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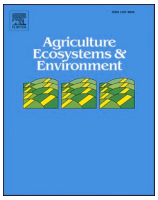

Effect of 20-years crop rotation and different strategies of fertilization on weed seedbank

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ABSTRACT

Crop rotation is thought to reduce weed density and maintain species diversity, preventing the domination of few competitive weeds. In this work rotations of 1, 2, 4 and 6 years length have been compared in a long-term experiment since 1976. In order to detect the effect of rotation length and fertilization on weed community evolution, a specific study was performed on weed seedbank with soil sampling in 1993 and 2012.
Results: show that weed density was not affected by rotation length or fertilization type or rate, and that about 98% of weed species were indifferent to the factors applied. The dominance of *Portulaca oleracea* and increment of a few grass weeds were consequences of an inadequate management of late emergence and post-harvest weeds. Furthermore, no significant changes in the distribution of seeds longevity groups occurred in the seedbank from 1993 to 2012. In cropping systems where herbicides are used according to best practices or to Integrated Weed Management principles, the effect of rotation on weeds is hard to detect even in long-term experiments. For this, the effect of herbicides must be included in a more general theory of rotational effects.

1. Introduction

One of the most important limiting factors for crop production is weed interference. Weeds cause significant losses in crop yield and quality, and changes in weed flora often occur in response to alterations of crop management practices, making their control a complex task (Karkanis et al., 2018; Knezevic et al., 2002; Zimdahl, 2007). The changes in weed flora could also trig for other changes of the agro-ecosystem, i.e. increasing or reducing the diversity of insects, arthropods, birds or mammals that feed on weed species or use them as shelter (Balfour and Ratnieks, 2022; Capinera, 2005; Norris and Kogan, 2005).

Manipulation of cropping systems to improve weed management requires a better understanding of the spatial and temporal dynamics of weed seeds, and it is widely acknowledged that one of the most important practices influencing seedbanks is crop rotation and the soil disturbance these rotations cause (Cardina et al., 2002; Hosseini et al., 2014).

Crop rotation is a planned sequence of crops grown in the same field year after year, which adds variability to the cropping system and increases its sustainability. Crop rotation puts a variable selection pressure on weeds, preventing anyone becoming problematic. Continuous cropping with the same crop is most likely to become infested with a few competitive weeds as they become adapted to the system. Many studies have documented changes in both weed flora and seedbanks in response to crop sequence (Blackshaw et al., 2001; Légère et al., 2005).

Since cropping sequence dictates other agricultural management practices, variations in weed communities between cropping systems may either be the direct result of crop rotation and the result of the different associated weed management practices (Al-Hajaj, 2021; Guareschi et al., 2020). Doucet et al. (1999) showed that the effect of crop rotation is generally not separated from that of weed management practices and the latter explained 37.9% of total variation, while rotation accounted for only 5.5%, a very low value. Crop rotation is known to modify seedbanks, especially their composition (Adhikary and Ghosh, 2014), but Liebman and Dyck (1993) showed that in 12 experimental cases where weed seed density was reported, density in crop rotation was lower in 9 cases and equivalent in 3 cases when compared to monocultures.

Rotating crops with different life cycles favours the natural loss of weed seeds over time because it can prevent the repeated addition of new seeds to the soil, reducing seedlings emerging in the following crops. Crop rotation design can also help with weed management in

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tilled systems, still the impact of crop rotation on weed control is lower in tilled systems compared to no-till systems because of a negative interaction between tillage and rotation effects due to the longer survival of buried seeds in soils. Soil management in tilled systems can lead to replenishing the soil seedbank burying the seeds disseminated on the surface and unearthing them in following seasons when they are in optimal conditions for germination (Cardina et al., 1991; Feledyn-Szewczyk et al., 2020; Jorgensen, 2018; Travlos et al., 2020). This can lead to more weed seedlings in following years, partially neutralizing the benefit of rotation. Also different levels of soil disturbance can cause the presence of different weed flora, where in more disturbed soil there is a prevalence of annual weeds, while in lesser disturbed soils it's possible to find more species with a longer life cycle (Hosseini et al., 2014; Sagar and Mortimer, 1976). From this example it is possible to see how different cropping systems can largely influence the stability of the agroecosystems, influencing the changes of the weed flora. In addition, each crop management practice imposes a specific selection pressure on weed flora, and the most important, possibly overcoming any other effect, are seedbed preparation and herbicide use (Swanton et al., 1999). Long-term rotation studies using conventional herbicide programmes show a striking trend: weed density increases if rotations consist of one cool-season crop followed by one warm-season crop, such as winter wheat (*Triticum aestivum*)–proso millet (*Panicum miliaceum*) (Anderson, 2007). Yet, it is of interest to study the relative importance of tillage disturbance, rotation cycle length and other relatively slow-evolving crop management practices on weed community when herbicides, a fast-evolving factor, are used regularly. The density of the above-ground weed flora is appropriate for measuring rapid response to herbicide use, whereas the seedbank composition is a more sensitive measure of long-term cumulative effects of a particular management approach. The seedbank represents a photograph of the weed community more or less masked by the environment and crop management practices, which acts as an evolutionary memory. The composition of the seedbank results from the temporal change in crops and associated crop management practices (Buhler et al., 1998; Gaba et al., 2014). It is also important to notice that the weed seedbank can be impacted by different ways of seed dispersal, such as wind dispersion of small and light seeds, which can be transported to great distances (Petit et al., 2013; Rahman et al., 2001). The role of dispersion in the composition and density of the seedbank has been reported by various studies in different environments and cropping systems (Bakker et al., 1996; Mall and Singh, 2014; Quintana-Ascencio et al., 2019; Walck et al., 2005), though in open field studies this interference cannot be avoided.

In order to evaluate the effect of crop rotation length and fertilization on weed seed community evolution, a study was performed within the "long-term rotation experiment" at the Experimental Farm of Padua University, where herbicides were used according to best agricultural practices. The aim of the study was to test the effect of rotation length, type of organic fertilization and amount of mineral fertilizer on the weed seedbank.

2. Material and methods

2.1. The long-term rotation experiment

The experiment has been underway since 1962, at the experimental farm of the University of Padua (Veneto region, NE Italy 45°21′ N; 11° 58′ E; 6 m above sea level). The soil is a Fulvi-Calcaric Cambisol, according to FAO-UNESCO classification (FAO, 2006), silty or sandy loam, with a pH of 7.8. The local climate is subhumid, with annual rainfall of about 850 mm. In the median year, rainfall is highest in June (100 mm) and October (90 mm) and lowest in winter (50–60 mm). Temperatures increase from January (minimum average: −1.5 °C) to July (maximum average: 27.2 °C). The reference evapotranspiration (ETo) is 945 mm with a peak in July (5 mm d^{-1}). ETo exceeds rainfall from April to September. The site has a shallow water table ranging from about 0.5–1.5 m in late winter–early spring to 1–2 m in summer based on the Regional Agency for Environmental Protection (ARPA) data. For all crops soil tillage is autumn ploughing at 40–45 cm, followed by standard seedbed preparation operations at different times according to crop.

2.2. Factors in comparison

Since 1976, four rotations (ROT) of different length (six, four, two and one year) with two different types of organic (ORG) and three rates of mineral fertilizer (MIN) have been compared.

The 6-year rotation (6-y), consisted in rotating crops as follows: maize (*Zea mays* L.), sugarbeet (*Beta vulgaris* L.), maize, wheat (*Triticum aestivum* L.), alfalfa (*Medicago sativa* L.), alfalfa.

The 4-year rotation (4-y), consisted in: sugarbeet, soybean (*Glycine max* (L.) Merr.), wheat and maize. The 2-year rotation (2-y) was maize, wheat. And the 1-year rotation (1-y) was continuous maize.

To test the effect of organic fertilizer, application of farmyard manure (MAN) was compared with application of slurry (SLU). MAN and SLU came from the cattle livestock on the experimental farm. All rotations receiving MAN had the crop residues removed for use as livestock litter.

MAN (average composition: 20% dry matter, 0.5% N, 0.25% P2O5, 0.7% K2O) was applied at an average rate of 20 t ha^{-1} per year; SLU (average composition 10% dry matter, 0.4% N, 0.3% P2O5, 0.4% K2O) was applied at an average rate of 40 t ha^{-1} per year. Both manure and slurry were applied prior to ploughing, generally in October.

To test the effect of mineral fertilizer, three mineral fertilizer rates were compared: no fertilization (0 M); 70, 70, 90 kg ha^{-1} of N, P2O5, K2O, respectively (1 M); 140, 140, 180 kg ha^{-1} of N, P2O5, K2O, respectively (2 M). No N was applied to soybean or alfalfa.

2.3. Experimental design and weed seedbank sampling

Experimental design was a split-plot with 3 replicates (REP) in 3 blocks, with ORG in the Main plots. ROT was not a balanced factor since each rotation included a number of crops equal to length of rotation. Since the experiment included 13 unique combinations of "Crop x Rotation" (CR-ROT) each with 3 levels of MIN, a total of $13 \times 3 = 39$ Sub-plots were randomly assigned in Main plots. In total, the field trial included 78 treatments in 234 experimental units (2 ORG x 13 CR-ROT x 3 MIN x 3 REP=234).

It is important to underline that within the 13 CR-ROT unique combinations (Maize-6y, Sugarbeet-6y, Maize-6y, Wheat-6y, Alfa-alfa,1st-6y, Alfa-alfa,2nd-6y; Sugarbeet-4y, Soybean-4y, Wheat-4y, Maize-4y; Maize-2y, Wheat-2y; Maize-1y), for each specific crop the soil preparations (ploughing and harrowing) and chemical weed control were the same. For example, in a given year maize seedbed was prepared with the same equipment and treated with the same herbicides in the 6-y, 4-y, 2-y, 1-y rotations. Furthermore, herbicide and application rate were selected according to "best options for the observed weed flora", that is according to the most abundant and/or most important weed species. So the effect of herbicides would have been the same as soil preparation, i.e. neutral across unique combinations.

In order to detect the effect of rotation length and fertilization type and rate on weed flora, for each of the 234 experimental units two seedbank samplings and evaluations were done:

1) first evaluation in October 1993, after 17 years, i.e. end of first period (from 1976 to 1993);
2) second evaluation in October 2012, after a further 19 years, i.e. end of second period (from 1994 to 2012) or final evaluation.

2.4. Evaluation of seedbank

In October 1993 and 2012, before the autumn sowings, 10 soil

samples were randomly taken in the 0–30 cm horizon in the central part of each plot (4 ×5 m) with a core sampler 3 cm in diameter. This number is considered sufficient for estimating the semi-quantitative composition of the seedbank for densities above 50–60 plants m^{-2} (Dessaint et al., 1996; Mickelson and Stougaard, 2003). The 10 soil cores were mixed and placed in a cold glasshouse, arranged in drained trays half-filled with sterilized sand and with a sheet separating the soil from the sand. The sheet allowed the periodic turning of the soil in the tray. The trays were kept in larger trays which were regularly supplied with water to maintain the soil at field capacity. Seed germination was stimulated by stirring the soil periodically and with two 15-days periods of drought. The experiment lasted for 18 months. The seedlings from each tray were counted and the seedbank was expressed as number of seeds m^{-2}.

2.5. Analysis of seedbank longevity

Weed were classified in 3 groups according to seed longevity: up to one year (L1), up to three years (L2) and over three years (L3) (Otto et al., 2012). In order to evaluate changes in weed community, for each soil sample a Weed Potential Dangerousness Index (WPDI) was calculated from weed density (plants per m^2, pp m^{-2}) and longevity (group):

$$WPDI = \frac{\sum(Density * Longevity)_i}{\sum Longevity}$$

For each soil sample, the WPDI is therefore a mean of longevity according to the proportion of density for each species (*i*). The Weed density-WPDI correlation was calculated to analyze weed community transformation. When longevity groups are not uniformly distributed between densities, then density and WPDI are not correlated and WPDI can add bi-variate information on weed community structure, i.e. a shift to a less dense but more persistent seedbank. The WPDI is a synthetic index similar to the "Longevity index" used by Albrecht and Auerswald (2009).

2.6. Statistical analysis

Seedbank was analyzed with analysis of variance (ANOVA) to test differences in the number of seeds in the 78 treatments. Differences in weed flora composition were analyzed using principal component and classification analysis (PCCA).

Weed flora transformations were analyzed with Weed density-WPDI correlation, Chi-square test for longevity groups distribution and Density-Rank graph.

Original weed count data were square root transformed and ANOVA performed on transformed data. Results, plots and discussion were based on untransformed data.

2.6.1. ANOVA

To test the significance effect of ORG in Main plots, error term used was the interaction (Block x Main plot). To test the significance effect in Sub-plot, error term used was the interaction (Main plot x Sub-plot. Other effects were analyzed using the randomized complete design. Analysis was performed with the module General Linear Models Statistica 10 (StatSoft, 2011) (fixed effects). To test effect of time, data were analyzed considering the survey in 1993 and 2012 as repeated measure in a factorial ANOVA, performed to test effect of time on ROT, MIN and the 13 CR-ROT combinations.

2.6.2. PCCA

To highlight general trends in weed flora composition, species densities (i.e. variables) were analyzed with PCCA to obtain a 2-dimensional representation of all information included in the data set. Supplementary variables were included in the PCCA to highlight grouping with variables not under analysis. A total of 8 PCCAs were performed: sampling years; rotations; Manure vs Slurry; rates of mineral fertilizer; ORG x MIN combinations; ROT x MIN combinations; ROT x ORG combinations; CR-ROT combinations. The weed species (i.e. cases) were also analyzed. The 1993 and 2012 data were analyzed separately. Analysis were performed with the module Principal Component and Classification Analysis of Statistica 10 (StatSoft, 2011).

2.6.3. Weed flora transformation

In order to analyze transformation caused by combined changes in species density and seedbank longevity The Weed density-WPDI correlation was calculated for the 78 treatments for the 2 sampling years and for the 4 rotation lengths for 2 sampling years combinations (means).

In order to analyse differences in the frequencies of longevity groups, four chi-square tests were applied to test differences: 1) between expected (uniform distribution) and observed frequencies within weed groups occurrence; 2) between rotations; 4) within rotations in 1993; 4) between 1993 and 2012.

In order to analyze transformation caused by changes in diversity and single species density, the Density-Rank graph was used. More precisely, it is used to assess the uniformity of relative density of the first 10 most abundant species in each rotation, and to assess species shift from 1993 to 2012. In the Density-Rank graph, when one or few species are of higher density the line is very steep (i.e. the weed flora is less balanced), when total density is quite uniformly distributed in all species the line is not steep (i.e. the weed flora is more balanced). Comparison of species found in 1993 and 2012 allows the detection of weed transformation.

2.7. Chemical load

In the cropping system under study, herbicides selection and application timing was done according to best technical-economic options in that moment. Furthermore, treatment to a crop was done irrespective of position in rotations, i.e., in a given year, maize was treated in the same way in both the 1-y and 6-y rotation. For each rotation, the total amount of herbicides (g ha^{-1}) was calculated, with specification of the mode of action (MoA) according to HRAC herbicides classification.

3. Results and discussion

3.1. Main characteristics of the weed flora

A total of 62 species were counted both in the 1993 and 2012 sampling years. In 1993 the mean density was 9814 pp m^{-2}, in 2012 it was 20138 pp m^{-2}. The mean density in 2012 was more than double with respect to 1993 (Table 1), and about the same ratio was observed for WPDI. From this data alone it is possible to observe the extent of transformation of this agroecosystem in the 20 years span.

In all rotations the distribution of longevity groups was very similar: most species were of high (45%) or medium (30%) seed longevity, while only 25% of species were of short longevity.

In all rotations, on average, at least the 16 most abundant weeds were detected with a density above 50 plants m^{-2}, i.e. with an acceptable precision (Dessaint et al., 1996).

The most abundant weed in all rotations was *Portulaca oleracea* (POROL, Longevity=3) in both 1993 (24% of seedbank) and 2012 (49% of seedbank), with high density in all rotations and crops, i.e. considering that each year has 13 CR-ROT combinations, *P. oleracea* was the most abundant in 12 CR-ROT combinations in 1993, and in 11 CR-ROT combinations in 2012. *Arabidopsis thaliana* (ARBTH) in 1993, *Digitaria sanguinalis* (DIGSA), *Echinochloa crus-galli* (ECHCG) and *Panicum dichotomiflorum* (PANDI) in 2012 were also widely present.

In both 1993 and 2012 the most abundant weeds were characterised by medium or high seed longevity. Even if a large number of species was found, ANOVA results were unexpected: after 20 years of treatments, application of manure or slurry was without effect on weed density, effect of mineral fertilizer rate was not clear, also effect of rotation was low and unclear. This was mainly due to:

Table 1
Longevity group and density (pp m^{-2}) of the 62 weeds observed in 1993 and 2012 in the seedbank of the 4 rotations.

n	Species	Code	Long. group	1993				2012				Total
				6-y	4-y	2-y	1-y	6-y	4-y	2-y	1-y	
1	Abutilon theophrasti Medicus.	ABUTH	3	4	0	0	0	0	0	0	0	4
2	Alisma plantago-aquatica L.	ALSPA	3	0	0	0	0	3	0	0	0	3
3	Alopecurus myosuroides Hudson	ALOMY	1	0	0	9	0	3	5	10	0	28
4	Amaranthus retroflexus L.	AMARE	3	449	667	589	996	133	319	555	461	4169
5	Anagallis arvensis L.	ANGAR	3	1090	776	726	582	63	58	73	42	3410
6	Arabidopsis thaliana (L.) Heynh.	ARBTH	2	1624	842	1486	1482	84	173	188	209	6087
7	Arenaria serpyllifolia L.	ARISE	3	119	26	117	0	10	5	10	147	434
8	Bidens frondosa L.	BIDFP	2	0	0	0	47	0	0	0	0	47
9	Bromus sterilis L.	BROST	1	0	0	0	0	0	10	0	0	10
10	Capsella bursa-pastoris (L.) Medicus	CAPBP	3	965	363	525	364	646	769	890	775	5296
11	Cardamine hirsuta L.	CARHI	2	0	10	0	0	0	21	10	0	41
12	Centaurium pulchellum (Swartz) Druce	CTIPU	3	149	145	158	310	35	63	199	0	1059
13	Cerastium holosteoides Fries	CERVU	2	713	515	510	689	1009	675	680	607	5400
14	Chaenorhinum minus (L.) Lange	CHNMI	1	826	632	418	305	185	99	272	84	2821
15	Chenopodium album L.	CHEAL	3	19	24	13	0	31	10	398	921	1417
16	Chenopodium polyspermum L.	CHEPO	3	18	27	53	0	0	0	10	0	109
17	Cichorium intybus L.	CICIN	2	0	0	0	0	3	0	0	0	3
18	Cirsium arvense (L.) Scop.	CIRAR	2	6	16	10	23	3	0	10	0	69
19	Convolvulus arv. L. (seed)	CONARs	3	4	0	0	0	3	0	0	0	7
20	Conyza canadensis (L.) Cronq.	ERICA	1	108	114	179	256	10	0	0	0	668
21	Cyperus esculentus L.	CYPES	3	0	0	0	0	3	5	0	0	9
22	Datura stramonium L.	DATST	3	4	0	0	0	0	0	0	0	4
23	Digitaria sanguinalis (L.) Scop.	DIGSA	2	867	320	150	373	3950	2157	6156	1738	15711
24	Echinochloa crus-galli (L.) Beauv.	ECHCG	2	126	26	49	0	555	2790	3779	147	7472
25	Eleusine indica (L.) Gaertner	ELEIN	3	0	0	0	0	3	0	0	0	3
26	Epilobium tetragonum L.	EPIAD	1	8	10	28	0	0	0	0	0	45
27	Erigeron annuus (L.) Pers.	ERIAN	1	0	0	0	0	28	37	63	63	190
28	Erophila verna (L.) Chevall.	ERPVE	2	0	0	0	0	70	178	293	461	1002
29	Euphorbia peplus L.	EPHPE	2	0	0	13	0	0	0	0	0	13
30	Juncus bufonius L.	IUNBU	3	16	20	35	0	0	0	0	0	71
31	Kickxia elatine (L.) Dumort	KICEL	3	4	0	12	0	0	5	0	0	22
32	Lactuca serriola L.	LACSE	2	0	0	0	0	0	5	0	0	5
33	Lamium purpureum L.	LAMPU	3	16	11	0	23	7	16	10	0	83
34	Matricaria chamomilla L.	MATCH	2	308	242	731	74	318	361	419	84	2536
35	Medicago lupulina L.	MEDLU	3	8	0	0	0	0	0	10	0	18
36	Medicago sativa L.	MEDSA	3	22	0	10	25	0	0	0	0	57
37	Oxalis corniculata L.	OXACO	1	62	8	0	0	10	16	0	0	96
38	Panicum dichotomiflorum Michx.	PANDI	2	0	0	0	0	1176	293	1047	649	3165
39	Papaver rhoeas L.	PAPRH	3	147	149	270	101	91	162	241	21	1182
40	Phytolacca americana L.	PHTAM	3	0	0	0	0	3	0	0	0	3
41	Pimpinella anisum L.	PIMAN	1	0	0	0	0	0	0	0	21	21
42	Plantago major L.	PLAMA	3	447	199	121	181	230	199	63	21	1462
43	Poa annua L.	POAAN	2	102	15	18	25	108	246	63	188	765
44	Poa trivialis L.	POATR	2	82	43	98	0	84	204	136	63	709
45	Polygonum aviculare L.	POLAV	3	58	16	33	0	24	10	31	0	173
46	Polygonum persicaria L.	POLPE	3	103	82	35	0	0	0	0	0	220
47	Portulaca oleracea L.	POROL	3	2242	2055	3072	2867	9143	9030	13191	9443	51043
48	Rorippa sylvestris (L.) Besser	RORSY	1	0	0	0	0	94	0	0	0	94
49	Sambucus nigra L.	SAMNI	1	0	0	0	27	0	0	0	0	27
50	Senecio vulgaris L.	SENVU	2	5	5	0	0	0	0	0	0	9
51	Setaria glauca (L.) Beauv.	SETPU	3	26	0	0	23	133	173	188	126	668
52	Setaria viridis (L.) Beauv.	SETVI	3	10	0	0	0	0	0	0	0	10
53	Solanum nigrum L.	SOLNI	2	109	152	92	51	129	183	168	251	1135
54	Sonchus oleraceus L.	SONOL	2	20	5	0	27	10	5	21	21	110
55	Sorghum halepense (L.) Pers.	SORHA	3	8	0	12	0	3	5	21	0	50
56	Stellaria media (L.) Vill.	STEME	3	226	148	146	281	147	188	52	126	1313
57	Taraxacum officinale Weber	TAROF	1	2	0	14	0	7	5	10	0	38
58	Trifolium repens L.	TRFRE	3	33	7	0	0	7	5	31	0	83
59	Verbena officinalis L.	VEBOF	2	3	0	0	0	0	0	0	0	3
60	Veronica hederifolia L.	VERHE	2	35	37	22	0	14	5	94	126	333
61	Veronica persica Poiret	VERPE	3	117	26	51	51	45	42	136	84	551
62	Vitis vinifera L.	VITVI	1	0	0	13	0	0	0	0	0	13
	All species			11308	7730	9816	9185	18618	18536	29533	16876	121602

1) great variability between crops within the rotation;
2) great increase of one or a few weed species that may have masked other effects. This is consistent with the results of Wilson (1988) showing that 70–90% of the total seedbank belongs to a few dominant species. This point is of great interest since rotation is considered an important balancing factor. In this study the most abundant species was *P. oleracea*. Its high abundance was observed across all crop-rotation combinations and years, likely because of an inadequate weed management between main crops in the case of 6-y, 4-y, 2-y rotations (inter-cropping management), or incomplete control in continuous maize (1-y), since in the Po Valley *P. oleracea* emerges after the standard post-emergence treatment.
3) very strong interference from chemical weed control. It is likely that effect of rotation length was overcome by the effect of herbicides. The similar weed density in the 6-y and 1-y rotations suggest that a rational use of chemicals can substitute rotation as a disturbance

factor, i.e. varying active ingredient and application timing can effectively manage weeds even without crop rotation. This also shows the strong effect of herbicides on weed flora transformation. Conclusions based on weed density can be affected by environmental factors (rainfall, temperature, soil nutrients). Nevertheless, results can still be reliable as long the effect of these factors is the same across plots, especially because results are mainly related to *P. oleracea* and *A. thaliana*, both barochorous species.

3.2. ANOVA

3.2.1. Main plot: effect of organic fertilizer (Manure vs Slurry)

All ANOVA results are in Table 2. Main plot effect was not significant in either 1993 or 2012. In 1993 (end of first period) on average, treatments with MAN had 8999 ± 687 pp m^{-2}, treatments with SLU had 10630 ± 523 pp m^{-2} (mean+st. error). In 2012 (end of second period) on average, treatments with MAN had 20545 ± 1894 pp m^{-2}, treatments with SLU had 19730 ± 1640 pp m^{-2} (mean+st. error). This result highlights that even if the total number of seeds doubled from 1993 to 2012, the effect of organic fertilizer was not significant since the increase was observed across treatments. This is opposed to what has been found by Saulic et al. (2022), where authors indicate that the use of organic fertilizers increases the weed infestation. It is possible that different management of manure and slurry is responsible for the different effects observed.

3.2.2. Sub-plot effect

Sub-plot effect was highly significant ($p < 0.01$) in both 1993 and 2012. Weed density in the 39 unique combinations (CR-ROT x MIN) in each year differed greatly, close to a factor of ten: in 1993, weed density ranged from 2524 to 25791 pp m^{-2}, in 2012 it ranged from 7412 to 69850 pp m^{-2} (Fig. 1) and this great variability was maintained across rates of mineral fertilizer, while a relationship between density and rate of mineral fertilizer was not observed.

3.2.3. Other effects

Significant effects ($p < 0.01$) were found for rotations in 1993 and 2012. Those effects were mainly due to the difference between 6-y and 4-y in 1993, and between 2-y and 1-y in 2012. It is important to note that ranking of rotations differed between 1993 and 2012 (Fig. 1), and that in both years the mean weed density in 6-y was similar to 1-y (1993: 11308 vs 9185; 2012: 18618 vs 16876), i.e. it was very similar in the two rotation lengths where the greatest difference was expected.

Effect of mineral fertilizer was not clear because, even if significant in 1993 ($p = 0.021$) density was not proportional to fertilization rate.

3.2.4. Effect of time

Repeated measures ANOVA performed on the 2 sampling years highlight that from 1993 to 2012 weed density changed significantly ($p < 0.01$), as expected since the general mean increased from 9800 to 20000 pp m^{-2}. This increase was in all rotations, for 12 out of 13 CR-ROT combinations, for all levels of mineral fertilizer. It is worth noting that weed density was inversely proportional to mineral fertilization rate in 1993 for all rotations, and weakly directly proportional in 2012, with variability between rotations. In brief, repeated measures ANOVA performed on the 13 CR-ROT combinations highlight that the effect of time is highly significant but not related to rotation length.

3.3. PCCA

The two sampling years were strongly correlated with the first principal component (first factor) (85–95% of variance), which is representative of the density of *P. oleracea*, very abundant in both 1993 and 2012. The second principal component (second factor), of very minor importance (5–15% of variance) is correlated with the abundance of *A. thaliana* in 1993, and *D. sanguinalis*, *E. crus-galli*, and, to a lesser extent, *P. dichotomiflorum* in 2012.

The 4 rotations were strongly correlated with the first factor, but grouped independently of rotation length.

Table 2
Analysis of variance results (DoF: degrees of freedom; SS: sum of squares, MS: variance, F=MS of effect/MS of Error, P = probability).

Main Plot effect (2 levels of Org. fert.)	DoF	1993	1993	1993	1993	2012	2012	2012	2012
Source of variation		SS	MS	F	P	SS	MS	F	P
Block (Random)	2	3500	1750	1256	0443	8624	4312	1223	0450
Main Plot (Fixed)	1	5768	5768	4142	0179	284	284	0081	0803
(Block x Main Plot) (Rand.) (Error)	2	2785	1393			7051	3526		
Sub-plot effect (39 levels of CR-ROT x Min. fert.)	DoF	1993	1993	1993	1993	2012	2012	2012	2012
Source of variation		SS	MS	F	P	SS	MS	F	P
Main Plot (Fixed)	1	5768	5768	8588	0006	284	284	0164	0688
Sub-plot (Random)	38	55997	1474	2194	0009	153859	4049	2334	0005
(Main Plot x Sub-plot) (Random) (Error)	38	25519	672			65923	1735		
Other effects (Random)	DoF	1993	1993	1993	1993	2012	2012	2012	2012
Source of variation		SS	MS	F	P	SS	MS	F	P
Rotation	3	16569	5523	10,784	< 0001	38152	12717	7316	< 0001
Mineral Fertilizer	2	4004	2002	3909	0021	2885	1442	0830	0438
Rotation x Mineral Fertilizer	6	2311	385	0752	0608	5811	968	0557	0764
Error	222	113702	512			385887	1738		
Repeated measures for Rotation and Min. Fert.	DoF	All	All	All	All				
Source of variation		SS	MS	F	P				
Year	1	143780	143780	140,329	< 0001				
Year x Rotation	3	28789	9596	9366	< 0001				
Year x Mineral Fertilizer	2	6623	3311	3232	0041				
Year x Rotation x Mineral Fertilizer	6	5121	853	0833	0546				
Error	222	227460	1025						
Repeated measures for CR-ROT combinations	DoF	All	All	All	All				
Source of variation		SS	MS	F	P				
Year	1	180363	180363	206,42	< 0001				
Year x CR-ROT	12	75076	6256	7160	< 0001				
Error	221	193099	874						

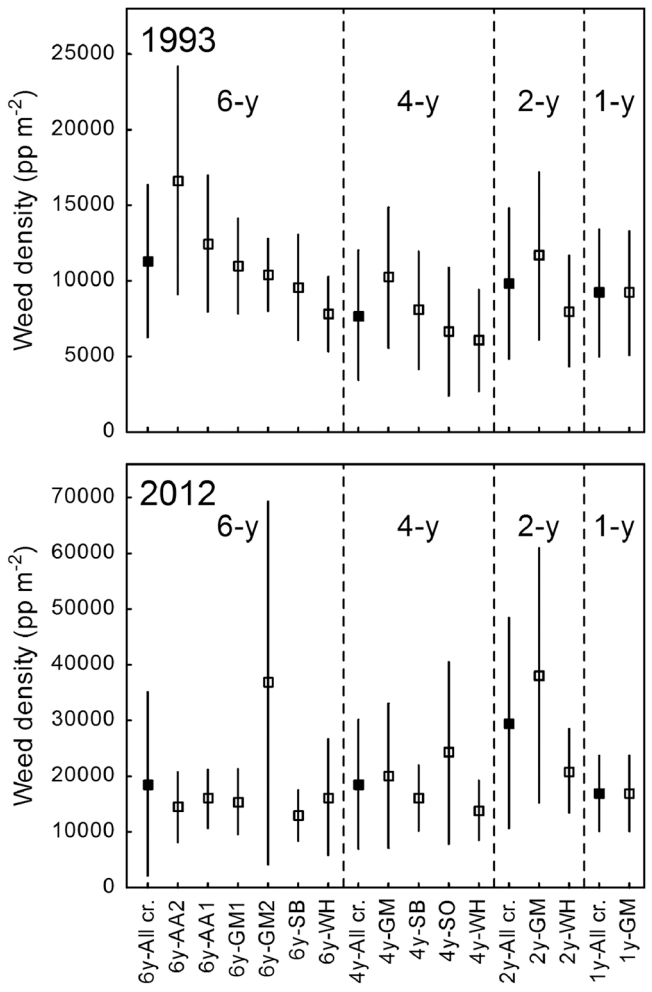

Fig. 1. Mean and standard deviation (empty marker and bar) of weed density in the 13 crop-rotation (CROP-ROT) unique combinations, in 1993 and 2012. The 4 rotations are shown from left to right according to decreasing length, and the general mean for each rotation length is shown with full marker. In 1993 the 13 CR-ROT combinations are in descending order within rotation, and the same order is used for 2012 to simplify comparison. Crop codes: AA1: alfalfa 1st year; AA2: alfalfa 2nd year; GM1: grain maize 1st year; GM2: grain maize 2nd year; SB: sugarbeet; GM: grain maize; SO: soybean; WH: wheat; All cr.: mean of all crops in the rotation.

In 1993 the density of *P. oleracea* was correlated with MAN, and *A. thaliana* with SLU. In 2012 this correlation was not observed because *A. thaliana* had disappeared. Densities of *D. sanguinalis* and *E. crus-galli* were almost independent of the type of organic fertilizer.

In 1993 and 2012 the 12 (ROT x MIN) combinations were all correlated with the first factor independently of rotation length. No particular grouping was observed, and both MAN and SLU were correlated with the first factor only. Weed species composition in the 13 CR-ROT combinations were very similar, and in all one to three species were of greatest importance, with *P. oleracea* being the most important.

In brief, even using all main combinations of variables and supplementary variables, results of PCCA did not show any particular grouping related to rotation length. The effect of type and rate of fertilizer was very low. After 20 years only minor changes had occurred to weed seedbank composition, variability across rotation length was always low, only a few species were slightly correlated with fertilization, but not with rotation length. *P. oleracea* was correlated with manure and medium-high rate of mineral fertilizer, A. *thaliana* with slurry and low rate of mineral fertilizer, *D. sanguinalis* with low rate of mineral fertilizer. Indeed, the main result was that 96–99% of weed species were indifferent to the factors applied. The very low effect of mineral fertilizer on weed flora had already been observed by McCloskey et al. (1996), showing that cultivation and weed management treatment were much more important factors, as also shown by the results of Saulic et al. (2022). Ruisi et al. (2015) found that after 18 years the continuous use of no-till in different crop rotations did not result in substantial changes to weed seed diversity. Other recent studies pointed out that crop diversification provides regulation of pests and weeds (Lechenet et al., 2014; Weisberger et al., 2019). Yet, in the present study weed communities were very similar across rotation lengths, and this unexpected and important result shows that herbicide use levels out differences. Still, the disappearance of *A. thaliana* signals how different factors observed can influence the changes in the agroecosystems. Considering how the only two significant factors related to this species presence were the application of slurry and chemical weed control, it is possible to assume that there were less to none seeds of this species in the animal's diet and that the chemical weed control was effective enough to prevent further seedbank build-up. Furthermore, this observation ads up to what was previously stated about the strong influence of herbicide utilization on the weed presence and on the changes occurring in the agroecosystems.

3.4. Weed flora transformation

3.4.1. Weed density-WPDI correlation

For the 78 treatments x 2 years= 156 combinations, WPDI and density were highly significantly correlated: Pearson's $r = 0.874$ with $p < 0.01$. For the 4 rotation lengths for 2 sampling years= 8 combinations, WPDI and density were highly significantly correlated: Pearson's $r = 0.871$ with $p < 0.01$.

These significant correlations mean that the inclusion of longevity acted as a scale factor. The WPDI change from 1993 to 2012 was mainly caused by a density shift, particularly the increased density of *P. oleracea*. For this, only the 1-y rotation in 2012, with the highest *P. oleracea* density (13191 pp m^{-2}) was outside the 95% confidence regression band.

Given the significant Weed density-WPDI correlation, results from multivariate analysis based on correlation (as PCCA) performed on density or WPDI are very similar, and in this study analyses were performed on density.

Correlation analysis provided no hard evidence for a relation between WPDI and rotation length. Likely the disturbance caused by tillage was similar across rotations, except the 6-y that was slightly less disturbed, so the selection of longevities was similar. This is consistent with the conclusions of Albrecht and Auerswald (2009) on the importance of tillage frequency for the increase in longevity. Dependency of weed presence on different tillage type was also observed by Hosseini et al. (2014).

3.4.2. Chi-square tests on longevity groups

Results of the first chi-square test show that longevity groups are not uniformly distributed within species since most have seeds with medium-high longevity, i.e. the general proportion is about 20% of L1, 32% of L2 and 48% of L3. This is typical of agricultural soils, where high longevity seeds are selected (Albrecht and Auerswald, 2009). Results of the second chi-square test show that the proportions observed in the four rotations in 1993 are very similar to the general proportion, the 6-y being less similar given the highest proportion of group L3 (59%). Results of the third chi-square test show that in 1993 frequencies of longevity groups are very similar across rotations. Results of the fourth chi-square test show that no significant changes in groups frequencies occurred from 1993 to 2012, as the application of rotations acted as a neutral (not significant) factor even after 19 years of application.

The rotation length did not affect the distribution of longevity groups in the seedbank, which remained almost unchanged across rotations after 20 years, and dominated by species with medium or high longevity. This is consistent with results of previous research showing that a

persistent seedbank is a basic requirement for success in arable habitats, and longevity of seedbank is proportional to tillage frequency (Albrecht and Auerswald, 2009; Armengot et al., 2016). But much more important is that herbicides (main) and tillage (minor) were more important than rotation length in this study.

3.4.3. Changes in diversity and single species density

The Density-Rank graph (Fig. 2) shows that most of the density was concentrated in a few species: four species accounted for 52–65% of total density in 1993 and 76–82% in 2012. In general, density distribution was therefore very unbalanced.

Comparison between the 4 rotations show that in 1993 the 6-y and 1-y were the most balanced rotations, while 2-y was less so.

In 2012 all rotations were less balanced with respect to 1993, and the 1-y the least balanced overall.

From 1993–2012 several weed species shifts common to all rotations were observed. In both years and in all rotations the main species was *P. oleracea*. *A. thaliana*, the second most abundant in 1993 had disappeared in 2012 (except in 1-y, with 1.2% of total density), while the density of the grasses *D. sanguinalis*, *E. crus-galli* and *P. dichotomiflorum* increased from 1993 to 2012. In all rotations weed flora was very unbalanced because of the general high density of *P. oleracea* in both 1993 and 2012. Some changes in the most abundant weeds were observed from 1993 to 2012, particularly *E. crus-galli* and *P. dichotomiflorum*, but it is interesting to note that modifications were similar in all rotations. Likely, the effects of herbicides were similar across crop-rotation combinations.

P. oleracea was the most important weed in both 1993 and 2012. A similar increase of *P. oleracea* was observed by Graziani et al. (2012). *P. oleracea* is a continuously fruiting annual weed that shed seeds over much of the growing season and life span (Otto et al., 2007). It is a late-spring emerging weed with a relatively low base-temperature for germination (13.6 °C) (Steinmaus et al., 2000), has a long emergence period and can partly escape post-emergence treatments in maize. After a standard treatment, according to IWM principles based on competitiveness and critical period, there is no practical need to apply further control measures later in the season since seedlings emerging after mid-July are not competitive. Indeed, *P. oleracea* seeds can ripen within 39–57 days after emergence, so also plants emerging in August may rapidly produce mature seeds as the days became shorter, demonstrating the adaptation of *P. oleracea* to an intensive agricultural system (Feng et al., 2015). Within a mid-term perspective it is thus important to control this weed until mid-July.

Importance of *E. crus-galli* increased from 1993 to 2012. *E. crus-galli* emerges constantly from June to September, is a thermophilous species whose abundance can increase with higher temperatures (Keller et al., 2014), has a flowering period strictly dependent on short photoperiod (Montegut, 1975; Norris, 1996) and can quickly ripen seeds until September, before ordinary autumn ploughing. As for *P. oleracea* late emerging plants are tolerated, but the seedbank can increase since longevity of *E. crus-galli* seeds is up to three years.

This same behaviour can explain the increasing importance of *P. dichotomiflorum*. This weed flowers from June to October, reproduces from seeds maturing in late summer and autumn and its density in the Po Valley has been increasing since the 1980 s (Zanin et al., 1992).

It is known that the time between August and the first frost provides a favourable environment for grasses to emerge, establish, and replenish the soil seedbank. In soybean, these post-harvest weeds are becoming major problems in the lower Mississippi River valley. Most growers perform various tillage operations to prepare the seedbed following soybean harvest. Yet, in some cases, frequent tillage favours grasses and species with a late and/or short emergence period (Colbach et al., 2014). So, if conditions are favourable, tillage alone cannot completely prevent grass weeds replenishing the seedbank. On the contrary, herbicides can

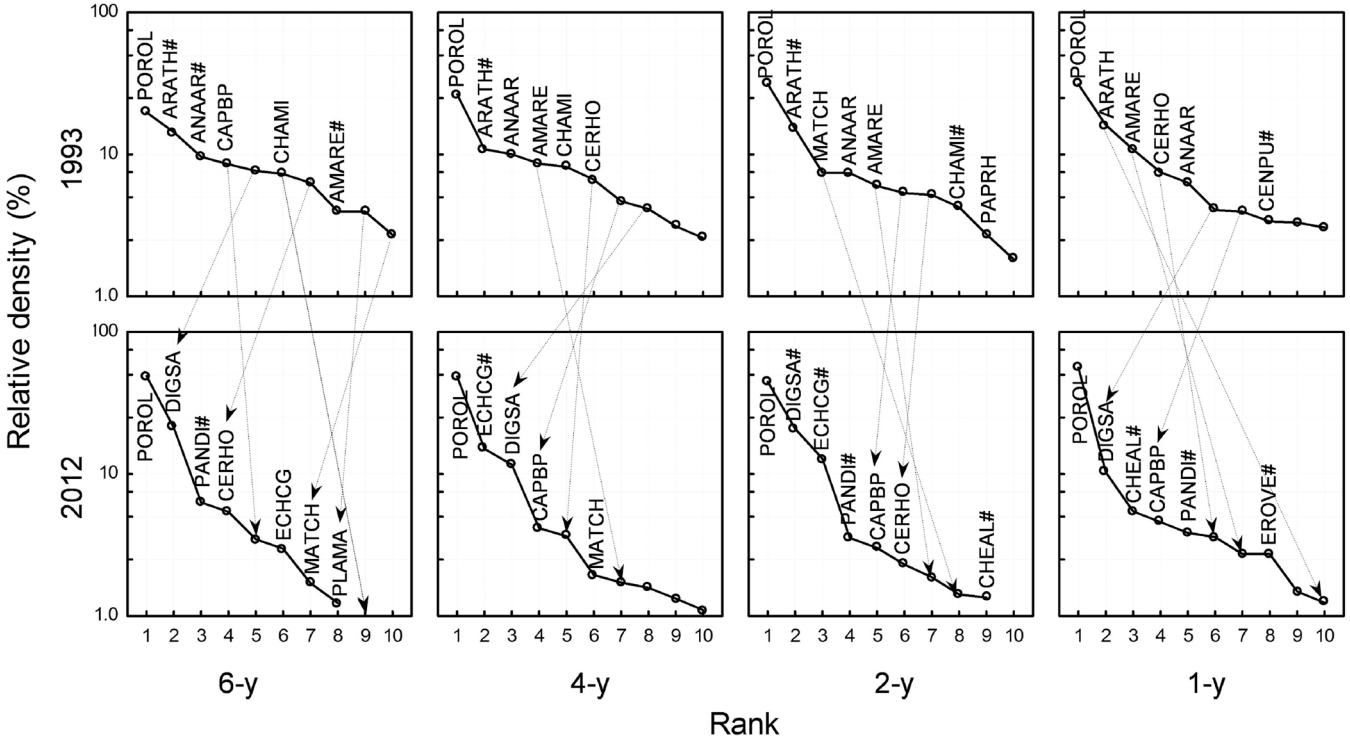

Fig. 2. The relative density (in percentage) of the 10 most abundant species (vertical axis, logarithmic scale) are plotted against the Rank, for 1993 and 2012 and for the 4 rotation lengths. Only markers for species with relative density> 1% and labels for interesting species are shown in sub-plots. The symbol "#" indicates species present in the top 10 ranks in 1993 but not in 2012, and vice versa. Arrows connect species detected in both years but in different rank position, and the label is always where the rank was higher. For example, for the 6-y rotation CAPBP (*Capsella bursa-pastoris*) was detected in both 1993 (rank 4) and 2012 (rank 5), so the label is in the 1993 sub-plot and the arrow points to 2012 sub-plot. For Code specification see Table 1.

effectively control these weeds (Reddy et al., 2015). Clearly, new tools complementing early season weed control and late weed seed production are needed.

The importance of *Anagallis arvensis* (ANGAR) and *Chaenorhinum minus* (CHNMI) decreased markedly from 1993 to 2012, *A. arvensis* in all rotations. These species are typically adapted to winter wheat, emerge over a relatively short period in late winter and can complete development when herbicides are not used, showing occasional spread (Holm et al., 1977). On the contrary, in other crops they can be easily controlled with various MoA, once again showing the influence that herbicides can exert on agroecosystems.

3.5. Chemical load

The mean yearly rate of chemicals applied to maize remained almost stable, decreasing from 2050 to 1850 g ha^{-1} from the first period (from 1976 to 1993) to the second period (1994–2012). Yearly application rates varied greatly in all rotation lengths, according to the fact that weeds were managed with the best chemical option (herbicide and rate) for the observed weed flora. For wheat the applied rate was 1290 g ha^{-1} in the first period and 600 g ha^{-1} in the second period, showing a great reduction because of the increasing use of sulfonylurea and 4 untreated years from 2000 to 2005 due to low weed density. On the contrary, the rate applied to sugarbeet increased from 2800 to 3600 g ha^{-1}, and a big increase was also observed for soybean, in which the rate increased from 370 to 1300 g ha^{-1} from the first to the second period.

These variations in single crops affected the total amount applied in the 4 rotation lengths.

Considering the whole period from 1976 to 2012, in the 1-y rotation, i.e. continuous maize, the annual applied rate was about 2000 g ha^{-1}. It is worth noting that the number of MoA increased steadily, from 1 to 3. From 2005 this is mainly due to isoxaflutole and mesotrione (MoA=F2) and nicosulfuron (MoA=B).

In the 2-y rotation, wheat-maize, the applied rate was 1500 g ha^{-1}, and the number of MoA almost double with respect to the 1-y rotation. In the 4-y rotation the applied rate was 1700 g ha^{-1}, and the number of MoA was from 4 to 6 in the 2005–2012 period. In the 6-y rotation the applied rate was about 1200 g ha^{-1}, given the low rate of herbicide applied to alfalfa. In total, 12 MoA were used from 1976 to 2012 (A, B, C1, C2, C3, F2, K1, K3, L, M, N, O) and the number of MoA was similar to that of the 4-y rotation.

It is interesting to note that the 2-y rotation received a rate slightly lower than the 4-y rotation, showing that the effect of a single crop, here the high herbicides demanding sugarbeet, overcomes that of the rotation length.

The number of MoA increased from the 1-y to 6-y rotation, and this can be considered an important rotational effect since the number of MoA is proportional to the number of crops. Indeed, a good weed management can also be obtained with a rational use of very few MoA when applied according to Integrated Weed Management principles. In this framework, a crop rotation cannot guarantee high variability of MoA if crops in the rotation are managed using herbicides with the same MoA.

The effect of herbicides on species richness (i.e. diversity) is generally secondary to that on weed density, since herbicides generally affect relative importance (weed-shift) more than species composition. Furthermore, Otto et al. (2012) showed that most of the density can be concentrated in very few dominant species even in weed communities with relatively high diversity. Changes in dominant species may have more implications for weed control management than actual changes (or lack of) in the diversity of the weed flora, i.e. when the incomig dominant species is more competitive or less sensible to chemical or mechanical weeding. Diversity ignores the total and individual species abundance, and species traits. So, whether weed species richness is desirable in weed management remains a question.

Previous studies pointed out the importance of tillage (Hosseini et al., 2014; Giuseppe Zanin et al., 1997) and other crop management practices on weeds (McCloskey et al., 1996; Saulic et al., 2022), and the present study highlights that herbicide levels out differences due to other (classical) rotational effects on weed density. In this study herbicides selection and application timing was done according to best options in that moment, and was the same for each crop irrespective of position in rotations. This had three main consequences:

1) positive effects of rotation length on weed flora were not exploited by a reduction in chemical input;
2) inadequate treatments against a certain weed had similar consequences in all rotation lengths. For example, if a late spring application of herbicide in maize partially failed to control *P. oleracea*, its density increased whatever the rotation;
3) in terms of chemical load, the main advantage of the 6-year rotation is that alfalfa was treated with 113.6 g $ha^{-1}$of herbicide in 36 years, reducing the annual average input.

This highlights the prominent role of herbicides for weed management in standard farming systems from the 1970 s to date, when the low cost of herbicides encouraged the steady use of full rate, masking possible beneficial effects produced by long cycle crop rotations.

Today the use of herbicides is decreasing due to limits set by national and supranational regulations (European Parliament, 2009; FAO/WHO, 2016). This could reduce efficacy and the effect of herbicides on seedbank. Yet, if the new technologies of precision weed control are applied, as proposed by Nikolić et al., (2021, 2022), chemical weed control could continue to have an important impact on weed flora and as a consequence also on weed seedbank.

4. Conclusions

Effects of rotation and tillage on weed seedbank reported previously are very variable and not completely in agreement with this study. Results of the present study show that herbicide effect must be included in a more general theory of rotational effects on weeds, because the time scale of flora reaction to herbicides (year) is much shorter than that of crop rotation (decades), and the effects more striking. When properly used, herbicides can effectively manage weed communities even with a reduced number of modes of action. In some cases, due to specific functional attributes certain weeds can exploit low selection pressure moments, such as during inter-cropping or the latest part of the cropping cycle, and increase their abundance.

Rotational effects, i.e. variation of mechanical disturbance, can indeed be very important for the management of a heavy weed flora shift, for example due to the spread of perennial weeds, such as *Equisetum* or *Cyperus*, or the spread of herbicide-resistant weeds.

Furthermore, the effect of new technology with robots for precise weed control on weed flora composition need to be address.

Lastly, the role of crop rotation is of great interest in organic farming, where herbicides are not used.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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