Functional Ecology

British Ecological Society

Functional Ecology 2015, **29**, 1560–1568 doi: 10.1111/1365-2435.12466

Diversity effects on root length production and loss in an experimental grassland community

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Summary

1. Advances in root ecology have revealed that root standing biomass is higher in species-rich plant communities than in species-poor communities. Currently, we do not know whether this below-ground diversity effect is the result of enhanced root production or reduced root mortality or both, which is essential information to understand ecosystem functioning, as it determines C sequestration and N dynamics in soil.

2. Minirhizotron observations were combined with root coring in five different plant communities (four monocultures and the respective mixture). Molecular markers were used to quantitatively determine species abundance in mixed root biomass samples in order to track shifts in below-ground species composition. In addition, a litterbag experiment was performed to study root decomposition independent of root mortality.

3. Root length production was greater and root length loss was lower in the mixture than expected from monocultures in all years. Simulations suggest that at least two species must have had reduced losses in mixture compared to monoculture. However, the diversity effects on root mortality may partially be explained by selection effects as the species with the longest root life span became dominant in the mixtures. Root length loss from minirhizotrons was very low; the combination of minirhizotron length measurements with root biomass estimates from coring suggested underestimation of root loss in minirhizotrons over time. Root decomposition was not affected by diversity.

4. Diversity enhanced root length production and decreased root loss, resulting in below-ground overyielding. With decomposition unaffected, our results suggest that root mortality is reduced with increasing diversity. Future studies have to reveal the generality of our observations in larger scale biodiversity experiments by using species having a wider variety of root traits.

Key-words: biodiversity experiment, minirhizotron, root decomposition, root mortality, root production, root turnover

Introduction

Plant roots are an essential component of ecosystems (Bardgett, Mommer & De Vries 2014), both in numbers (Poorter *et al.* 2012) and in function (Casper, Schenk & Jackson 2003; Fornara & Tilman 2008; Padilla *et al.* 2013; Cong *et al.* 2014). Several biodiversity experiments have shown that root standing biomass is higher in mixtures compared to monocultures (Tilman *et al.* 2001; Dimitrakopoulos & Schmid 2004; Fornara, Tilman & Hobbie 2009; Mommer *et al.* 2010; Ravenek *et al.* 2014). However, the underlying processes are unknown, as data on root production and root mortality in biodiversity experiments do not exist. The lack of accurate estimates of root turnover is a major omission in our understanding of how biodiversity affects ecosystem functioning, since roots are expected to be a major driver of soil carbon accumulation in grasslands (Aerts, Bakker & de Caluwe 1992; Fitter *et al.* 1997; Allard *et al.* 2005; Bai *et al.* 2010).

Above-ground biomass, typically measured in grassland biodiversity experiments by annual or biannual clipping, is a quick and accurate measure of annual above-ground production. In contrast, below-ground biomass, typically

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determined using relatively small soil cores usually taken once a year, at the peak of the growing season, is a measure of standing biomass, which results from the balance between root production *and* root loss over a longer time period. There is limited information about the effect of diversity on root production, but it appears that annual root production in diverse communities is promoted compared to monocultures, at least in some stages of the experiments (Fornara & Tilman 2008; Mommer *et al.* 2010; Mueller *et al.* 2013; Ravenek *et al.* 2014). Direct measurements on root mortality are even scarcer. There is a potential for differences in root mortality between different plant communities, since root life span estimates for grassland species are highly variable, ranging from 50 to 100 days up to several years (Fitter *et al.* 1997; Gill & Jackson 2000; Milchunas & Lauenroth 2001; Pärtel & Wilson 2002; Van der Krift & Berendse 2002; Gao *et al.* 2008). Some studies suggest that root mortality increases with plant diversity in grasslands, but this is based on decomposition of roots or carbon accumulation in soil (Hattenschwiler, Tiunov & Scheu 2005; Fornara & Tilman 2008; Steinbeiss *et al.* 2008; Fornara, Tilman & Hobbie 2009) rather than on direct measurements of root loss.

Here, we provide one of the first estimates of root production and mortality from a controlled grassland biodiversity experiment using four different plant species in monoculture and mixture (Mommer *et al.* 2010). We used nondestructive minirhizotron observations to characterize root length dynamics at the plant community level. In addition, the application of molecular markers on mixed root samples to quantitatively determine species abundance (Mommer *et al.* 2008) allowed us to determine changes in species' biomass in the mixtures over time and compare them with the root length dynamics observed using the minirhizotrons.

A complicating factor when investigating root mortality in minirhizotrons is that root decomposition is intermediate between root mortality and root disappearance. Consequently, estimates of root disappearance from minirhizotrons (root loss) incorporate both root death and decomposition. Potential diversity effects on decomposition (e.g. Fornara, Tilman & Hobbie 2009) may thus affect root loss estimates. Therefore, in a separate litterbag study, we quantified relative differences in root decomposition of these four species used in this study. In addition, we quantified the soil biodiversity effects on root decomposition, albeit in another grassland biodiversity experiment (i.e. the Wageningen biodiversity experiment; van Ruijven & Berendse 2009).

Materials and methods

EXPERIMENTAL SET-UP: NIJMEGEN BIODIVERSITY EXPERIMENT

We investigated root growth and production over a 3-year period in a biodiversity experiment in the Nijmegen Phytotron, a facility designed to study below-ground processes under near-ambient conditions. Full details of the experimental design are described in Mommer *et al.* (2010). Here, a brief description is given.

Two grasses, *Anthoxanthum odoratum* L. and *Festuca rubra* L., and two forbs, *Leucanthemum vulgare* Lamk. and *Plantago lanceolata* L., were grown in replicated monocultures and 1 : 1 : 1 : 1 mixtures in a substitutive design in plots (50 × 50 cm; 70 cm depth; grouped by 3 in polyethylene containers). Plant density of all plots was equal: 36 seedlings were planted in each plot giving a plant density of 144 m^{-2}). Soil depth was 64 cm, divided into a 52-cm-thick layer of a rich soil mixture [2 : 1 : 1 (v : v : v) sand: loamy sand: potting soil] and a 12-cm layer consisting of nutrient-poor riverine sand below. In Mommer *et al.* (2010), this soil arrangement has been referred to as the nutrient-rich topsoil layer treatment. All plots were kept moist using an automated irrigation system every other day (PRIVA, de Lier, The Netherlands), aided by a 4-cm bottom layer containing pebbles that allowed drainage via an outlet at the base. In this experiment, we measured root length nondestructively via minirhizotron tubes (see below) and root biomass destructively via soil coring and root washing (Mommer *et al.* 2010; see Appendix S1, Supporting information).

MINIRHIZOTRON OBSERVATIONS: NONDESTRUCTIVE SAMPLING

We used minirhizotron tubes to monitor root production and loss nondestructively from June 2006 to June 2009 in monocultures and mixtures through transparent minirhizotron tubes placed horizontally in each container at soil filling. The minirhizotron system did allow tracking of individual roots (fragments), but species identification was not possible from visual inspection of the root images in mixtures. The minirhizotron tubes were installed in the containers at a depth of 18 cm in a horizontal position, to ensure that all four species were tracked to the same distance to the tube. The depth of 18 cm was selected, since this allowed the development of a proper root mass above the tube. Apart from a lower overall root length density at this depth, the soil layer was representative of the species proportions of the top soil layer. Tubes were 68 cm long with a 6·4 cm internal diameter and made of PMMA (polymethyl methacrylate; Vink Kunststoffen B.V., Didam, the Netherlands). Flexible PVC caps glued with sealant prevented water infiltration in the bottoms of the tubes, and PVC collars and caps prevented light and water penetration in the tube. Root images (21·6 × 19·6 cm, 300 dpi) were collected with a specific scanner (CI-600 Root Scanner, CID Inc., Camas, WA, USA). From the scanned images, a subarea of 21·6 × 3 cm corresponding to the top of the minirhizotron tube (positioned at 18 cm depth) was cropped and analysed (root length) in each image (values are given per m^2 of minirhizotron image throughout) using the WINRHIZOTRON V2005A software (Regents Inc., Québec, Canada).

Root images were taken every 58 days on average, but the averaged time interval between censuses in spring and summer (April–September) was 44 days, with a minimum interval of 22 days in the first censuses, for a total of 19 censuses. We may have missed some short-lived roots that died between censuses, but the majority of roots remained visible in minirhizotron tubes much longer, suggesting that the intervals were not too large for our species. Observable root segments on each date were digitized, coded and followed from appearance (birth) to disappearance; birth date was estimated by calculating the date midway between the first observation and the previous one. Similarly, disappearance date was estimated as the midway between the last observation and the next one (Van der Krift & Berendse 2002; Bauerle *et al.* 2008). Complete root disappearance was used as end point for defining root longevity in monocultures and mixtures (Fransen & de Kroon 2001; Johnson *et al.* 2001; Van der Krift & Berendse 2002) as changes in root colour seen in minirhizotron images may not be indicative of root vitality (Hendrick & Pregitzer 1996; Eissenstat & Yanai 1997). Root longevity defined in this way will be greater than measurements based on changes in root colour, since it comprises both root mortality and subsequent decomposition.

ROOT LENGTH-BASED CALCULATIONS

Minirhizotron observations allowed us to record on each date standing root length and related length rates, such as root length production and root length loss following Padilla *et al.* (2015). Root length production for each year (m m^{-2} image) was calculated as follows: $(Lo2-(Lo1-(Lg2-Lg1)))/(Time2-Time1)$,

where *Lo*2 and *Lo*1 are total observable root lengths at the end (*Time2*) and beginning (*Time1*) of the year, respectively. The difference between *Lg2* and *Lg1* is the length of roots that has disappeared within the same period (i.e. dead and decomposed roots). This length has to be accounted for in the calculation of root production rate, since new root length production does depend not only on the observable root length at *Time 2* but also on the length of pre-existing roots at *Time1* that has disappeared within the *Time2*−*Time1* period (see further explanation in Appendix S1, Supporting information). Production rate calculated in this way is always ≥0 and prevents negative rates if observable length on *Time2* is lower than on *Time 1*; in these latter cases, root production equals zero and it is root loss rate that actually shows decreases in root length. Root length loss (m m^{-2} image) within a year was calculated from lengths of disappeared roots at the end and beginning of the year following *(Lg2*−*Lg1)/(Time2*−*Time1)*.

Root length turnover (expressed here as % $year^{-1}$) was calculated at the end of the experiment by dividing the lost root length by the maximum observable root length detected in the monitoring in minirhizotrons (Ferguson & Nowak 2011), relative to the time elapsed. This value shows the averaged percentage of the maximum standing root length that is lost on a yearly basis.

ROOT-COUNT SURVIVAL ANALYSIS

Based on root counts from the 2006 cohort (the one with longest time span), survivorship functions were calculated by applying the Kaplan–Meier product-limit method (Kaplan & Meier 1958), considering the roots that did not disappear at the end of the study as incomplete or censored responses. Separate functions were fitted for the different monocultures and the mixture. Subsequently, expected root longevity in mixtures was calculated from root-count data of the four monocultures correcting for the actual species proportions below-ground (i.e. weighted average) in 2006 as obtained from molecular analyses on root cores in the first harvest, being 56%, 12%, 19% and 14% for *A. odoratum*, *F. rubra*, *L. vulgare* and *P. lanceolata,* respectively (Mommer *et al*. 2010). This approach was only possible for the 2006 cohort and not for cohorts of subsequent years, since for the later years, we did not know the species-specific proportions of the *newly produced* roots in the mixture. Root counts from 2006 were grouped in 48 root longevity intervals, each of 22 days each (the shortest time frame between two consecutive observations). We grouped the roots that were visible in minirhizotrons 22 days, 45 ≥ days > 22, and so on until 1057 > days > 1035 and ≥1057 days (the longest time frame considered in the experiment), and computed the fraction of visible roots within each class. We estimated the contribution of the species to the function of the mixture by simulating zero disappearance of a given species (or combination of species) as extreme cases. The resulting simulated curves were compared to the observed curve of the mixture (Appendix S1, Supporting information).

ROOT DECOMPOSITION

In a litter bag experiment using the same four plant species, we investigated the species-specific component of root decomposition in a different biodiversity experiment in Wageningen (van Ruijven & Berendse 2005, 2009). Roots in the litterbags originated from separate plants (n = 4 for each species) that had been growing outdoors on sand with Osmocote® fertilizer (6 g kg^{-1}) for 6 months. These plants were watered three times a week. In September, all roots from the four plant species were harvested; roots were washed, cut in small pieces of 2 cm and weighed into 'monoculture' portions of 2 g fresh weight. We did not mix the roots of different species in the litterbags. Subsamples of the roots were taken to analyse dry-matter content. Litterbags (mesh size 250 μm) were incubated at 0–7 cm depth in plots of the Wageningen biodiversity experiment. In this experiment, we used the monoculture plots of our four plant species and eight-species mixture plots (including four additional herbs and grasses; *Centaurea jacea*, *Rumex acetosa*, *Agrostis capillaris* and *Holcus lanatus*), each replicated six times. The litterbags were incubated from September 2009 until April 2010. The winter period was included, since most root death will occur in winter.

In spring, litterbags were collected; the roots were taken out, cleaned by gently brushing of soil particles, dried at 70 °C and weighed. Root mass loss was calculated as the difference between start and end dry weight, divided by the start weight. Dry weight at the start was calculated by multiplying the fresh weight with the dry-matter content (%).

STATISTICS

Standing root length, root length production and root length loss were analysed by RM-ANOVA, using time as a within-subjects factor and diversity as between-subjects factor. Time was 19 censuses, every 58 days on average, for standing root lengths (see above) and 3–4 years for the other variables. The plant treatments were included as between-subjects factors. Diversity effects were tested in two separate ways. First, we tested whether the mixture performed better than the average monoculture (overyielding) by including diversity (one or four species) and species identity nested within diversity (to test for differences between monocultures) as fixed factors. Comparing mixtures to the average monoculture is the common procedure in biodiversity research. In order to further investigate the differences among monocultures and the mixture, in a next step, we included only plant species composition (five levels: the mixture and the four monocultures) as a fixed factor. LSD *post hoc* tests were used to test for significant differences between the five levels. When necessary to meet the assumption of sphericity, Huynh–Feldt-adjusted degrees of freedom were used for within-subject effects. Prior to ANOVA, all data were log-transformed in order to improve homoscedasticity of variables.

As for root-count measurements, differences between monocultures of the 2006 cohort in root disappearance functions and the risk of root loss were determined by running proportional hazard Cox regression. Similarly, Cox regression was used to test for differences between observed and expected mixtures. In these data, the 'expectation' was calculated from actual species proportions, as determined by the molecular analysis in the first growing season (see Fig. S1, Supporting information). When Cox regression results were significant in monocultures, pairwise Cox-Mantel tests were used to compare the specific curves. In short, proportional hazard Cox regression tests the influence of the predictor variable on the time elapsed from root birth until the event of death (Agresti 2002). The significance of the variable is given by the Wald statistic of its regression coefficients: if significant (P-value of Wald <0·05), the variable is useful for the model and it can be concluded that it has an effect on root longevity.

Root decomposition data were analysed using a mixed linear model, in which plot was included as a random factor, to incorporate the fact that the litterbags of the four different species were included in the same plots. Species identity of the root litter and species composition of the plot where litter bags were incubated identity were included as fixed factors, with four and five (four monocultures and the diverse mixtures) levels, respectively.

All analyses were run with IBM SPSS STATISTICS 19 (IBM Corporation, Armonk, NY, USA). Data are presented as means ± one standard error throughout (n = 3–4 in the Nijmegen experiment; n = 5–6 in the Wageningen root decomposition experiment).

Results

ROOT LENGTH PRODUCTION AND LOSS

Root biomass in mixtures was greater than the average monoculture, and monocultures differed significantly from each other (Fig. S2, Supporting information; Table S1, Supporting information), when considering the complete soil profile as well as the soil layer just above the minirhizotron tubes (12–18 cm depth). This pattern was also apparent in the minirhizotron tubes as standing root length

increased with time (Fig. 1; Table 1) and was significantly larger in mixtures than in the average monoculture (i.e. overyielding; see Fig. 1; Table 1), except for the first two censuses (57 and 77 days after transplant) as indicated by a significant time × diversity interaction (Table 1). Standing root lengths were significantly different among the four species in monoculture (Table 1). Overall, mixtures and *F. rubra* monocultures showed significantly higher standing root lengths than the other three monocultures (Fig. 1), but did not differ significantly from each other (*post hoc* test: $P = 0{\cdot}30$). The significant interaction between time and species in monoculture (Table 1) was due to *A. odoratum*, which did not show a significant increase in standing root length with time ($F_{18,36} = 0{\cdot}6$; $P = 0{\cdot}86$).

Root length production was significantly greater in the first year (2006) compared to the other years (Fig. 2a; Table 1). Mixtures produced more root length than the average monoculture (Table 1), particularly in 2006, in which mixtures produced nearly twice the length of roots than expected from the averaged monocultures (Fig. 2a). Root length production also differed between monocultures, but this effect depended on year (Table 1). In all years except for the first one, *A. odoratum* monocultures produced less root length than the other species (Fig. 2a). Root length production in mixture was not significantly different from the *F. rubra* monoculture in any year (Fig. 2a).

Root length losses were considerably lower than root length production rates. Whereas root length production at the end of each year ranged from 15 to 428 m m^{-2} image (Fig. 2a), root length loss varied between 1 and 63 m m^{-2} image (Fig. 2b). Overall, root length loss was greater in the second year than in the other years. This effect was independent of diversity or species identity. Root length loss was significantly lower in mixture than in the average monoculture (Table 1; Fig. 2b). Root length loss differed between species in monoculture (Table 1; Fig. 2b), as *F. rubra* monocultures and mixtures showed significantly lower root length loss rates than the other three monocultures. This pattern did not change when calculating annual root length turnover, from lost root length and maximum standing root length. There were significant differences among the plant communities (ANOVA $F_{4,14} = 17{\cdot}37$, $P < 0{\cdot}001$): mixtures and *F. rubra* monocultures had the lowest root length turnover at 1·3 ± 06 and 2·1 ± 0·3% year^{-1}, respectively, which contrast sharply

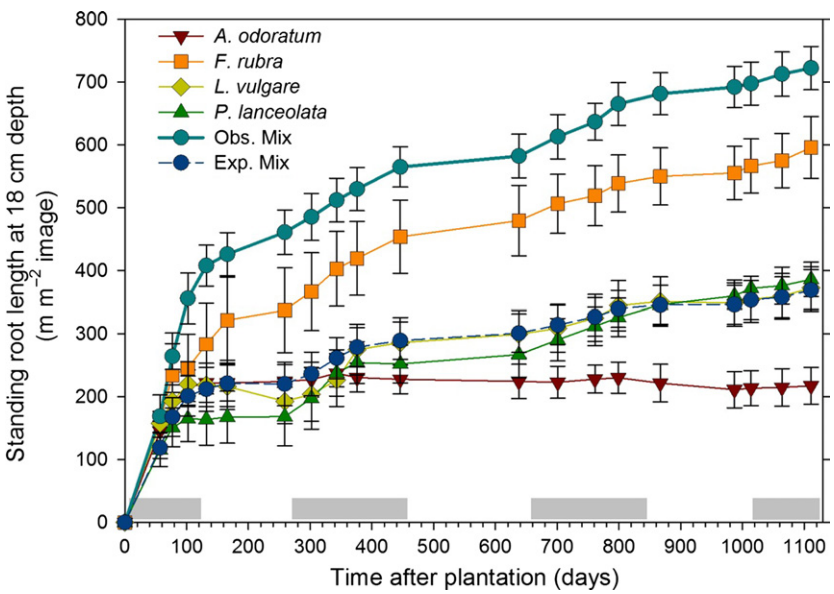

Fig. 1. Observable root length per m^2 of minirhizotron image of monocultures of *Anthoxanthum odoratum*, *Festuca rubra*, *Leucanthemum vulgare* and *Plantago lanceolata*, and of mixtures of the four species (Obs. Mix), evaluated over time through minirhizotron images at 18 cm depth. Expected mixture values (Exp. Mix) were calculated from averaged monocultures. Grey rectangles delimit the respective growing seasons, that is summer 2006, spring + summer 2007, spring + summer 2008, spring 2009. Data are means (n = 3–4) ± 1SE.

Table 1. Effects of diversity, species identity in monoculture and time on root length data from minirhizotron tubes. The diversity effect (Div) compares mixtures with the average monoculture. Differences among monocultures are tested by species identity nested in diversity. Significant effects are highlighted in bold

	Standing root length			Root production			Root loss		
Effect	d.f.	*F*	*P*-value	d.f.	*F*	*P*-value	d.f.	*F*	*P*-value
Between-subjects									
Div	1	35·3	**< 0·001**	1	26·7	**< 0·001**	1	21·8	**< 0·001**
Species (Div)	3	10·1	**0·001**	3	25·6	**< 0·001**	3	12·4	**< 0·001**
Error (MS)	12	0·7		14	0·1		14	0·8	
Within-subjects									
Time (T)	2·7	54·7	**< 0·001**	3	96·2	**< 0·001**	3	5·1	**0·004**
Div × T	2·7	2·0	0·132	3	0·5	0·666	3	1·5	0·236
Species (Div) × T	8·2	4·0	**0·002**	9	2·7	**0·015**	9	1·5	0·185
Error T (MS)	32·8	0·2		42	0·1		42	0·7	

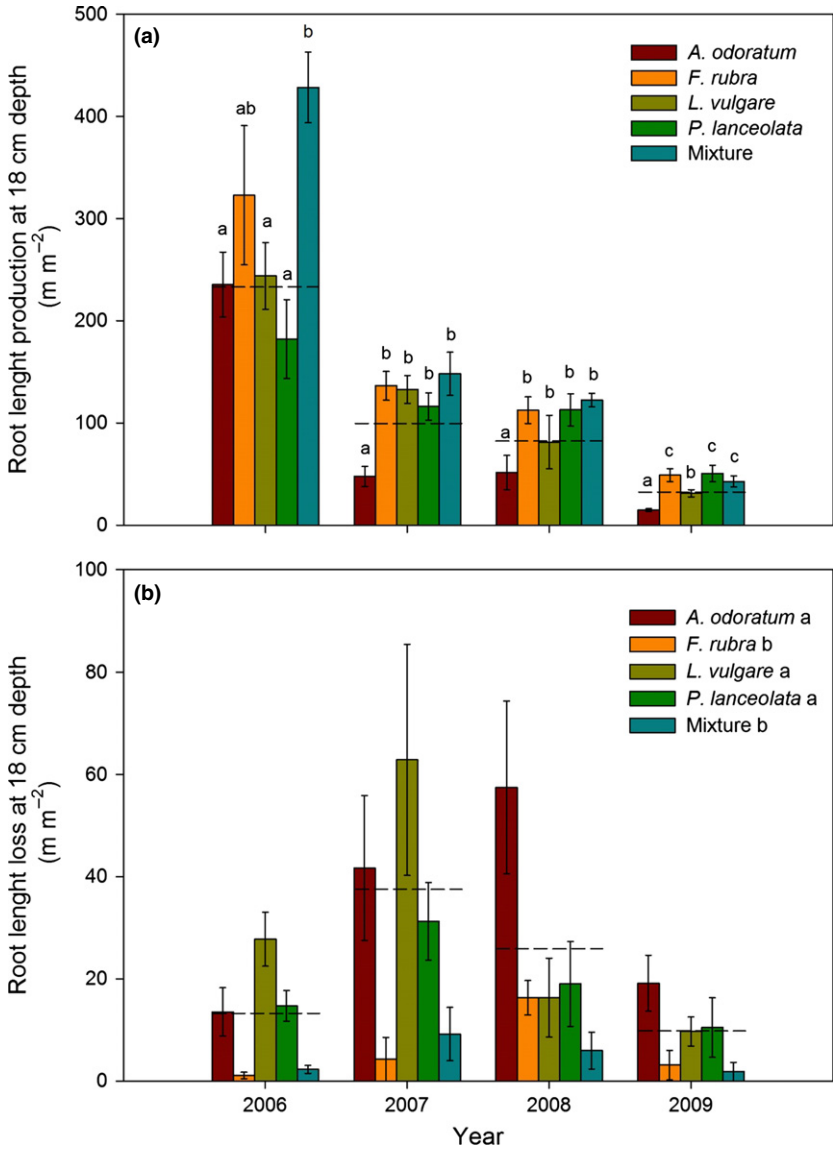

Fig. 2. Root length production (a) and root length loss (b) of monocultures of *Anthoxanthum odoratum*, *Festuca rubra*, *Leucanthemum vulgare* and *Plantago lanceolata*, and of mixtures of the four species (Obs. Mix), evaluated over time (years 2006, 2007, 2008 and 2009) through minirhizotron images at 18 cm depth. The dotted line represents the averaged monoculture per growing season. Different letters indicate significant differences between plant treatments (LSD *post hoc* tests on log-transformed data). Data are means (n = 3–4) ± 1SE.

with the higher root length turnover found in *A. odoratum*, *L. vulgare* and *P. lanceolata* monocultures (26·0 ± 6·3, 16·9 ± 4·7 and 10·0 ± 0·7% year^{-1}, respectively).

ROOT-COUNT SURVIVAL ANALYSIS

The first root cohort (2006) allowed analysing root loss over time based on counts of remaining (living) and disappeared root fragments. This survival analysis confirms the differences in root loss among monocultures (Fig. 3; Wald = 219·44, $P < 0·001$). *F. rubra* monoculture clearly had the lowest loss rate (i.e. longer root life span), followed by *P. lanceolata* and *L. vulgare*. *A. odoratum* had the largest loss rate with <60% surviving after 3 years (Fig. 3). Proportional hazard (Cox) regression showed a significant biodiversity effect on root survival in the 2006 cohort (Wald = 204·93, $P < 0·001$). Based on actual species proportions in the 2006 mixtures, approximately 34% of the root fragments in the expected mixture had disappeared over the 3-year study period, but as little as ≈4% disappeared in the actual mixture (Fig. 3). Root life spans were >3 years in all communities, and median root life span at the 50th percentile could not be calculated in any community because root survivorship was >50% after 3 years of monitoring. It would have been very interesting to explore how root life spans of the other cohorts would have responded to diversity, but this was not possible. In the first year, the species proportions in mixture as determined by the sampling of root biomass represents the first cohort followed in the minirhizotrons. In the following years, however, the species-specific abundances in mixture apply to the total standing root biomass, not to the *newly produced* roots in the following years. Thus, we could not reliably estimate the relative abundance of each species in these cohorts. As a consequence, it was not possible to accurately predict the root dynamics in mixture of these cohorts.

Which of the species were responsible for the increase in root longevity in mixtures? In order to explore this, we simulated extreme situations for the 2006 cohort with zero loss for one of each of the four of the species (Fig.

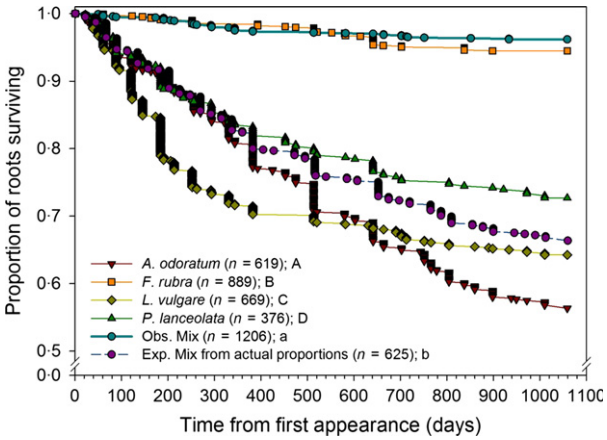

Fig. 3. Kaplan–Meier survival functions of roots born in 2006 of monocultures of *Anthoxanthum odoratum*, *Festuca rubra*, *Leucanthemum vulgare* and *Plantago lanceolata*, and of mixtures of the four species (Obs. Mix), evaluated through minirhizotron images at 18 cm depth. Expected mixture values (Exp. Mix) were calculated from the actual species root mass proportions. Numbers in brackets are total root counts (both complete and incomplete responses). Uppercase letters show differences between monocultures and lowercase letters between ob. and exp. mixtures, after pairwise Cox-Mantel tests.

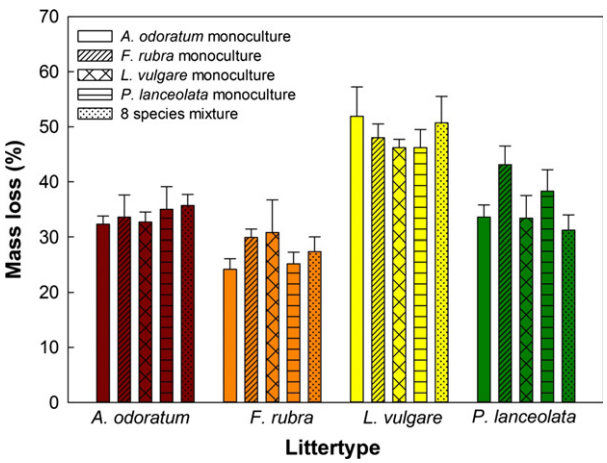

Fig. 4. Root mass loss of the four species from litterbags in monoculture plots of each of the four species and in eight plant species mixtures. Colours indicate the root litter origin; patterns (as indicated in the legend) indicate the plant composition of the plots where the litterbags were incubated. Data are means ± SE, n = 5–6.

S3, Supporting information). In none of the cases, this zero root loss was sufficient to approach the root survivorship observed in mixtures. This suggests that root life span of at least two of the species has likely been increased in mixtures. However, even assuming zero loss of three of four species together (*F. rubra*, *L. vulgare* and *P. lanceolata*) was not sufficient to achieve the survivorship observed in mixtures, suggesting that the root life span of the species not included into this simulation, *A. odoratum*, has been increased in mixtures. *L. vulgare* and *P. lanceolata* are likely the other species that had increased root life span in mixtures, rather than *F. rubra* that had inherently the longest root life span. Indeed, zero root loss of *F. rubra* hardly increased the life span of expected mixtures.

ROOT DECOMPOSITION

In the litterbag experiment in the Wageningen biodiversity experiment using the same four plant species, root mass decomposition differed significantly between root litter type (Fig. 4; $F_{3,92}$ = 34·8; P < 0·001). *L. vulgare* showed the highest decomposition rate (49% root mass loss), followed by *P. lanceolata* and *A. odoratum* (36% and 34%, respectively) and, finally, *F. rubra* at 27% (Fig. 4). Root decomposition was not affected by species composition of the plot where the litterbags had been placed (Fig. 4; $F_{4,92}$ = 0·6; P = 0·69) nor by the interaction between litter type and plot composition ($F_{12,92}$ = 1·0; P = 0·44). A potential bias in our litterbag experiment may be that soil legacies of four nontarget species also occurring in the plots of the biodiversity experiment (but not present in the minirhizotron study) may have affected the decomposition rate of the four target species. However, as we did not detect any significant effects of plot and the litter type × plot interaction on decomposition in the biodiversity experiment, such a bias is unlikely.

Discussion

We observed positive biodiversity effects on root standing length using minirhizotrons. This enhanced standing root length resulted from both enhanced root length production and decreased root length loss in mixture compared to monocultures. A survival analysis of root counts of the first cohort suggested that roots of two or three species must have had lower loss rates in mixtures than in monocultures. It should be noted that the diversity-dependent root mortality, particularly in later years, may be explained by selection effects as the species with the longest root life span, *F. rubra*, became dominant in the mixed plant communities.

BIODIVERSITY EFFECTS: INTERPRETING ROOT LENGTH PRODUCTION AND ROOT LENGTH LOSS

As described in Mommer *et al.* (2010), root biomass was higher in mixtures than in monocultures already in the first year and even preceded above-ground biodiversity effects. The strength of the current analysis is that we could disentangle the biodiversity effects on root length production and root length loss, thereby providing insight into mechanisms underlying the development of below-ground overyielding. The positive biodiversity effect on root length production was particularly strong in the first growing season, but remained significant in all years. Earlier work suggested that it is unlikely that the diversity-dependent increase in root growth is driven by nutrients only (Mommer *et al.* 2010; Hendriks *et al.* 2013; Padilla *et al.* 2013). Therefore, alternative explanations for the biodiversity effects on root growth consider built-up of soil-borne pathogens (Maron *et al.* 2011) and/or the exudation of allelopathic compounds (Bais *et al.* 2006; Mazzoleni *et al.*

2015) in monocultures or positive interactions with symbionts (van der Heijden *et al.* 1998; Bever, Platt & Morton 2012) and/or heterospecific positive effects of root exudates (Bednarek & Osbourn 2009; de Kroon *et al.* 2012).

The biodiversity effect on root loss was negative as root length loss estimates and root-count survival analyses revealed lower losses in mixtures compared to the average monoculture. One of the potential explanations for the reduced root loss in mixtures could be decreased pathogenic soil biota in mixtures as compared to monocultures, which would be consistent with current developments in biodiversity research on this issue (Maron *et al.* 2011; Schnitzer *et al.* 2011; de Kroon *et al.* 2012; Kulmatiski, Beard & Heavilin 2012). For each of the four species used in this experiment, Hendriks *et al.* (2013) showed that root biomass *production* was reduced in monoculture soils (i.e. with 'own' soil biota) and also that nutrient uptake of the roots in patches of this soil was decreased (Hendriks *et al.* 2015). Data of the present research are the first to suggest that such reduced root growth in monocultures may be accompanied by higher root loss (i.e. decreased root life span) due to negative effects of 'own' soil biota. Indeed, research in tree plantations has shown that root mortality of citrus trees was increased with increasing population density of *Phytophthora nicotianae* (Kosola, Eissenstat & Graham 1995) and that application of insecticide and fungicide to the soil decreased root mortality of roots in peach (Wells, Glenn & Eissenstat 2002).

An important question in biodiversity research is whether the observed diversity effect is due to complementary or selection effects. Although we could not apply the additive partitioning method to disentangle these two effects (Loreau & Hector 2001), because we did not have data on the contribution of individual species to root length production and loss in mixtures, it is clear from our results that mixtures were increasingly dominated by the species with the highest root biomass and lowest root loss rates in monoculture (*F. rubra,* see Fig. S1, Supporting information). This selection effect may be an additional explanation for the reduced root loss in mixtures compared to monocultures, as the performance of the mixture was not different from this monoculture. It should be noted, however, that this selection effect does not apply to enhanced root production in the first year which was mainly due to enhanced root production of the less productive *A. odoratum*, whereas the three other species produced similar root lengths as in monocultures (Mommer *et al.* 2010). Similarly, the selection effect probably does not apply to the survival analysis on root counts of the 2006 cohort, because in this cohort *F. rubra* was not yet dominant. This seems to be confirmed by our simulation studies, which showed root life spans of at least two out of the four investigated species, *A. odoratum* being one of them, must have been higher in mixtures compared to their respective monoculture in order to explain the low root turnover in mixtures. The shifts in species contribution over time may shed light on important species traits contributing to ecosystem functioning. In the short term (e.g. after a disturbance), high root production and turnover (*A. odoratum*) may be important, whereas in the long term, low root turnover rates, consolidating established root densities (*F. rubra*) may be more important (Mommer *et al.* 2011). This suggests that interactions between plant species with these different root traits, particularly in a changing environment, may affect ecosystem functioning. Clearly, one has to be careful when drawing conclusions regarding biodiversity and root turnover from an experiment with just four species. However, these species are common in temperate grasslands and they represent the two dominant functional groups in these grasslands, grasses and nonleguminous forbs. Therefore, we expect that our experiment based on the interactions among these four species provides insights into the functioning of temperate grassland ecosystems.

METHODOLOGICAL CONSIDERATIONS

In a meta-analysis with fine tree roots, Strand *et al.* (2008) concluded that minirhizotrons overestimated root loss because they mainly observed lower order roots, which have more rapid mortality. Our comparison points to the opposite: *under*estimation of root loss of grassland species on minirhizotron images rather than *over*estimation. However, the two studies are not fully comparable, since the criteria for root loss included a combination of shrinkage/fracturing/colour change (Strand *et al.* 2008) as opposed to the definition we used here (i.e. total root disappearance).

The time interval between censuses to the expected root life span of the study species also deserves consideration, particularly in periods when root turnover is presumably high, such as the end of the growing season. In our study, the observed root length losses were very low in all growing seasons (1–26% per year). Although we did track a few short-lived roots that disappeared within 22 days, the vast majority of roots remained visible in minirhizotron tubes much longer. It is possible we missed some short-lived roots between censuses, but the mean time interval used in spring and summer (44 days, in some cases 22 days) does not seem too long for grasses and forbs. In a meta-analysis by Chen & Brassard (2013), the lowest root life span for these functional groups was 100 and 60 days, respectively; Arnone *et al.* (2000) reported 100–225 days in *Bromus erectus*, and Van der Krift & Berendse (2002) found 100–400 days in *Lolium perenne*, *Nardus stricta* and *Molinia caerulea*.

The application of the molecular markers on the mixed root biomass samples is of great value in identifying the discrepancy between the two methods. Using the molecular technique, we identified a major shift in below-ground species composition over time. Initially, *A. odoratum* was the most productive species, accounting for more than 50% of root biomass in the mixture in the first year and the others each representing approximately 15% [see Fig. S1, Supporting information and Mommer *et al.* (2010)]. However, after the first year, *F. rubra* became particularly dominant, increasing from 50% in the second year to 95% of the root biomass in the final year. Applying these percentages to root length data, this means that from 2006 to 2009, root length of *F. rubra* in mixtures should have increased from approximately 70 m m^{-2} in the first year (15% of 400 m m^{-2} in the mixture) to almost 700 m m^{-2} in 2009 (95% of 700 m m^{-2} in the mixture),that is roughly 200 m m^{-2} per year. This is in fact similar to the average yearly estimate of root length production in mixture in this period

(mean ± SE: 185 ± 84 m m^{-2}), suggesting that estimates of net root production are rather similar for root biomass measurements and minirhizotron observations. Based on shifts in root biomass in mixtures, root loss of *A. odoratum* (the species that was initially dominant) in mixture from 2006 to 2009 should be 200 (50% of 400) – 28 (4% of 700) = 172 m m^{-2}. Thus, based on this species alone, root loss in mixtures should be at approximately 60 m m^{-2} per year. However, the average yearly loss rate observed in the minirhizotrons was only 5 ± 2 m m^{-2} (see also Fig. 2b), which is approximately ten times less. This matches the estimates of the iterative model we used, which suggested that root length loss rates in mixtures had indeed to be increased at least tenfold, suggesting that minirhizotrons underestimated root mortality in our experiment.

ROOT MORTALITY VS. ROOT DECOMPOSITION

Detection of root loss involves the following steps: root death + decomposition + loss of root residues from the images. A severe limitation when studying root loss is that the differences among species may be caused not only by species-specific differences in root mortality, but also by additional species-specific differences in root disappearance, that is decomposition. To incorporate the decomposition aspect of root turnover, we determined root decomposition rates of the four species of interest in a separate litterbag experiment. *F. rubra* showed the lowest decomposition rate in the litterbags and the lowest root length loss on the minirhizotron images. Similarly, *L. vulgare* showed the highest root mass decomposition rates and, together with *A. odoratum*, the highest root loss rates on minirhizotron images. Thus, the observed root loss differences among species observed in minirhizotrons are consistent with their differences in decomposition rate. However, incorporating the observed root decomposition rates in the iterative model to understand the species shift in mixtures increased the estimated root loss rate only twofold, which is still not sufficient to reach the loss rates based on the shifts in root biomass of the species in mixtures. Although we acknowledge the experimental difficulties associated with estimating decomposition rates of dead roots, we do think it is important to incorporate differences in decomposition among species in estimates of root turnover based on minirhizotron images.

Conclusion

In summary, root length production was higher and root length loss was lower in the four-species mixture compared to their respective monocultures. As root decomposition rates did not differ between monocultures and mixtures, this result suggests that root mortality is decreased with increasing species diversity. Future studies have to reveal the generality of this observation and the underlying root traits as well as interactions with soil biota. Progress in this field will require a multidisciplinary approach in which root production, root decomposition, root loss and soil biota are simultaneously taken into account. This is necessary to achieve a mechanistic understanding of the relationships between plant productivity, root biomass, root turnover and C sequestration, and to predict the consequences of changes in biodiversity and environmental conditions on ecosystem functioning in grasslands in the long term.

Acknowledgements

The authors would like to thank Gerard van der Weerden, Gerard Bögemann, Jan van Walsem and Frans Möller for technical support. LM was supported by a NWO-VENI grant, FMP acknowledged the support from postdoctoral and Juan de la Cierva grants from the Spanish Ministry of Education and Science. All authors state that there are no conflict of interests to declare.

Data accessibility

Data are deposited in the DRYAD Data Repository: http://dx.doi.org/10.5061/dryad.pd730 (Mommer *et al.* 2015).

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Received 13 January 2014; accepted 13 March 2015
Handling Editor: Shuli Niu

Supporting Information

Additional Supporting information may be found in the online version of this article:

Appendix S1. Supplementary Materials and Methods and Results.
Fig. S1. Relative species abundance in mixed root samples above and belowground over time (2006, 2007, 2009).
Fig. S2. Destructive harvests in plant communities over time: (a) standing root mass at 0–64 cm depth; (b) standing root mass at closest depth of minirhizotron tube (12–18 cm); (c) shoot production of monocultures of *Anthoxanthum odoratum*, *Festuca rubra*, *Leucanthemum vulgare* and *Plantagolanceolata*, and of mixtures of the four species (Obs. Mix).
Fig. S3. Expected Kaplan & Meier survival functions of roots born in 2006 in mixtures after simulating zero mortality of each species independently or of species combinations together.
Table S1. Effects of diversity, species identity in monoculture and time on annual shoot biomass and standing root biomass at 0–64 cm depth and 12–18 cm depth.