional group identity for the old monoculture over the full period ($F_{3,23} = 0.53$, $p = 0.77$; Fig. 2), as well as for the old monoculture over the selected period ($F_{3,23} = 1.27$, $p = 0.31$), and for the young monoculture ($F_{3,22} = 0.05$, $p = 0.98$). In the young monoculture all sample species experience yield decline except two species, *Plantago lanceolata* and *Sanguisorba officinalis*, with a pronounced increase in biomass over time. Those trends are due to the poor establishment of those two species in the first and second year of the experiment. In addition, *Bellis perennis* did not establish well in the young monoculture and did not reach the 3 cm height of the frame used for the biomass collection. Therefore, the yield decline of this species could not be quantified and the species was excluded from further analysis on the young monocultures.

## Leaf and fine root sampling

The sampling campaign was conducted from 18 May to 5 June 2020, after the plots were weeded. Sampling was restricted to the morning from 7:00 to 11:00 h to minimize chemical trait shifts during the day. Twenty-seven species were sampled
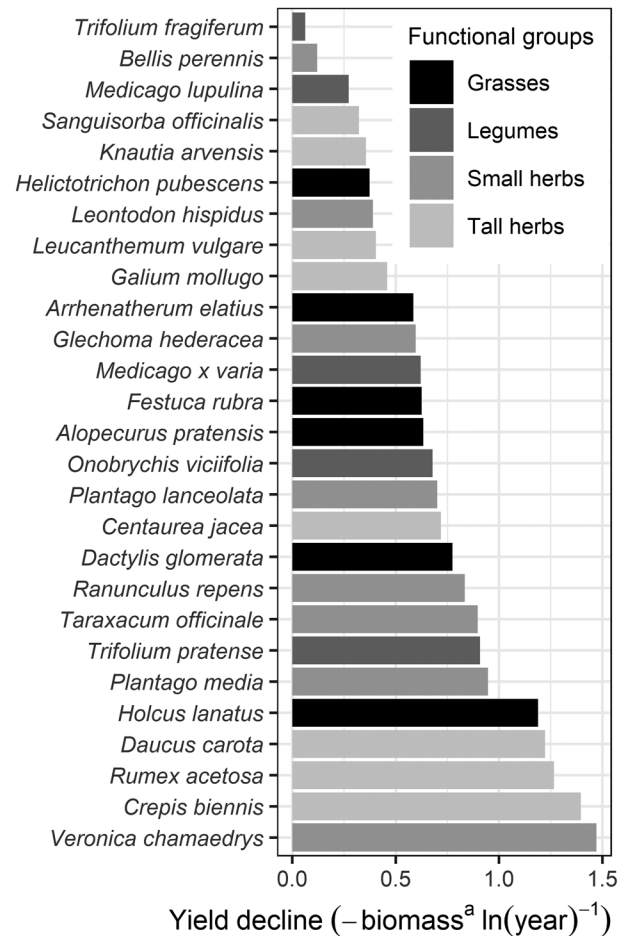


Figure 2. The extent of yield decline for the sampled species in old monocultures over the full period. Yield decline is expressed as the slope of a linear regression with scaled aboveground plant biomass as response variable and the natural logarithm (ln) of year as explanatory variable. Biomass scaling ([a]) was done by dividing species annual biomass by the species mean biomass in the period 2003–2020. Slopes were multiplied by '-1', so that higher values depict higher yield decline. For each species a separate linear regression was constructed using old monocultures' data from 2003 to 2020 (year of trait measurement). Shades of grey depict different plant functional groups.

in both monoculture types (young and old) for a total of 54 plots. In each of the monocultures, we sampled the above- and below-ground part of 3–5 individuals to account for intraspecific trait variation. We first harvested the aboveground plant part by cutting the stem 1–2 cm above the ground. Each plant individual was stored in a separate, sealed plastic bag with a wet paper towel to ensure leaves were rehydrated to full potential before trait measurements (Pérez-Harguindeguy et al. 2013). We then sampled the roots of each individual by collecting a 5 × 10 cm (diameter × depth) soil core with the remaining part of the stem in the centre of the core. The cores of individual root systems were stored together in a sealed plastic bag. All sampled material was stored in a dark cooling box. Samples were stored at 4°C in the lab for a maximum of 6 h after sampling. Sample processing started 6 h after the collection of the

first sample and ended within 26 h. Above- and below-ground samples were processed in parallel.

## Measurements of leaf morphological traits and leaf antagonists damage

All fully-expanded and undamaged leaves of each individual were separated from the rest of the aboveground portion of the plant, and rachis and petioles were removed. One or a few leaves (depending on leaf size) attached between the 3rd and 5th internode from the top of each individual were processed separately. For grasses without flowering stems, this was not possible, and random leaves were taken instead. The rest of the leaves were pooled at the plot level and used to measure the fresh weight and leaf area with a flatbed Epson Expression 11000XL scanner at 600 dpi resolution. Leaves were then frozen in liquid nitrogen and stored at −80°C until the end of the sampling campaign. Leaf dry weight was measured from freeze-dried samples. We calculated leaf mass per area (LMA; g m$^{-2}$) as dry weight divided by the leaf area and leaf dry matter content (LDMC; g g$^{-1}$) as the dry weight divided by the fresh weight (Pérez-Harguindeguy et al. 2013). We measured leaf damage (%) caused by antagonists as the proportion of damaged leaf area (damaged or infested leaf area/ undamaged leaf area) using leaf scans in ImageJ ver. 1.53a (Schneider et al. 2012). The proportion of leaf damage was estimated separately for chewers, miners and raspers, and pathogen infestation (leaf spot and rust diseases). Due to difficulties in differentiating damage caused by miners and raspers, the two categories were grouped together (Meyer et al. 2017). To estimate the undamaged area, we summed the leaf area from the scan with the leaf area lost due to chewing damage.

The separated leaves from each individual were used to measure leaf water repellency, hair density, and mean hair length as well as leaf toughness. We measured those traits on one leaf per individual in the widest part of the lamina between the main vein and the leaf edge.

We assessed water repellency (WR; deg.) as a proxy for epicuticular waxes by measuring the left and right contact angle of a 10 or 5 μl water droplet on the leaf adaxial and abaxial surface of one leaf per individual (Pérez-Harguindeguy et al. 2013; for additional details see the Supporting information). All values (left and right, adaxial and abaxial, and individuals) were averaged at the plot level. High contact angle values and thus high water repellency is associated with crystalline waxes (Barthlott and Neinhuis 1997), which are known to reduce attachment of plant antagonists to the leaf surface (Gorb and Gorb 2017).

To measure leaf hair density and mean hair length, we collected images of the adaxial and abaxial surface using a dissecting microscope equipped with a camera at 4.5× magnification (Di-Li 2009-16). To keep the leaf flat during the collection of images, we gently pressed a microscope slide on the top of the leaf. We used ImageJ ver. 1.53a (Schneider et al. 2012) to count all the hairs within the image frame, measured the length of ten random hairs, and calculated the area

of the leaf image. Hair density was calculated as the number of hairs divided by the leaf area (no. of hairs mm$^{-2}$) and the hair length as the mean of the 10 measurements (mm). All values (adaxial and abaxial, and individuals) were averaged at the plot level.

We measured leaf toughness on each leaf with the shearing test (Pérez-Harguindeguy et al. 2013). Leaves were mounted on a motorized vertical test stand equipped with a Sauter FH 50 dynamometer and a surgical blade type 24. The motorized vertical test stand was operated at a constant speed of 15 mm min$^{-1}$. One cut per leaf was done perpendicular to the main vein and towards the edge of the leaf avoiding the main vein. The maximum force registered was recorded and divided by the thickness measured with a digital calliper at the side of the cut. Leaf toughness was calculated as the ratio of the maximum force to shear to the thickness (N mm$^{-1}$), and values were averaged at the plot level.

## Fine root morphological traits and root mycorrhizal colonisation

We washed roots from the soil by soaking soil cores in cold water for 15 min. We then removed the soil by gently massaging the core inside a bucket filled with water to avoid the rupture of roots. We refreshed the water in the bucket by filtering the water with soil debris into a sieve and collected fine root fragments. We repeated this procedure until the roots were completely free of soil particles. Only fine roots attached to the stem of the correct species or large fine root fragments that were unequivocally identified as being from the same species using dissecting microscopes were kept for further processing. We bulked the fine roots of each individual at the plot level and discarded all coarse roots with a diameter larger than 2 mm. Fine roots with a diameter lower than 2 mm were separated into three random subsamples: 1) one subsample was used to measure morphological traits, 2) a second subsample was stored in 75% ethanol at 4°C for the quantification of arbuscular mycorrhizal (AMF) colonisation rate (Freschet et al. 2021a), 3) the remaining fine roots were frozen in liquid nitrogen and stored at −80°C to be used for chemical analyses.

For the morphological trait measurements, we scanned fine roots (flatbed Epson Expression 11000XL) at 600 dpi and measured the fresh weight after carefully drying the roots with a paper towel. We then dried the scanned fine roots for 48 h at 70°C. We used WINRHIZO (Regent Instruments) to retrieve root length and mean root diameter (RD; mm). We calculated specific root length (SRL; m g$^{-1}$) by dividing root length by the root dry weight and root dry matter content (RDMC; g g$^{-1}$) by diving the dry weight by the fresh weight (Freschet et al. 2021a). We measured root toughness on five random root fragments with the shearing test using a similar approach as for leaves. Root fragments were cut perpendicular to the length, and root thickness was measured at the edge of the cut. Root toughness was calculated as the ratio of the maximum force to shear to the thickness (N mm$^{-1}$), and values were averaged at the plot level. We additionally

measured AMF colonisation rate as a proxy of plant mutualist collaboration using the method developed by Trouvelot et al. (1986); additional details on the measurement of AMF colonisation rate can be found in the Supporting information.

## Leaf and fine root chemical analyses and untargeted metabolomics

We freeze-dried and ground the samples for chemical analyses with a zirconium kit in a ball mill (MM400, Retsch). To avoid overheating, samples were shaken at 30 Hz for 1 min and cooled at −20°C for 1 or 2 min. The procedure was repeated until the samples were reduced to powder. The samples were then frozen at –80°C and freeze-dried once again before further measurements.

We measured leaf and root nitrogen content (N, % of dry weight) on 10 mg of each sample with an elemental analyser (VarioEL II, Elementar), at the RoMA laboratory of the Max Planck Institute for Biogeochemistry in Jena, Germany. We quantified cellulose content (% of dry weight) on 10 mg of sample by sulfuric acid digestion and anthrone solution dye (Viles and Silverman 1949), with a spectrophotometer (V730, Jasco) at 630 nm (for additional details see the Supporting information). Due to limitations in sample material, N (24% of samples, 5 leaf and 17 fine root samples) and cellulose content (14% of samples, 5 leaf and 10 fine root samples) were predicted using near-infrared spectra measured with a Multi-Purpose FT-NIR-Analyzer (MPA, Bruker Corporation) coupled with a bootstrapped CARS-PLSR models procedure calibrated with the rest of the data. This was done following the procedure developed by Elle et al. (2019) with minor modifications as described in Volf et al. (2022). Model validation statistics confirmed the high accuracy of both models ($R^2 = 98\%$ for nitrogen content and $R^2 = 75\%$ for cellulose content). A detailed description of the procedure and validation statistics is reported in the Supporting information.

We extracted silicon (Si; % of dry weight) by adding 30 ml of alkaline solution of 0.1 M $Na_2CO_3$ to 30 mg of sample material. The sample was incubated in a water bath at 85°C for 5 h and shaken every 30 min (Katz et al. 2021). We filtered the extract with a 0.45 μm syringe filter and analysed the extract with an ICP-OES (IRIS Intrepid II XSP, Thermo Fischer Scientific).

We measured protease inhibitor activity against trypsin (nmol/mg; nmol inhibited trypsin per mg of extracted protein) using the radial diffusion assay as described in Jongsma et al. (1993, 1994). Protein extracts from 10 mg of sample material were tested for trypsin-inhibiting activity in gel diffusion assays stained with Fast Blue B salt (scbt) and N-acetyl-DL-phenylalanine-beta-naphthyl ester (APNE; Sigma-Aldrich). The full description of the method is provided in the Supporting information.

We measured phytochemical diversity using an untargeted metabolome analysis by calculating the feature richness (number of features; i.e. the numbers of mass signal of putative secondary metabolites) in each sample. While

secondary metabolites have many functions in plants such as pollination, response to abiotic or biotic stress or allelopathy, a higher phytochemical diversity, generally promotes defence against a larger pool of antagonists (Whitehead et al. 2021). Polar metabolites were extracted using methanol (75% v/v) and water acetate buffer (25% v/v) extraction. The untargeted metabolome analysis was performed using an ESI-UHR-Q-ToF-MS (maXis impact, Bruker Daltonics) in positive mode, following the procedure described in Weinhold et al. (2022) with some minor modifications. The full description of the method is reported in the Supporting information. The raw data were processed in Bruker Compass MetaboScape Mass Spectrometry Software ver. 5.0.0 (Build 683; Bruker Daltonics). The MetaboScape's T-ReX algorithm was used to perform mass recalibration, peak alignment, peak picking, region complete feature extraction, grouping of isotopes, and adduct and charge states (all settings are reported in the Supporting information). After features from blanks (2149) were removed, our final data matrix contained 16 330 features and was used to calculate the number of features in each sample.

## Soil available phosphorus and total nitrogen measurement

To evaluate the role of nutrient depletion on yield decline we measured soil available phosphorus in the rhizosphere soil of the sampled species and soil total nitrogen in the bulk soil. Rhizosphere soil was collected from the same core used for the root sampling. Prior to root washing, we first removed the non-root associated soil by gently shaking the cores, and discarded the loose soil. We then collected the rhizosphere soil attached to the roots of the target species with clean tweezers and a spoon. The rhizosphere of each of the 3–5 cores was pooled and frozen at −20°C. The soil was thawed and homogenized by thorough mixing with a clean spatula. Afterwards, the soil was frozen at −20°C again, freeze-dried and ball-milled. Soil available phosphorus was measured with the calcium-acetate-lactate extract (PCAL) method according to Schüller (1969) from 1 g of dry soil.

For soil total nitrogen measurements, in each plot we collected and pooled three soil cores of 2 × 10 cm (diameter × depth). Soil cores were quickly stored in a cooling box and frozen at −20°C upon arrival to the laboratory. The soil was than thawed, homogenized by sieving (2 mm mesh size), dried at 40°C and ball milled. Soil total nitrogen was measured with an elemental analyzer at 1150°C (Elementaranalysator vario Max CN).

## Missing value imputation and variable reduction (PCA)

To avoid missing values in our trait data matrix due to limitation of sample material (Si) and errors during the measurements of some sample (WR, SRL, RDMC, N and features richness), we imputed those missing values with a phylogenetically informed *missForest* algorithm 'missForest' R package

ver. 1.4 (www.r-project.org, Stekhoven and Bühlmann 2012) as those traits could not be well predicted with the NIR procedure. Except for the Si dataset, with 12% of missing data points, the remaining traits had only 1–3 missing data points (overview of missing data points is shown in the Supporting information). Prior to the imputation, we added the first three phylogenetic eigenvectors to the full trait matrix (11 leaf and 9 fine root traits) as described in Debastiani et al. (2021). We obtained the phylogenetic tree (Supporting information) with the 'V.Phylomarker' R package and the 'GBOTB. extended.tree' as backbone (ver. 0.1.0; Jin and Qian 2019).

We summarised plant defence traits in both the old and young monocultures by running one principal component analysis (PCA) for leaves and one for fine roots, with the data of both monocultures combined. We additionally performed a PCA for the old and young monocultures separately, to evaluate a possible bias of the combined PCA used in our further analyses. To increase interpretability of the fine root trait PCA, we applied a varimax rotation, so that traits with the highest loading lay parallel to the rotated components (R package 'psych' 2.2.3, Revelle 2022). The full list of traits included in the PCAs and their roles in plant defence is reported in Table 1. For further analysis we used the scores, hereafter called 'defence strength', of the old and young monocultures extracted from the first two principal components (PCs) of the leaf defence traits PCA and the first two rotated components (RCs) of the fine root defence traits varimax rotated PCA. We additionally calculated the difference between the scores of the old and young monocultures using the PCAs calculated with the data of both monocultures combined, hereafter called 'delta defence'. Delta defence was used as a proxy of temporal change in defence response between 18- and 4-year-old monocultures. Positive values of delta defences indicate an increase, while negative values indicate a reduction along the components.

### Statistical analysis

All statistical analyses were performed in R ver. 4.3.0 (www.r-project.org). We validated the effect of the two leaf trait PCA components as defence by testing the effect of the two leaf defence components on foliar damage caused by chewers, miners and raspers, and pathogen infestation. Similarly, we examined how root defence traits influenced mutualists and antagonists, by testing the effect of the two varimax rotated principal components of fine root traits on AMF colonisation rate and abundance of root-feeding nematodes, respectively. Note that the abundance of root-feeding nematodes was collected in 2014 in the old monocultures (previously published in Dietrich et al. 2020). In this case, we used only the RC scores of the old monocultures, as nematode data for the new monoculture was not available. To meet linear model assumptions, chewer, miner and rasper damage, pathogen infestation and root-feeding nematode abundance were transformed with the natural logarithm. A pseudo-count of 0.1 was added prior to the log transformation (except for root-feeding nematode abundance). The effects of plant defences on foliar and

root antagonists and on root mutualists were tested separately for old and young monocultures, using linear mixed models with the experimental block as random intercept using the scores extracted from the PCAs calculated with the data of both monocultures combined.

We tested the effect of plant defences on yield decline in the old and young monocultures separately, using linear mixed models with experimental block as random intercept and assessed significance levels with ANOVA type II sum-of-squares ('lmerTest', Kuznetsova et al. 2017). Models were performed using the scores extracted from the PCAs calculated with the data of both monocultures combined as well as the PCAs calculated separately for the old and young monocultures.

We tested the effect of delta defences (temporal change) on yield decline of the last 14 years in the old monocultures using multiple linear regressions and assessed significance levels with ANOVA type II sum-of-squares ('car' R package ver. 3.0-12, Fox and Weisberg 2019).

Given the strong link between the collaboration gradient and AMF (Bergmann et al. 2020) we additionally tested if the potential effect of the collaboration gradient on yield decline is mediated by AMF, using linear mixed models with yield decline as response variable and the AMF colonisation rate as independent variable. We additionally tested if the potential effect of root trait gradients or AMF on yield decline is driven by their role on nutrient uptake rather than protection against antagonists. This was done using linear mixed models with yield decline as response variable and soil phosphorus availability or soil total nitrogen as independent variable. For both AMF colonisation rate and soil nutrients we constructed separate models for the old and the young monocultures using the scores extracted from the PCAs calculated with the data of both monocultures combined. Experimental block was included as random intercept.

## Results

### Relationships between leaf defences and antagonists

The first and second component of the leaf trait PCA based on old and young monocultures combined, explained 35 and 18% of the variation in leaf traits, respectively (Fig. 3, Supporting information). The first component was characterised by a tradeoff between physical (toughness and leaf dry matter, cellulose and silicon content) and mostly chemical defences (leaf feature richness but partly also hair length), hereafter referred to as 'leaf physical versus chemical defence tradeoff'. This first component was positively correlated with foliar damage caused by chewers (marginal $R^2 = 24$ %, $p = 0.0077$; Fig. 4, Supporting information) as well as raspers and miners (non-significant) and negatively to damage caused by pathogen infestation (marginal $R^2 = 29\%$, $p = 0.0033$; Fig. 4, Supporting information) in the young monocultures. In the old monocultures, we found similar, but weaker, trends with only marginal significance for foliar
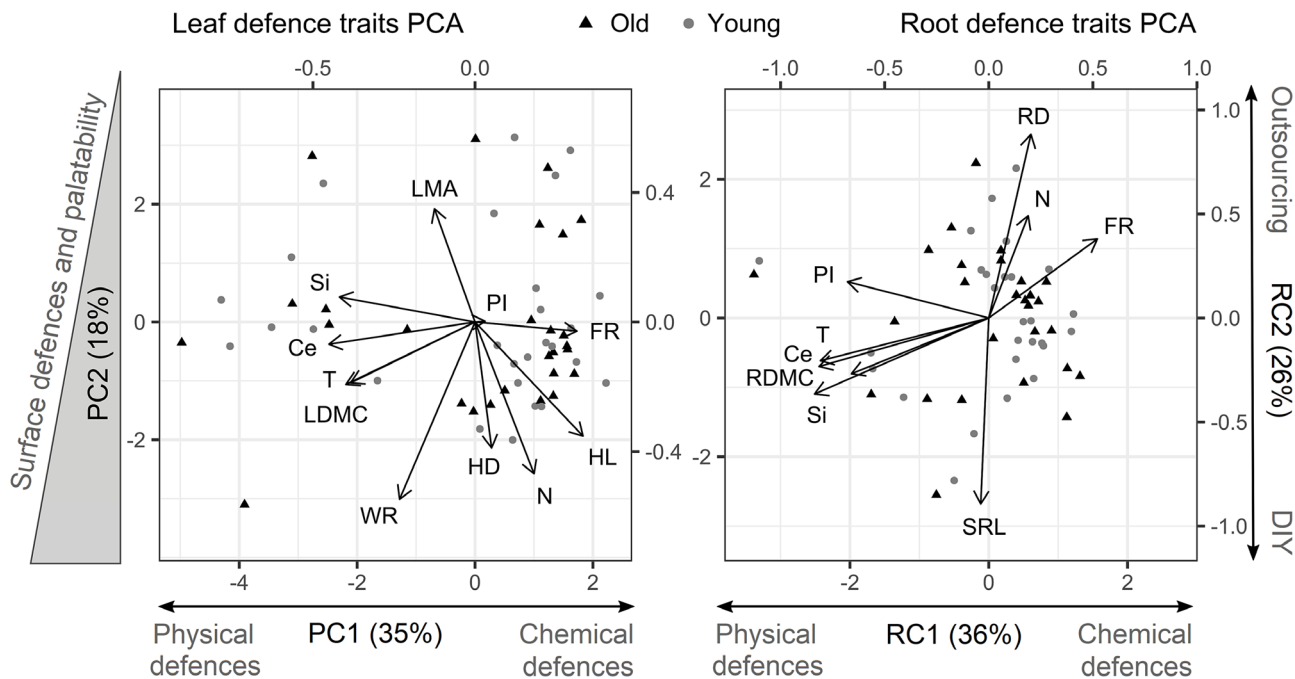
Figure 3. Biplot of the first two components of the leaf trait PCA (on the left) and the root trait varimax rotated PCA (on the right) for the old (black triangles) and young (grey circles) monocultures. Variation explained by each component is reported on axis labels. Note that we applied a varimax rotation to the root PCA and refer to these components as rotated component (RC) rather than principal component (PC). Axes scales on the left and bottom refer to the scores while scales on the right and top refer to the loadings. Abbreviations: LMA = leaf mass per area, FR = feature richness, HL = hair length, N = nitrogen content, HD = hair density, PI = protease inhibitor, WR = water repellency, LDMC = leaf dry matter content, RDMC = root dry matter content, T = toughness, Ce = cellulose content, Si = silicon content, RD = root diameter, SRL = specific root length.

damage caused by chewers (marginal $R^2 = 10\%$, $p = 0.0933$; Fig. 4, Supporting information) and by pathogen infestation (marginal $R^2 = 11\%$, $p = 0.0897$; Fig. 4, Supporting information). Thus, leaves with high leaf toughness and silicon, cellulose and dry matter content and with low feature richness were less damaged by chewers, but had higher pathogen infestation, but this effect of plant defences on foliar antagonist is less pronounced in the old monocultures. The second component was characterised by a negative correlation between

leaf mass per area (LMA) and leaf surface defence defined by leaf N, hair density and length, and water repellency. We named this second component 'leaf surface defence and palatability'. The leaf damage caused by raspers and miners along this component was slightly higher for plant species with low palatability (high LMA and low leaf nitrogen content) and lower for plant species with high surface defence (high hair length and density and water repellency) in young monocultures (marginal $R^2 = 14\%$, $p = 0.0461$; Fig. 4, Supporting
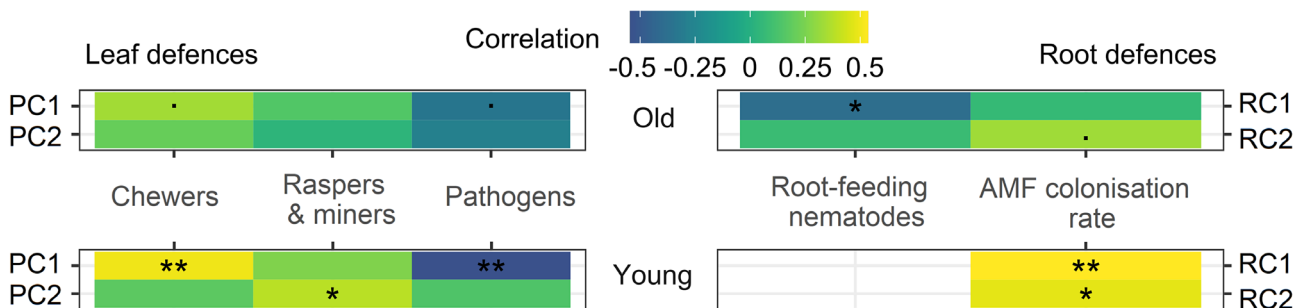


Figure 4. Correlation (Pearson's $r$) heatmap for the first two components of the leaf PCA and leaf foliar damage caused by three major classes of leaf antagonists (on the left) and the first two components of the root PCA and AMF colonisation rate and abundance of root-feeding nematodes (on the right) for the old (top) and the young (bottom) monocultures. Foliar damage (%) variables and root-feeding nematodes abundance (individual $g^{-1}$ of soil) were log transformed (natural base) For foliar damage variables a pseudo-count of 0.1 was added before the log-transformation. Note that data on root-feeding nematodes was measured six years before the current study only in the old monoculture. Significance levels are reported with asterisks or dots: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; . $p < 0.1$.

information) but not in old monocultures. Comparing these PCA results based on old and young monocultures combined to separate PCAs for only young or only old monocultures revealed very similar patterns (Supporting information).

## Relationships between root defences and antagonists and mutualists

The varimax rotated root-trait PCA based on old and young monocultures combined, explained 36 and 26% of the variation in fine root traits by the first and second component, respectively (Fig. 3, Supporting information). Comparable to the leaf PCA, the first component of the fine root PCA showed a tradeoff between physical and chemical defences, hereafter referred to as 'root physical versus chemical defence tradeoff': species with high fine root toughness, dry matter, silicon and cellulose content (but also high proteinase inhibitors) had lower feature richness. This component was also correlated to the first component of the leaf PCA (Pearson's $r = 0.704$; Supporting information). The root physical versus chemical defence tradeoff was negatively correlated with the abundance of root-feeding nematodes measured in 2014 (marginal $R^2 = 15\%$, $p = 0.0435$; Fig. 4, Supporting information) in the old monocultures, and positively with AMF colonisation rate, as measured in this study in the young monocultures (marginal $R^2 = 28\%$, $p = 0.004$; Fig. 4, Supporting information). Thus, the abundance of root feeding nematodes in 2014 was lower for species with high fine root physical defences and lower for fine roots with high feature richness. On the other hand, the abundance of AMF was higher in species with high fine root feature richness and lower in fine roots with high physical defences, but only in the young monocultures. The second component of the root PCA showed the 'collaboration gradient' of the recently defined root economics space (Bergmann et al. 2020) with a negative correlation between root diameter (RD) and specific root length (SRL) and was slightly correlated to the first component of the leaf PCA (Pearson's $r = 0.493$; Supporting information). This component was significantly positively correlated with AMF colonisation rate in the young monocultures (marginal $R^2 = 22\%$, $p = 0.0101$; Fig. 4, Supporting

information) and only marginally in the old monocultures (marginal $R^2 = 11\%$, $p = 0.093$; Fig. 4, Supporting information). Thus, in line with the root economics space, outsourcing species with high fine root diameter and low specific root length had higher AMF colonisation rates than DIY species (Bergmann et al. 2020). Comparing these PCA results based on old and young monocultures combined to separate PCAs for only young or only old monocultures revealed again similar patterns, albeit somewhat less consistent than for the leaf traits comparison (Supporting information).

## Effects of plant defence strength and its temporal change on yield decline

Testing the effect of plant defences of the four main PCA axes of leaf and fine root defence traits on yield decline in the old monocultures revealed significantly negative effects for the root collaboration gradient (Table 2, Fig. 5) and a significantly positive effect of the leaf physical versus chemical defence tradeoff (Table 2). In the young monocultures, none of the plant defence trait axes had a significant effect on yield decline (Table 2). These results were irrespective of the PCA from which the scores were extracted (Supporting information). The negative effect of the collaboration gradient on yield decline in the young monocultures indicates that species on the outsourcing side of the root economics space, and thus with high fine root diameter and low specific root length, experienced lower yield decline than species on the DIY side of the root economics space. The positive effect of the leaf physical versus chemical defence tradeoff on yield decline in the young monocultures indicates that species with high physical defences experienced lower yield decline than species with high chemical defences. However, the effect of the leaf physical versus chemical defence tradeoff on yield decline was weaker than the one of the collaboration gradient with a coefficient of 0.125 (95% CI = 0.022, 0.229; p-value = 0.037) and −0.320 (95% CI = −0.478, −0.167; p-value = 0.001), respectively.

Testing the effect of a change in defences (delta defences) on yield decline in the last 14 years of the old monocultures

Table 2. ANOVA table based on type II sum of squares for the linear mixed model with yield decline as response variable and plant defences as explanatory variables in the old and young monocultures (modelled separately) and the linear model with yield decline of the last 14 years in the old monocultures as response variable and plant delta defences (temporal changes) as explanatory variables. The table reports beta coefficient (Estimate), F statistic (F) and the marginal and conditional $R^2$ for the mixed linear models and the $R^2$ and adjusted $R^2$ for the linear model. Significance levels are reported with asterisks: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

| Explanatory variable | Estimate | | | F | | |
|---|---|---|---|---|---|---|
| | Old | Young | Delta | Old | Young | Delta |
| (Intercept) | 0.696 | 0.702 | 0.63 | – | – | – |
| Leaf defences | | | | | | |
| Physical versus chemical defences tradeoff (PC1) | **0.125** | 0.025 | −0.003 | **4.96*** | 0.02 | 0.00 |
| Surface defence and palatability (PC2) | 0.014 | 0.047 | −0.132 | 0.11 | 0.11 | 0.95 |
| Root defences | | | | | | |
| Physical versus chemical defences tradeoff (RC1) | −0.098 | −0.012 | −0.401 | 1.02 | 0.00 | 1.33 |
| Collaboration gradient (RC2) | **−0.32** | 0.045 | **−0.241** | **14.58***** | 0.03 | **5.7*** |
| Marginal $R^2$; Condition $R^2$ (%) | 38; 46 | 2; 2 | – | | | |
| $R^2$; Adjusted $R^2$ (%) | – | – | 22; 8 | | | |
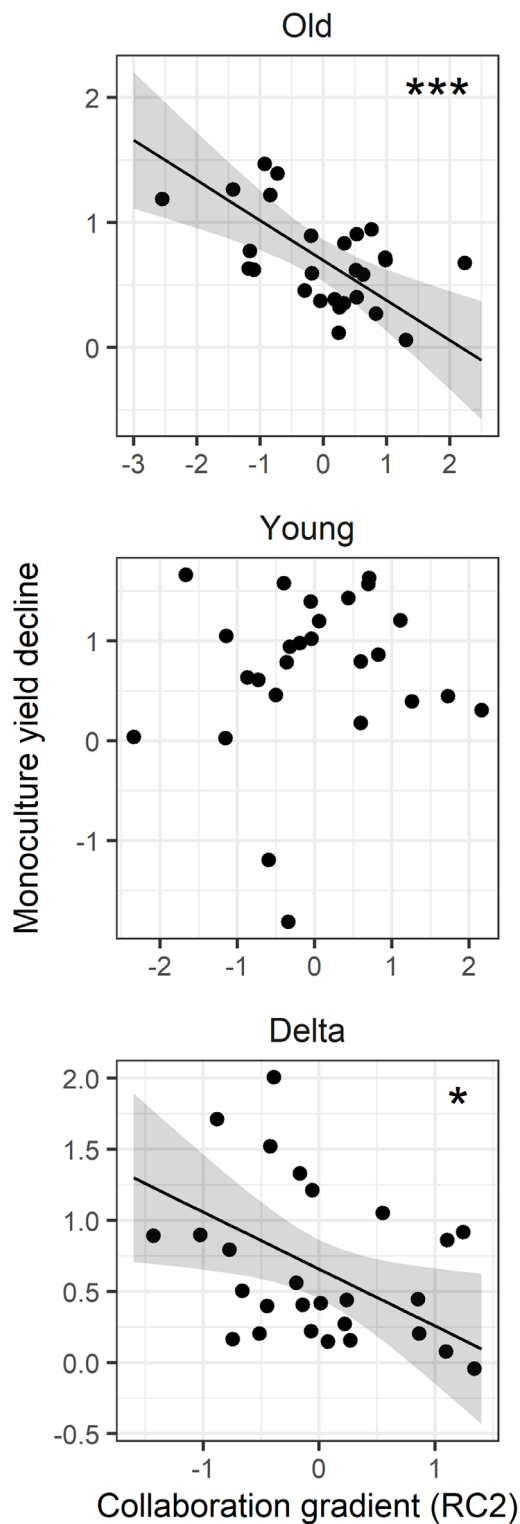
Figure 5. Scatterplots of yield decline against the collaboration gradient in the old and young monocultures as well as yield decline in the last 14 year of the old monocultures against delta collaboration gradient (temporal changes). Significant slopes and 95% confidence intervals are reported as solid line and grey band. Significance levels are reported at the top right corner with asterisks: ***p < 0.001; ** p < 0.01; * p < 0.05.

revealed a significantly negative effect of the delta collaboration gradient on yield decline with a coefficient of −0.241 [95% CI −0.478, −0.167; p-value = 0.026] (Table 2, Fig. 5). The negative effect of the delta collaboration gradient on yield decline, indicates that, under long-term selective pressure in monocultures, species that increased fine root diameter and at the same time reduced specific root length, experienced lower yield decline than species that reduced fine root diameter and increased specific root length. In addition, old and young monocultures were not significantly different along the four main PCA axes (Supporting information), suggesting that plants growing in monoculture for longer periods of time do not change their traits in the same direction, but rather that trait changes are species specific.

Our results further showed that AMF colonisation rate, soil available phosphorus and soil total nitrogen had no effect on yield decline in both the old and young monocultures (Supporting information), suggesting that AMF and nutrient depletion do not have a direct effect on yield decline differences among species in neither the short nor long term.

## Discussion

In this study, we investigated the predictive power of a comprehensive set of 20 physical and chemical defence traits of leaves and fine roots on monoculture yield decline of 27 grassland plant species. Our aim was to compare the effects of differing aboveground versus belowground defence strategies and their changes through time on yield decline, in young (4 years) and old (18 years) monocultures, using principal components of leaf and root traits. Our results revealed that none of the expected leaf and root defence trait gradients were significant predictors of yield decline in young monocultures. Instead, leaf traits defining the physical versus chemical defence tradeoff did significantly predict yield decline in old monocultures. However, fine root anatomical traits defining the root collaboration gradient of the root economics space, as well as their change over 14 years of selection in a monoculture, were the strongest predictors of yield decline in the old monocultures, highlighting the importance of belowground mechanisms in this grassland system.

### Yield decline response to the collaboration gradient and its temporal changes

The key results of our study thus support our first and fourth hypothesis that plant root traits should be stronger predictors of monoculture yield decline than leaf traits and that plant traits are better predictors of yield decline in long- rather than in short-term monocultures. In addition, our results support our fifth hypothesis that both differences in defence strength and their temporal changes under long-term selective pressure in monocultures, as indicated by the strength and delta parameters of the root collaboration gradient, were important predictors of monoculture yield decline in 18-year-old monocultures. We were able to show that plant species with

low specific root lengths and high root diameters, and thus species on the 'outsourcing' side of the root collaboration gradient of the root economics space, experienced substantially lower monoculture yield decline over 18 years than species on the 'do-it-yourself' (DIY) side of the gradient.

Additionally, we could show that not only the expression of specific root length and root diameter was important, but also their temporal changes under long-term selective pressure in monocultures: species that increased root diameter and reduced specific root length over time (delta collaboration gradient), experienced yield decline to a similar extent as species that were on the outsourcing side of the collaboration gradient in the first place (old and delta collaboration, Fig. 5). Despite the fact that not all species changed their traits in the same direction, these species-specific shifts along the collaboration gradient highlight that long-term monoculture growth exerts a strong selective pressure against DIY species. These trait temporal changes may be due to phenotypic plasticity (Ojha et al. 2022). Alternatively, the genetic and epigenetic trait divergence previously found in the same monocultures of this study (van Moorsel et al. 2018, 2019) suggest that the shift along the collaboration gradient may be partially due to plant microevolution in response to belowground processes, such as a potential accumulation of root antagonists (Didiano et al. 2014). Moreover, the weaker link between leaf defence gradients and yield decline compared to root defence gradients, suggests that belowground antagonists or other belowground processes are more important drivers of monoculture yield decline than aboveground processes (Bennett et al. 2012, Benitez et al. 2021).

**Yield decline response to leaf and root physical and chemical defences**

Our second hypothesis was only partly supported by our data: despite the fact that root collaboration predicted monoculture yield decline, there was no indication that root physical defences were more important than root chemical defences. Even though the first components of both the root and leaf PCA showed a similar tradeoff between physical and chemical defences (Fig. 3), only the leaf component was a significant predictor of yield decline. Contrary to our third hypothesis of higher importance of chemical compared to physical defences aboveground, we have found that species with high leaf physical defence experienced less yield decline than species with high leaf chemical defences in 18-year-old monocultures.

The similar tradeoff between physical and chemical defences in leaves and roots (Fig. 3) highlights that while some species are primarily defended through physical barriers other species are rather defended through chemical compounds (Eichenberg et al. 2015). The second component of the leaf PCA showed a gradient from non-palatable species (high leaf mass per area and low leaf nitrogen content) to palatable species (low leaf mass per area and high leaf nitrogen content) that are well defended through leaf surface barriers including hair density, hair length and water repellency

(Fig. 3). The two extremes of this gradient, non-palatable and non-defended species to palatable but well defended species reflect two of the defence syndromes identified by Agrawal and Fishbein (2006) in 24 milkweeds species (*Asclepias* spp.). Overall, these defence tradeoffs may suggest that either different plant species can deploy different defence strategies to cope with similar antagonists (Agrawal 2007, Moore and Johnson 2016, Hervé and Erb 2019, Whitehead et al. 2021) or that plant species use different defence strategies to cope with different groups of antagonists. Our analysis on foliar damage showed that each defence strategy was effective against only a restricted group of antagonists but not against other groups of antagonists. This suggest that plant species that deploy different defence strategies may suffer from the accumulation of different groups of antagonists. For instance, plant species with pronounced leaf physical defences were well protected against foliar chewers, but at the same time they were more susceptible to foliar pathogens, while the opposite was true for species with high leaf chemical defences (Fig. 4). Similarly, species with high root physical defences and low chemical defences were well protected against root-feeding-nematodes and potentially other root chewers (Hanley et al. 2007, Johnson et al. 2010, Caldwell et al. 2016, Freschet et al. 2021b), but not against other groups of root antagonists. Thus, species defended against root-feeding nematodes and chewers through physical defences might experience the accumulation of root pathogens, while others well-defended against pathogens through chemical defences may face the accumulation of root-feeding nematodes and chewers; yet, the variety of different options might preclude strong trait-based responses of either individual or combined trait axes.

**Possible drivers of yield decline in relation to the leaf physical vs chemical defence tradeoff**

At first glance, our analysis on foliar damage may suggest that the effect of the leaf physical versus chemical defence tradeoff on yield decline arises from its defensive role against foliar chewers, and possibly that foliar chewers may be more important at driving yield decline than leaf pathogens and raspers and miners. Indeed, a previous study at our site found that foliar chewers cause substantially more damage than other leaf antagonists (Loranger et al. 2012). However, we have found that the relationship between leaf traits and foliar damage is stronger in the young compared to the old monocultures. In contrast, the effect of the leaf psychical versus chemical tradeoff component is evident only in the old monocultures. This changing importance of individual trait components over time might hint towards trait-multifunctionality relationships: single traits and components of multiple traits could be related to multiple functions such as defence, nutrient uptake or stress resistance. For example, leaf dry matter content which is part of the leaf physical versus chemical tradeoff, is linked to plant relative growth rate, resource conservation and drought resistance (Garnier et al. 2004, Hodgson et al. 2011, Blumenthal et al. 2020). Species with high physical defence

and low yield decline have low relative growth rates, high conservation of resources, and are more resistant to drought. Species with low physical defences and hence higher growth rate lead to a faster depletion of soil nutrients, resulting in higher yield decline especially in the early stage of the experiment. This is in line with findings that fast species with high yield decline had higher concentrations of leaf nitrogen in the early stage of the experiment and a quicker reduction of leaf nitrogen over time than slow species (Dietrich et al. 2020). However, our results show that soil available phosphorus and soil total nitrogen did not explain yield decline in young or old monocultures (Supporting information). Concurrent with multiple trait–function relationships, the importance of possible drivers of yield decline may change over time. For instance, prior to our study the monocultures experienced recurrent droughts (2014–2015 and 2018–2020; Hari et al. 2020, Rakovec et al. 2022). Given that plants with high physical defence are better equipped to deal with herbivores, nutrient depletion and drought at the same time (Blumenthal et al. 2020), the effect of leaf physical defences on yield decline may be due to a response to these multiple stressors, potentially in concomitance or at different points in time.

### Possible drivers of yield decline in relation to the root collaboration gradient

Our results suggest that belowground processes related to the root collaboration gradient may be key to drive yield decline. In a previous study on the same site, Dietrich et al. (2020) found the diversity of root-feeding nematodes and the abundance of predatory nematodes to be strong drivers of monoculture yield decline, thus supporting knowledge about nematodes as key antagonists in several crop species (Bennett et al. 2012, Jones et al. 2013, Grabau and Chen 2016, Wilschut et al. 2019). In addition, recent studies found several links between the root-feeding nematode community and the traits defining the root collaboration gradient. Otfinowski and Coffey (2020) found that the abundance of root-feeding nematodes increases with specific root length for a grass species *Bouteloua gracilis* along an ecological restoration gradient. At our study site, Dietrich et al. (2021) found that the diversity of root-feeding nematodes increases with specific root length at the community level (community weighted mean). At the same time, Ristok et al. (2022) found the abundance of root-feeding nematodes to be higher in plant communities with higher root length density, a trait generally positively correlated with specific root length (Freschet et al. 2021b). However, in our study, the abundance of root-feeding nematodes was not affected by the collaboration gradient, but rather by the root physical and chemical defence tradeoff. This suggests that high root physical defence, and lower root tissue quality, are strong deterrents against root-feeding nematodes (Fig. 4) as they are for insect herbivores (Hanley et al. 2007, Johnson et al. 2016b). The apparent discrepancy between our results and other studies, at the same site, may be due to the different dynamics between root traits and nematodes at the community and at

the species level. Alternatively, the mismatch might be due to the six years delay between the data on abundance of root-feeding nematodes and our trait measurements. This suggests that the effect of the collaboration gradient on yield decline does not arise from its defensive role against root-feeding nematodes but potentially from other functions.

Considering the robust association between the collaboration gradient and AMF (Bergmann et al. 2020, Fig. 4), and the observed positive influence of AMF in mediating the collaboration gradient's impact on plant–soil feedback in a prior study with a comparable set of species (Cortois et al. 2016), we expected AMF to similarly mediate the effect of the collaboration gradient on yield decline in our study. However, comparable to the results of leaf defences and foliar damage, we found a stronger link between root defence traits and AMF in young compared to old monocultures, but no effect of AMF on yield decline irrespective of monoculture age as did Dietrich et al. (2021) (Supporting information). Even though our measure of AMF colonisation rate does not directly reflect the efficiency or nature of the relationship with plants, which could be parasitic and promote yield decline (Bennett et al. 2012), our results suggest that the effect of the collaboration gradient on yield decline is not mediated by AMF. Nonetheless, our findings align with those of Wilschut et al. (2023), who observed that plant species with high specific root length experience stronger negative plant soil feedback than species with low specific root length, and that this effect was not mediated by relative abundance and community composition of AMF.

Similar to leaf physical effects on yield decline, the effect of the collaboration gradient on yield decline may be mediated via other stresses, such as nutrient depletion in early years of the experiment, and recurrent droughts in later years. Indeed, when nutrients are limiting, species may increase specific root length to explore a larger volume of soil per unit carbon and increase nutrient uptake (Ho et al. 2005), potentially explaining the high yield decline in species with high specific root length in the early years of the experiment. On the other hand, outsourcing species with thicker roots have higher penetration strength through soil and often also deeper roots which could promote resistance to drought (Freschet et al. 2021b). This may explain the low yield decline in species with high root diameter in the late years of the experiment. Overall, we were not able to link the importance of the collaboration gradient for yield decline to root-feeding antagonists nor to soil nutrient depletion or other processes controlled by AMF. We speculate that the effect of the collaboration gradient on yield decline emerges as a response to multiple stress factors that occurred concomitantly or consecutively over time.

### Speculations on alternative roles of the collaboration gradient in relation to antagonists

An alternative mechanism potentially linking the collaboration gradient to the accumulation of soil antagonists might be that roots with high specific root length explore a larger

volume of soil and expose a larger surface per unit carbon than species with low specific root length (Ho et al. 2005). While this allows species to explore the soil for nutrients, it may also increase the chance to encounter root antagonists. The large root surface exposed in DIY species may increase the area available for pathogen infection (Laliberté et al. 2015). Thus, higher specific root length could increase yield decline in DIY species (current study) and negative plant–soil feedback (Wilschut et al. 2023), independent of its relationship with AMF. Despite data on root antagonists is lacking, we speculate that this mechanism would promote the accumulation of any group of root antagonists independently of their taxonomic group or feeding guilds and would be in line with our suggestion that the groups of antagonists responsible for yield decline differ between species with different defence strategies.

## Conclusion

Our study demonstrates that the collaboration gradient and the plastic response of roots along this gradient of the root economics space as well as the tradeoff between physical and chemical defence in leaves, are significant predictors of yield decline for 27 plant species in a long-term grassland experiment. Our study further indicates that plants can deploy a large variety of defence strategies, which may be effective only against a restricted group of antagonists and also relevant to address other stressor that plants are experiencing in long-term monocultures. The complexity of these relationship might mask a generalisable relationship between plant defence traits and yield decline. When species are growing in mixtures, this diversity of plant defence strategies may promote complementarity in defence, responses to drought, and nutrient depletion, which could support the increasingly positive biodiversity effect on ecosystem functioning through time (Eisenhauer et al. 2019). Such complementarity might also help to counteract yield decline in agricultural settings, e.g. via increased genetic diversity in crops or diversification of crop rotations, or increased spatial diversity of different crops. While the mechanism relating the collaboration gradient to yield decline still obscure, the present findings stimulate research on the relationship between root traits and different groups of plant antagonists and mutualists in natural or seminatural systems.

## Author contributions

**Leonardo Bassi**: Conceptualization (equal); Data curation (lead); Formal analysis (lead); Investigation (lead); Methodology (equal); Project administration (lead); Visualization (lead); Writing – original draft (lead); Writing – review and editing (lead). **Justus Hennecke**: Data curation (supporting); Investigation (supporting); Methodology (equal); Visualization (supporting); Writing – original draft (supporting); Writing – review and editing (supporting). **Cynthia Albracht**: Data curation (supporting); Investigation (supporting); Methodology (supporting); Writing – original draft (supporting). **Maximilian Bröcher**: Data curation (supporting); Investigation (supporting); Methodology (supporting); Writing – original draft (supporting); Writing – review and editing (supporting). **Marcel Dominik Solbach**: Methodology (supporting); Writing – original draft (supporting); Writing – review and editing (supporting). **Jörg Schaller**: Conceptualization (supporting); Data curation (supporting); Investigation (supporting); Methodology (supporting); Writing – original draft (supporting); Writing – review and editing (supporting). **Van Cong Doan**: Data curation (supporting); Methodology (supporting); Writing – original draft (supporting); Writing – review and editing (supporting). **Heiko Wagner**: Data curation (supporting); Methodology (supporting); Writing – original draft (supporting); Writing – review and editing (supporting). **Nico Eisenhauer**: Conceptualization (supporting); Methodology (equal); Project administration (equal); Resources (supporting); Supervision (supporting); Writing – original draft (supporting); Writing – review and editing (supporting). **Anne Ebeling**: Methodology (supporting); Project administration (supporting); Resources (supporting); Supervision (supporting); Writing – original draft (supporting); Writing – review and editing (supporting). **Sebastian T. Meyer**: Conceptualization (equal); Methodology (equal); Writing – original draft (supporting); Writing – review and editing (equal). **Nicole M. van Dam**: Conceptualization (lead); Funding acquisition (lead); Methodology (equal); Supervision (supporting); Visualization (supporting); Writing – original draft (supporting); Writing – review and editing (equal). **Alexandra Weigelt**: Conceptualization (lead); Funding acquisition (lead); Methodology (equal); Project administration (equal); Supervision (lead); Visualization (equal); Writing – original draft (equal); Writing – review and editing (equal).

## Data availability statement

Data and R code are available from the Jena Experiment database (https://jexis.idiv.de/): https://doi.

org/10.25829/1V4X-5N25 (Bassi et al. 2023a) and https://doi.org/10.25829/9Q4N-GT79 (Bassi et al. 2023b). The raw and processed metabolome data are available from MetaboLights (https://www.ebi.ac.uk/metabolights/) under the study identifier MTBLS4286.

## Supporting information

The Supporting information associated with this article is available with the online version.

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