The importance of multi-species grassland leys to enhance ecosystem services in crop rotations

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Abstract

The ongoing simplification (temporal and spatial) of agricultural production systems has resulted in severe negative consequences, ranging from losses in soil organic carbon and biodiversity to a high dependency on external inputs to maintain high yields. This paper will identify how grassland leys in crop rotations are vital to mitigate these effects, by conserving soil organic carbon and enhancing nutrient efficiency. In particular, grasslands containing legumes enhance these benefits by providing nitrogen. In crop rotations, these grasslands transfer some of the acquired nitrogen to arable follow-on crops, thereby reducing the necessity for external inputs, while at the same time providing additional benefits, such as improvement of soil quality and reduction of weed pressure. However, our knowledge about the possibilities of enhancing these ecosystem services by optimising the community composition of the leys remains patchy. While the benefits of multi-species grasslands for the grassland crop have been shown repeatedly and across a large gradient of environments, further research is required to determine the benefits for follow-on crops, particularly across environments. Here, we emphasize the importance of multi-site research, such as in the research network LegacyNet. A further important question is the potential role of the functional plant diversity of the leys to achieve a range of objectives in different types of rotations, and this paper will explore the state of knowledge about this role. Finally, management techniques that are optimised for both ecosystem services and agronomic performance will be presented for cut and grazed systems. For the latter, an outlook will also be presented as to how the inclusion of bioactive plant species can additionally enhance animal health and lower methane emissions in grazing ruminants.

Keywords: crop rotations, diversification, sustainability, nutrient cycling, circularity, LegacyNet

Simplification of agricultural systems increases input dependence

Agricultural production systems have continuously become more specialized, thereby enabling an intensified production, and today less than 10% of the agricultural area in the United States and Western, Central and Northern Europe are used as Integrated Crop Livestock Systems (Garrett *et al.*, 2020). However, this led to an ongoing simplification of production systems at both temporal (i.e. monocropping) and spatial (monocultures and simplified landscapes) level. As a result, many regions developed high densities in either continuous cropping of arable crops, or livestock production with high-energy, low-fibre rations that often contain a large share of grain. Due to the arising spatial separation, manure often needs to be processed and transported to regions with lower animal density at substantial cost to avoid excessive nitrogen loads to groundwater. The processing and enhanced storage duration of manure increases ammonia volatilisation, thereby reducing the nitrogen (N) efficiency of dairy farms below 30% (Löw *et al.*, 2020). Simultaneously, 72 kg of nitrogen in the form of mineral fertilizer are applied per hectare and year on average across the EU, with large regional differences (De Vries *et al.*, 2021). A more diversified approach based on integrated crop livestock systems would render these transports and high mineral N amounts unnecessary. Thus, while providing high yield increments, the simplification of agricultural systems has resulted in severe negative consequences, ranging from losses in soil organic carbon to a high dependency on external inputs (Lemaire *et al.*, 2015). For example, global cereal yields have increased by almost 4-fold between 1961 and 2016, without expanding the production area, yet during the same time the global mineral N fertilizer production has increased by almost a factor of 10, and arrived at 123,000,000 Mg N annually in 2018 (Kopittke *et al.*, 2019). This is problematic for environmental reasons, as each kg of N fertilizer produces greenhouse gas (GHG) emissions in the range of 1.3-4 kg CO_{2eq} kg^{-1} N for urea, and 3.5-10.3 kg CO_{2eq} kg^{-1} N for ammonium nitrate (Walling and Vaneeckhaute, 2020).

Similarly, the specialisation of agriculture has resulted in substantial losses of soil organic carbon in the regions dedicated to crop production. Moreover, the increasing amount of concentrate feed in ruminant production systems is understood to have resulted in reduced grassland use throughout Europe (Van den Pol-van Dasselaar *et al.*, 2020), and thus have contributed to losses of soil organic carbon. This has direct implications for soil organic carbon stocks for three reasons: (a) grasslands have a dense rooting system, where more of the total biomass is stored belowground compared to annual crops, and even more so in species rich grasslands, (b) grasslands have a year-round soil cover with no bare ground being exposed to erosion, and (c) the amount of mechanical disturbance is reduced substantially in grasslands, thereby allowing less soil organic carbon oxidation and mobilisation. Thus, crop rotations with grassland leys are advocated to be a viable alternative that produces both arable crops and forage, while also maintaining carbon stocks. However, little is known about the general applicability of these findings across environmental conditions, leys community composition and management types. Consequently, the goal of this review paper is to address the following research questions:

- Can higher diversity in grassland leys increase ecosystem service performance and affect agronomic performance of the follow-on crop?
- How can multisite experiments help better investigate the impact of management and environmental conditions on the effects of grassland leys?
- What is the effect of more diverse plant functional traits in grassland leys on performance at the system level? and
- What is the future outlook for diverse grassland leys?

Spatial diversity: diverse grasslands are an important tool to enhance circularity expectations of leys

Historically, leys hugely contributed to improve agricultural production by replacing the grazed fallow of the medieval three-field system (Stebler, 1895). It is therefore obvious that expectations of leys include a high production of high-quality forage, as well as the maintenance of an adequate soil structure for cropping (Hoeffner *et al.*, 2021). As an important element of the crop rotation, it is moreover expected that leys suppress crop weeds and soil-borne diseases (Martin *et al.*, 2020). For instance, Dominschek *et al.* (2021) observed that weed biomass in maize (*Zea mays*) was more reduced after three years of ley than after a sunflower (*Helianthus annuus*)-maize-sunflower sequence because the relative abundance of fast-growing weeds was suppressed. Because ley cropping allows the selection of a mix of forage species and varieties with specific properties, it allows the design of forage mixtures that prioritise various functions, types of utilisation and pedo-climatic conditions (Lüscher *et al.,* 2019). For commercial seed mixtures, the range of utility nevertheless needs to remain broad enough for economic efficiency. Lately, resilience to severe weather events, resource use efficiency and multifunctionality received increased attention, as well as the potential role of plant diversity within the leys to target these multiple functions. Here, we review evidence of the effects of plant diversity on the main roles of leys.

Principles for the design of mixtures

A mixture may consist of a blend of varieties within a single species, multiple species or a combination of multiple species and multiple varieties within species. The benefits of associating contrasting complementary traits (e.g. N_2 fixing root system of legumes with capture-efficient root system of grasses) has been demonstrated in a number of studies (*e.g.* Finn *et al.*, 2013; Husse *et al.*, 2017; Nyfeler *et al.*, 2011; Suter *et al.*, 2021). Because such large contrasts in plant traits are usually not found among varieties within the same species, associating multiple species is an essential building block of forage mixtures. Emerging from experimental manipulations of plant diversity is a strong signal that (a) the identity of the plant species is crucial, and (b) more balanced mixtures tend to maximise the benefits of synergistic interspecific interactions and deliver the strongest yield responses (Lüscher *et al.,* 2019). Strong mixing effects have been achieved with four well selected species (Connolly *et al.*, 2018; Finn *et al.*, 2013) and the positive effect on biomass productivity of adding a supplementary species has been shown to decrease with the number of species already included in the mixture (Weisser *et al.*, 2017). Thus, a large number of constituent species may not be required for maximized agronomic performances. This could also be the case for multifunctionality (Gamfeldt and Roger, 2017), as observed in the experiment of Suter *et al.* (2021) in which four species (2 grasses and 2 legumes) were sufficient to induce concurrent diversity effects on yield, yield stability, weed suppression, symbiotic N_2 fixation and N efficiency, while maintaining forage quality. However, combining complementary varieties within species may further improve the agronomic performances of the mixture (Meilhac *et al.*, 2019; Prieto *et al.*, 2015).

Forage quality for livestock

Mixing grass and legume species allows for a more balanced forage in terms of energy and protein contents as compared to pure swards of either grasses or legumes (Lüscher *et al.*, 2014). Forage digestibility and protein content decrease with increasing plant maturity and the associated formation of structural tissues. Thus, for a given sward, forage digestibility and protein content usually decrease with increasing yield. Nevertheless, forage yield can be significantly increased without compromising forage digestibility and protein content by mixing grass and legume species (Sturludóttir *et al.*, 2014; Suter *et al.*, 2021). Moreover, positive mixture effects on forage voluntary intake by livestock have been observed with a binary mixture of perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*), compared to perennial ryegrass and chicory (*Cichorium intybus*) mixtures (Niderkorn *et al.*, 2017, 2019). Similarly, Soder *et al.* (2006, 2007) observed that the ingestive grazing behaviour of dairy cows (grazing time, biting rate, and grazing jaw movements) were similar with a mixture containing nine species (grasses, legumes, and chicory) as with a binary mixture of cocksfoot (*Dactylis glomerata*) and white clover (Soder *et al.*, 2006, 2007). This is in accordance with a study by Loza *et al.* (2021), in which dairy cows grazing a complex 8-species mixture had higher milk yields than those cows grazing a simple binary mixture, which was likely a result of their higher herbage intake.

Benefits to yield across environmental conditions

The role of plant diversity in increasing yields has been reviewed recently e.g. (Jaramillo *et al.*, 2021; Lüscher *et al.*, 2022) and we refer briefly to it here. Climate change is already affecting both the mean and variance in climate conditions, with consequent increases in the incidence of severe weather events, including drought. Higher plant diversity within productive grassland communities is associated with higher drought resilience than less diverse mixtures or monocultures (Hofer *et al.*, 2016; Komainda *et al.*, 2020; Skinner, 2005). The strength of the relationship varies across studies and the degree of overyielding can be sufficiently strong for the more diverse mixture communities under drought to have similar or greater yields than either the average of the monoculture yields in rainfed controls (Finn *et al.*, 2018; Grange *et al.*, 2021; Hofer *et al.*, 2016) or perennial ryegrass monocultures with higher nitrogen levels (Grange *et al.*, 2021). In related drought experiments, plant diversity in mixtures also increased yield stability (Grange *et al.*, 2022; Haughey *et al.*, 2018). Finally, synergistic interspecific interactions can also occur in drought conditions, and mitigate drought effects on yield (see Lüscher *et al.,* 2022).

Nitrogen efficiency and losses

At the same level of moderate N fertilization, positive diversity effects on yield are usually produced by the interaction between grass and legumes (e.g. Nyfeler *et al.,* 2009). Correspondingly, the presence of N_2-fixing legumes grown in association with grasses did not increase N leaching as compared to pure grass swards when levels of N fertilization were adapted to crop requirements (Bracken *et al.*, 2020). Moreover, legume-based multi-species leys may have a reducing effect on N_2O emissions (Peoples *et al.*, 2019). Cummins *et al.* (2021) have observed similar N yield and DM yield from a six-species mixture compared to a perennial ryegrass monoculture while reducing N_2O emissions by 41 and 24%, respectively. Other trait contrasts that result in positive diversity impacts on yield are the combination of fast establishing and persistent species or the combination of shallow and deep rooting species (Hofer *et al.*, 2017; Husse *et al.*, 2017), however with lesser extent and less robustness than the grass-legume interaction (Finn *et al.*, 2013). A further trait that could increase N efficiency of the ley is a more thorough capture of soil available N by vertical and temporal complementarity in N capture among the species of the community, induced by contrasting rooting depth and growth pattern (Husse *et al.*, 2017). At the crop rotation level, the months following the destruction of the ley should also be included, as N leaching is generally rather low during the period of ley cultivation (Valkama *et al.*, 2016) but may be large following ley destruction (Eriksen *et al.*, 2015; Hansen *et al.*, 2019). Furthermore, residual N from ley cultivation can significantly reduce the need for fertilizer by the following crop (Fox *et al.*, 2020).

Can grassland mixtures show yield benefits within the restricted temporal duration of a ley?

Permanent grasslands are defined by not being ploughed more than every five years. In multi-year experiments using special types of species-rich grasslands, the benefit of diversity generally increases over time; for example, Van Ruijven and Berendse (2005) found no effect of diversity in the establishment year, but the effect was significant from year 2. Grassland leys, in contrast, are characterised by their short duration. A key question, therefore, is: can the known benefits of productive grassland mixtures be evident within a period of twelve to eighteen months? Here, we look at field experiments that manipulate plant diversity in productive grasslands, and present evidence on the effect of mixture diversity within that time frame.

Overall, there are strong responses of mixture yields to diversity within an eighteen-month duration. In the AgroDiversity experiment that compared monocultures and mixtures of two grasses and two legumes across 31 different international locations, there were strong diversity effects and regular occurrence of transgressive overyielding in the first full year of yield measurements after sowing (Kirwan *et al.*, 2007). An associated experiment compared the effects of three nitrogen levels (Nyfeler *et al.*, 2011), and found strong responses to diversity in the first year of measurements. An experiment across three sites that included four-species mixtures of a grass, two legumes and a herb also found responses to diversity in the first year of harvest sampling (Hofer *et al.*, 2016). A six-species experiment investigating communities assembled from two grasses, two legumes and two herbs also found strong responses to plant diversity in the first full year of yield measurements after sowing (Grange *et al.*, 2021). Using three-species mixtures of a grass, a legume and a herb, Cong *et al.* (2018b) also showed strong yield responses to diversity in year 1, when the included herb was ribwort plantain (*Plantago lanceolata*). All of these studies were conducted under a harvesting regime and were not grazed.

With respect to the temporal duration of leys and diversity benefits, the temporal evolution of the botanical composition of the grasslands should also be considered. Indeed, more balanced mixtures tend to maximise the benefits of synergistic interspecific interactions and deliver the strongest yield responses. On the other hand, mixture compositions often tend to get less balanced over time. For instance, legume relative abundance often decreases from the first to the third year of ley cultivation in favour of grass relative abundance (Brophy *et al.*, 2017; Lüscher *et al.*, 2014), with the corresponding decrease in ley performance. On the other hand, several benefits in a crop rotation are generally considered to increase with increasing ley duration (Hu and Chabbi, 2022; Lemaire *et al.*, 2015). Thus, there is a need to assess the optimal ley duration for maximal benefits from synergistic interspecific interactions.

Nitrogen legacy effects of grassland leys

A substantial amount of research has investigated the conversion from grassland to arable crops (and *vice versa*) within crop rotations, and the abiotic and biotic consequences of this (Creme *et al.*, 2018; Hoeffner *et al.*, 2021; Martin *et al.*, 2020). A variety of legacy effects are possible that include the stocks and flows of specific nutrients (especially carbon and nitrogen); attributes of soil structure and hydrology; incidence of weeds, pests and pathogens; soil biodiversity, and crop or forage production. Yet there has been much less research on the legacy effects associated with the diversity of grassland species in a grassland ley.

Fox *et al.* (2020) used a systematically varying proportion of legumes across plant communities assembled from a grass, two legumes and a herb, to find that the yield of a follow-on monoculture of Italian ryegrass (*Lolium multiflorum*) (the legacy effect) was strongly related to the legume proportion. Mixture communities with >20% legume proportion had a significantly higher legacy effect than that from grass monocultures. Communities with 50% legume proportion in the preceding grassland exerted the same legacy effect as a 100% legume proportion (legume monoculture), and the legacy effect was evident for at least 12 months. Similarly, in a six-species experiment by Grange *et al.* (2022) an experimental drought was imposed across all communities, and compared with the rainfed control. Furthermore, all plots received 150 kg N ha^{-1} yr^{-1} of nitrogen fertiliser (150N) in the grassland phase, but were compared to a high N perennial ryegrass monoculture receiving 300 kg N ha^{-1} yr^{-1} of N (300N). They found a strong legacy effect that was related to the legume proportion in the grassland ley, and persisted across four successive yields over four months. The legacy effect was negatively affected by the drought, but to a modest degree in comparison to the effect of legume proportion. In this experiment, the legume proportion in the pre-crop was positively correlated with the benefits in the follow-on crop. Accordingly, at the crop rotation level, the legume monoculture resulted in the highest N transfer to the follow-on crop. This is different to the legume derived N benefits in the grassland ley itself, where a legume share of 25-33% has generally been sufficient to provide the maximum Nitrogen yield across a large gradient of environments (Suter *et al.*, 2015). Finally, Komainda *et al.* (2022) also observed that the yield of a follow-on crop of Italian ryegrass with a simple mixture as pre-crop was only 45% of the yield that was obtained when the pre-crop was a more complex lucerne-dominated multispecies grassland mixture.

Additional benefits of grassland leys in crop rotations

In the AgroDiversity experiment mentioned above, combining two grass and two legume species also strongly improved weed suppression during the period of ley cultivation, especially compared to the legume pure swards, but also compared to the grass pure swards (Connolly *et al.*, 2018). For the function of weed suppression, the interaction between grass and legume species had the strongest positive effect, like for yield, but the interaction between the fast and the persistent species played a major role as well. Thus, multi-species leys are more stable against weed invasion and might therefore help reducing the use of herbicide. Additional benefits of grasslands in crop rotations have for example been illustrated by Colombi *et al.* (2017), who showed that in compacted soils, soil macropores from tap-roots are a tool to provide a path of least resistance, as well as oxygen, thereby enhancing yields of wheat (*Triticum aestivum*), maize and soybean (*Glycine max*) as follow-on crops. However, Kautz *et al.* (2014) showed that even in non-compacted soils, taproots from chicory and lucerne (*Medicago sativa*) resulted in an increased density of biopores compared to tall fescue (*Festuca arundinacea*) when grown for up to 3 years as a ley. In the same time frame, anecic earthworms (*Lumbricus terrestris*) were unable to markedly increase the sub-soil's biopore density, indicating that in these ley durations, deep-rooting species with tap-roots have a stronger importance on the creation of biopores than earthworms (Kautz *et al.*, 2014). In grass-clover swards with added manure, however, it was identified that 3 years are sufficient to create biopores that aid the increased impact of the epi-anecic earthworm species *Aporrectodea longa* and *Lumbricus herculeus* (Krogh *et al.*, 2021). These benefits have been shown before and are especially prominent in no till systems, where the soil structure is not destroyed during the conversion of a follow-on crop (Alhameid *et al.*, 2020). Yet, while little research has been conducted on the potential of multispecies leys directly to increase macropores via the inclusion of forages with deep tap-roots, similar studies on multispecies cover crops indicated their suitability to establish macropores in even a shorter time frame, especially if these cover crops were grazed, thereby allocating more resources to form root biomass (Singh *et al.*, 2021). Thus, further research on the potential of multispecies grassland leys and their impact on macropore creation for follow-on crops would be promising.

Flower resources for pollinators

Pollination is important for maintenance of wildflower communities and crop production as many crops are partially or fully dependent on animal pollination for seed or fruit set (Klein *et al.*, 2006), but a historical decline is documented for wild pollinators in Europe (Biesmeijer *et al.*, 2006). However, multispecies grasslands can be designed to support a higher diversity of pollinators if plants with different pollinator profiles are included in flower mixtures to enhance flower-visiting insects (Cong *et al.*, 2020). In a comparison of grassland leys with increasing levels of diversity compared to a conventional monoculture of perennial ryegrass as permanent grassland, pollinator abundance increased drastically, with 541 wild bees of 10 species in the diverse grassland leys, compared to no wild bees in the permanent grassland due to the absence of flowers. While pollinator abundance was not affected by the grassland ley diversity, the simple mixtures consisting of perennial ryegrass and white clover were only visited by common generalist species, whereas the multispecies grassland ley increased the abundance of rare long-tongued bumblebee to 10% in grazed areas, and even to 20% in ungrazed exclosures (Beye *et al.*, 2022). Typically, legumes were visited mainly by large bees (honeybees and bumblebees) while some of the forbs attracted syrphids and other flies. Attracting pollinators with nectar delivering flowers must imperatively be combined with an adapted management, as mowing at an unsuitable time can amount to a fatal trap for the insects (Kenyeres and Varga, 2023). Spatially heterogeneous management can better support bees and hoverflies through delayed mowing and leaving uncut refuges in extensively managed grasslands (Meyer *et al.*, 2017). An alternative to wildflower strips to achieve this is to systematically leave strips of uncut multispecies grassland at each mowing date, to be included in the following harvest (Cong *et al.*, 2020). This may improve resource availability for pollinators considerably, with only marginal yield and quality loss.

Tailoring management for different ecosystem service priorities

Management is an important tool to steer the performance of swards and crop rotations between maximised yields and maximised ecosystem services. From an agronomic point of view, ploughing-in of grassland provided the highest benefits for the follow-on crop in a crop rotation, combined with a ley duration that spans several years. Accordingly, the incorporation of a three-year-old grassland provided higher gains compared to an annual grass-clover sward, arable legume (field bean) or the manure fertilization of previous crop (Kayser *et al.*, 2010). With regards to the reduction of environmental disservices, nitrate leaching was lowest if the arable crop in the crop rotation was undersown with a catch crop to ensure an additional uptake of nitrogen in autumn (Biernat *et al.*, 2020; Eriksen *et al.*, 2015; Hansen *et al.*, 2007). Accordingly, barley (*Hordeum vulgare*) with an undersown Italian ryegrass (the barley was harvested as whole crop silage to permit enough time for the Italian ryegrass to establish) was the only crop in a study by Eriksen *et al.* (2015) to result in low nitrate leaching of less than 10 kg N ha^{-1} yr^{-1}, whereas maize as a follow-on crop resulted in high nitrate leaching amounts of more than 50 kg N ha^{-1} yr^{-1} for five consecutive years.

Regarding the C inputs, grasslands leys and permanent grassland can actually exhibit similar carbon inputs, but permanent grasslands retain the carbon longer in the soil, thus only permanent grasslands result in substantially enhanced C stocks (Hu and Chabbi, 2022).

As a result, the modelling of changes in soil organic carbon (SOC) stocks, based on a 7-year experiment in Northern Germany and initial SOC stocks of around 50 Mg ha^{-1}, estimated annual losses across a 100 year time horizon to be on average 76 kg C ha^{-1} yr^{-1} for the monocropping of silage maize, fertilized with 240 kg N ha^{-1} $yr^{-1}$from cattle slurry, compared to gains in SOC of 413 kg C ha^{-1} yr^{-1} in an equally fertilized permanent grassland. In that same experiment, a crop rotation consisting of two years of grassland, followed by maize and winter wheat resulted in slight annual gains of 15 kg C ha^{-1} (Loges *et al.*, 2018). This is in accordance with a study by Jensen *et al.*, 2022, who identified that introducing a 2-year-old grass-clover ley into a six-year arable crop rotation increased carbon stocks by 5 Mg ha^{-1}, before a new steady-state condition was achieved after 20 years. Subsequent conversion to a 4-year grass-clover ley resulted in additional C stock increments of 4.2 Mg ha^{-1}, with no new steady state being achieved after 13 years. Similarly, Guillaume *et al.* (2022) have shown a clear benefit of leys for improved SOC, with a positive correlation between the proportion of leys in the crop rotation and SOC stocks (Figure 1). This is due to a combination of the negative impact of tillage on SOC stocks (Haddaway *et al.*, 2017) and the fact that the root biomass only reaches its maximum in the second year (Weisser *et al.*, 2017). After that peak, only grassland mixtures with a higher species richness were able to maintain high levels of root biomass, whereas grass monocultures had decreased their root biomass in year five by more than 50% compared to the first experimental year, and by approximately 75% compared to the maximum root biomass.

To balance soil C stocks and food production, Hu and Chabbi (2022) proposed the incorporation of grassland leys with high N application for three years embedded into a crop rotation. However, with regards to the high N fertilisation, this should not be combined with grazing, as the combination of grazing and slurry application has generally resulted in the highest leaching rates of grasslands (Eriksen *et al.*, 2015), whereas cutting generally produced the lowest leaching rates (Wachendorf *et al.*, 2004). However, reducing fertilizer to reduce nitrate leaching or gaseous emissions also reduces C sequestration, thereby resulting in a trade-off between C accumulation and N losses, as well as yields (Allard *et al.*, 2007)

Performance of diverse crop rotations is dependent on environmental and soil conditions

A recent meta-analysis from China has shown that the potential yield benefits of crop rotations are largest if: initial soil nitrogen concentrations are low; soil organic carbon stocks are intermediate; and soil textures are coarse to intermediate (Zhao *et al.*, 2020). This is to be expected, that yield gains are largest under conditions where nutrient statuses are most limiting. However, environmental conditions also affect the soil microbial conditions and with that the turnover of residual biomass and the transfer of nutrients in a crop rotation. For example, using laboratory conditions, a study by Taghizadeh-Toosi *et al.* (2021) found that soil moisture levels of 60% water-filled pore space (WFPS) resulted in higher N mineralisation rates of red clover residues compared to 40% WFPS. Furthermore, a previous cropping history of diverse crop rotations instead of cereal monocropping has been shown to result in up to 80% higher recovery of nitrogen in follow-on crops, both under drought and controlled conditions (Bowles *et al.*, 2022). As this is likely a result of changes in the soil microbial composition, soil-plant-microbial interactions will need to be analysed in detail to understand their impact on the N transfers. Repeated drying-rewetting cycles might have particularly long-lasting impacts on C and N dynamics and increase N losses from the system via enhanced nitrifier activity (Fierer and Schimel, 2002).

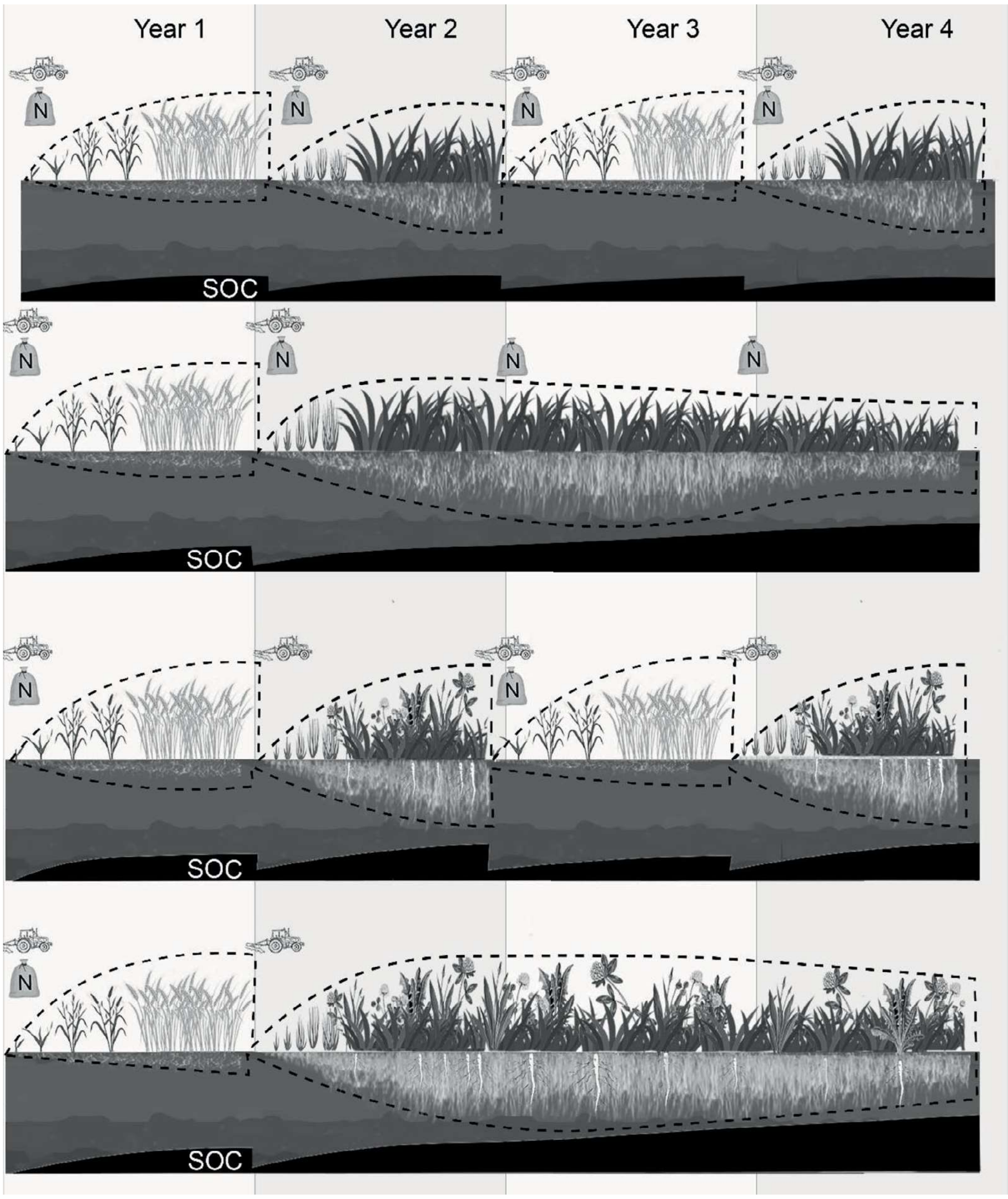

Figure 1. Conceptual representation of a crop rotation comprising a cereal crop followed by grassland leys of different duration (short ley: a,c; long ley: b,d) and species diversity (grass monocultures in the leys in panels a and b; multispecies grassland mixtures in panels c and d) on above- and belowground biomass production over time (dashed lines), as well as soil organic carbon (SOC) stock changes over time (black area at the bottom of each panel). Pictograms of tractors indicate ploughing events, while images of fertiliser bags of N indicate requirement of large amounts of external fertilizer applications. Overall, ley duration affects soil carbon stocks; legume-based mixtures reduce the requirement for nitrogen fertiliser inputs; mixtures maintain above- and below-ground biomass over time, and; grasslands have more root biomass than cereals.

New experimental approaches to account for multitude of factors affecting ecosystem services

The impact of environmental conditions, soil types and the resulting plant-soil-microbiome interactions require our experimental approaches to account for these levels of complexity. If an experiment is repeated at multiple sites, the inference and generalisability of the results from the experiment can be enhanced compared to a single-site study. If multiple sites are selected at random across a country (or alternative spatial scale) and a common experiment implemented at each site, the results can generalise to the scale of the country, whereas the results from a single-site study may be confounded with the conditions of the selected site. For example, in a grassland biodiversity experiment, the species diversity and other treatment outcomes may be affected by factors such as the soil type, previous land use history and climatic conditions at the site (Finn *et al.*, 2013). However, the impact of these differences across sites requires a revised understanding of experimental, as well as analytical approaches.

There are several ways to analyse data from multi-site experiments; for example, including site as a random effect in a linear mixed modelling framework would account for the hierarchical nature of the data. However, the effects from experimentally manipulated treatments could vary from site to site and in this case, random coefficient models, where individual model parameters are assumed to vary from site to site, may be more suitable. Such approaches account for site-to-site variation and appropriately model the correlations between observations from the same site.

One example of a multi-site international experiment that attempts to prioritize the potential generalization of the impact of grassland leys in crop rotations across environments and soil types is the LegacyNet experiment (LegacyNet 2023). In this study, a common experiment is implemented at 32 sites spread across Asia, Europe, New Zealand and North America. The experiment aims at understanding how increased diversity in grassland leys can enhance not only the performance of the ley itself, but also in a follow-on crop. Therefore, the experiment consists of a grassland ley phase of at least 18 months, followed by a follow-on crop phase. In the grassland stage, systematically varying combinations of six forage species (two grasses, two legumes, two herbs), are established and dry matter and botanic composition and nitrogen concentration will be measured. The follow-on crop is then a monoculture of either a grass 'model' crop, or a cereal. Yield, nitrogen yield and other quality variables are recorded on each plot in this phase, as the project assumes that even the recording of a small number of responses can lead to deep knowledge increase, via the value from the distributed and coordinated data collection effort across multiple sites.

Thus, this approach enabled considerable variation of sites across geographical, environmental and soil conditions, and the results of this study will be powerful in testing the robustness of species diversity.

Re-evaluating traditions and preparing them for the future

Bioactive herbs and legumes that contain tannins or other plant specialized metabolites (PSM) have been pursued as a promising route to be included in future grasslands. The PSM in these plants have been identified to act anthelmintic, reduce methane emissions from ruminants and potentially enhance carbon stabilization rates in the soil (Adamczyk *et al.*, 2017; Mueller-Harvey *et al.*, 2018). Consequently, their inclusion in grasslands could reduce the dependence on external inputs further, while generating healthy and nutritious diverse forages. However, to date, the agronomic potential of these bioactive species is often low and the bioactivity has been too variable as a result of environmental impacts and a large variability within species or even cultivars, due to lack of breeding efforts (Verma *et al.*, 2021). Therefore, further efforts will be required to achieve a large share of continuously high bioactivity.

In the future, grasslands may also play a more important role in stockless plant production systems. Not only for maintaining soil fertility and combat weed problems, but also for delivering bioenergy. There is an increasing interest in using grassland biomass for bioenergy production in Europe and North America (Prochnow *et al.*, 2009; Wahid *et al.*, 2018), which is further intensified with the current energy crisis. Biogas production can be enhanced through mixing of two or more substrates with complementary characteristics leading to improved efficiency of microorganisms involved in anaerobic digestion. It has been demonstrated that co-digestion of grass and forbs (chicory and plantain) synergistically enhanced methane yields (Cong *et al.*, 2018a). The synergistic effects were attributed to a more balanced nutrient composition (C/N ratio and micronutrients) in the grass-forb mixture promoting the utilization of multi-species grasslands for bioenergy production.

Conclusions

Increasing species richness has been shown to provide substantial benefits for grassland leys by reducing external inputs and enhancing ecosystem services. Similar benefits have been indicated at the systems level in crop rotations, but complex plant-soil-microbe interactions make it difficult to generalize and quantify these benefits. Future developments will, however, increase the importance of grasslands both in mixed and stockless plant production systems, making the research at the system level even more pressing. Therefore, more multisite experiments will be required to gain an understanding of the extent of these benefits across a gradient of soil and environmental conditions.

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