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Article

Minimal Necessary Weed Control Does Not Increase Weed-Mediated Biological Pest Control in Romaine Lettuce (*Lactuca sativa* L., var. Romana)

Alessandra Virili * and Anna-Camilla Moonen *

Center of Plant Sciences, Group of Agroecology, Scuola Superiore Sant'Anna, Piazza Martiri della Libertà 33, 56127 Pisa, Italy
* Correspondence: alessandra.virili@santannapisa.it (A.V.); camilla.moonen@santannapisa.it (A.-C.M.)

Abstract: Lettuce is one of the most consumed leafy greens. Depending on the variety, it is ready for harvesting 40–80 days after sowing, and therefore several growth cycles can be concluded within a growing season. Due to its high market standards, lettuce may require intensive management. This study implemented a critical period of weed interference (CPWI) trial to understand at which moment of the cropping cycle weeds can be tolerated without impacting crop yield to decrease the time needed for weeding and assess the potential support weeds can give to biological pest control in lettuce. Treatments represented two gradients of weed intensity: (1) increasingly weed-free, and (2) increasingly weedy. Dose–response curves were produced to find the CPWI based on lettuce relative yield. RLQ analysis was used to explore the relationships between weeding regime and weed functional traits for biological pest control. Yield was above the 5% acceptable yield loss threshold in all plots kept weed-free for 20 days or more, indicating a necessary weed-free period of 20 days from transplanting. However, the support of beneficial insects was not guaranteed at the end of the necessary weed-free period. We suggest that it is possible to limit intense weed management to the beginning of the growing season, reducing the cost of plastic mulches and increasing on-farm biodiversity, but field margins could be better suited to deliver conservation biological control in short-term crops where this service is of primary interest.

Keywords: critical period of weed interference; spontaneous vegetation; leafy vegetable; ecosystem services; functional traits

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1. Introduction

Italy is an important country in the horticultural sector, representing 31% of the total harvested area dedicated to vegetable production in Southern Europe [1]. Vegetable crops are grown in many regions and are sold for fresh consumption or for transformation in the food agri-business. Within this context, lettuce is certainly the most consumed and appreciated leafy vegetable. In 2019, Italy produced 75,898 tonnes of lettuce, coming fifth after China, USA, and India, and second in Europe only to Spain [2].

Lettuce is easy to produce and can be grown in the open field and in greenhouses, guaranteeing nearly year-round production. Like many vegetable crops, lettuce cultivation is often intensively managed, and it requires frequent soil tillage, abundant irrigations, and short or no crop rotations [3]. Lettuce is often transplanted on biodegradable or plastic mulch to retain moisture and minimize weed growth. Plastic mulches are effective but account for high operational costs; they take a long time to degrade in the soil and cannot be reused. Considering that lettuce has one of the shortest growing cycles among vegetable crops, the question is if weeds need to be managed so intensively.

When dealing with fresh produce, standardization is a major factor in determining the market type in which plants can be sold. For lettuce, head quality is greatly affected by insect injury, slime, and rot on the leaves, and the tolerance for these defects is reported to be no more than 5% of lettuce heads in any container (as per USDA standards). At the same time, consumers have an increasing interest in organic fruits and vegetables and pesticide-free products. In this context, it is relevant to develop agroecological pest control methods capable of reducing reliance on phytochemicals in these systems [4,5]. Even though lettuce is considered sensitive to weed competition [3], it could benefit greatly from the weed community, which could potentially control its most common pest [5–7]. Weeds can make the crop less attractive for insect pests by visual and sensorial interference, therefore limiting successful crop colonization, and the flowers they produce may attract natural enemies, thus facilitating aphid predation [4,8–11]. The question is which functional weed groups are most effective in supporting natural enemies without causing yield losses, and when do these weed functional groups offer their services and disservices to the crop [12–14]. Given the short growing cycle of lettuce and the constant turn-over of the soil, it is likely that after necessary weed management, most of the functional weed groups are not able to guarantee the expected agroecosystem services. However, there are fast-growing ruderal species that develop at low biomass and flower after a few weeks, such as *Papaver rhoeas, Cichorium intybus,* and *Portulaca oleracea* [13], which could be able to support natural enemies without competing with the crop. The question remains if it is possible to reconcile the presence of functionally relevant weeds without creating yield losses, and if so, what is the best temporal and spatial configuration at which we should aim. To answer this question, we need to collect information about (1) the critical weed-free period of the crop, (2) the weed community composition that develops early and late in the crop cycle, and (3) the functional traits of the dominant weed species. In the case that the land-sharing approach does not allow the crop to tolerate functionally relevant weeds, the option is to follow a land-sparing approach, separating the crop from the functionally relevant weeds, for example by managing the existing field margins or sowing flower strips. In this last case, no benefit will be obtained from a reduced cost for weeding operations.

This study aimed to determine if and when functionally relevant weeds can be tolerated in open-field romaine lettuce (*Lactuca sativa L.*, var. *Romana*), by establishing (1) in which period of the growing season weeds are tolerated by romaine lettuce; (2) which weed species develop when weeding is done at different moments of the crop cycle; and (3) if the functional traits of the dominant species in the weed community are relevant for the support to beneficial insects. Therefore, a critical period of weed interference trial was set up, in which different weedy and weed-free gradients were compared to calculate a curve of relative yield and estimate the moment within the crop's cycle in which weeding is necessary, based on the acceptable yield loss threshold [14]. A functional trait approach was then used to estimate the potential of the weed community left after the necessary weed management to support beneficial insects and provide biological pest control, compared to the weedy control plots.

The few studies that have reported on the critical period of weed interference in lettuce indicate that weed control in just the first couple of weeks after crop transplanting is crucial to avoid yield loss [3,15,16]. These studies provide valuable information regarding agronomic and economic aspects, but they do not investigate other positive externalities possibly related to optimizing weed control. Within the context of AES provisioning, the question remains which weed species are able to develop in the last 30 to 40 days of the crop cycle, and if these fast-growing species have the right traits to support beneficial insects. In order to shed light on this question, we established a trial to determine the critical period of weed interference and to examine the functional traits of the weed communities that develop under different weeding regimes.

We hypothesized that the weed community in the weedy treatment would develop the maximum potential to support beneficial insects but would result in a significant yield penalty. With an increasing length of the weed-free period, the service provision potential and competitiveness of the weed community were expected to decrease, while yield was expected to increase. We further expected that the weed community that develops after crop transplanting would be different from the one that develops when respecting the 20-day weed-free period after transplanting, and that the potential to support beneficial insects would differ accordingly.

2. Materials and Methods

2.1. Experimental Site and Layout

The critical period of weed interference (CPWI) trial was carried out at the Center for Agro-Environmental Research (CIRAA) of the University of Pisa, located in San Piero a Grado (Italy) (43.66258, 10.34837). In total, the experimental area occupied 400 m^2. Before the first year of the trial, the field had been left fallow for at least one year. In the second year, lettuce was transplanted on the adjacent field, which was previously cropped with lentil. The trial was performed from the 28th of May to the 17th of July in 2019 and from the 15th of May to the 7th of July in 2020. Weather data were retrieved from the local automatic weather station positioned at CIRAA. The average maximum and minimum temperatures during the 2019 cropping season were 29.7 °C and 15.6 °C, respectively, with a total precipitation of 64 mm. The average maximum and minimum temperatures during the 2020 cropping season were 26.4 °C and 13.6 °C, respectively, with a total precipitation of 178 mm.

Treatments were displayed in a completely randomized block design with five replications. Treatments represented two gradients of weed intensity: (1) increasingly weed-free, and (2) increasingly weedy (Figure 1). Weeding intensity changed every 10 days, and weeding was performed once a week for weed-free plots. Romaine lettuce was transplanted in square plots of 4 m^2 in a regular 30 cm × 30 cm grid (equal to 49 plants with 7 plants per row), amounting to a crop density of 9000 plants ha^{-1}. The outer two rows on each side were kept as a buffer and all measurements including harvest were taken in the central 1 m^2 of each plot, which included nine lettuce plants.

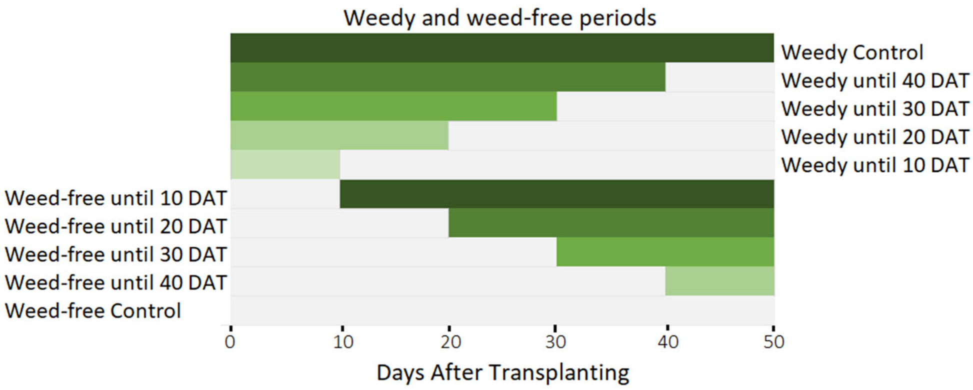

Figure 1. Weeding regimes applied to the experimental plots to create the increasingly weedy and increasingly weed-free gradients expressed in days after crop transplanting (DAT). Green bars represent the weedy intervals, grey bars represent the weed-free intervals.

Before the first cropping season, three soil samples were taken at random points of the field to obtain physical and chemical parameters. The soil was mainly sandy (800 g kg^{-1}) with 1.93% OM, 0.98 g kg^{-1} of total N, 32 mg kg^{-1} P_2O_5, and 130 mg kg^{-1} K_2O. Each year, two weeks before transplanting, we applied 1 kg m^{-2} of an organic amendment derived from a mix of bovine and equine manure (“HORTUS CLT®”, produced by “Centro Lombricoltura Toscano”, C:N = 12.5, organic matter = 44.4%). In addition, mineral fertilizer was applied within the first week of crop establishment, following indications for poor soils (130 kg ha^{-1} N, 100 kg ha^{-1} P_2O_5, 220 kg ha^{-1} K_2O). Water was provided to the plots with an overhead irrigation system made of six sprinkler heads, each with a flow rate capacity of 200 L ha^{-1} hr^{-1}, for two hours about three times a week, adjusted based on the local precipitation regime.

2.2. Data Collection

Weed species, cover, and density were determined in the weedy plots every 10 days from transplanting (DAT) within a 1 m^2 sampling frame positioned in the center of the plot. Cover was estimated by the projection of the weed species on the ground surface, expressed as percent covered by each species or by the overall weed community cover, to obtain the total weed cover. Bare soil cover, lettuce cover, and lettuce density were also assessed. When the weedy period ended, weeds were collected and separated by weed group (broadleaved species, grasses, *Cyperus spp*.), dried at 100 °C, and weighed. Weed groups were chosen instead of single species due to the patchy composition of the weed community. For each plot, detailed data about weed species cover and density were recorded at the same time as weed biomass sampling, and therefore no information was lost about the species composition.

Relative lettuce chlorophyll content, plant height, and circumference measurements were taken at the 25th, 35th, and 45th DAT on three numbered plants out of the nine central plants of each plot. Chlorophyll content measurements were taken with a handheld SPAD meter ("Soil Plant Analysis Development", Konica Minolta, SPAD-502Plus), which gives an indirect measurement of chlorophyll present in the leaves. As explained by Monostori et al., "the device measures the leaf transmittance red light at 650 nm (at which chlorophyll absorbs) and in near-infrared light at 940 nm (for the correction of leaf thickness). The ratio of these two transmission values is referred to as SPAD reading or SPAD value" [17]. Three leaves per plant were sampled for a total of nine points per plant, averaged in one SPAD value per plant.

Lastly, fresh and dry biomass of all nine central lettuce plants were taken at crop harvest. Marketable lettuce biomass was determined by removing any damaged leaves from the plant and weighing the remaining lettuce head.

2.3. Data Analysis

Weed species were classified based on their frequency of occurrence (<10%; 10–30%; and >30%) and their local cover (<5%; 5–10%; >10%), and the species falling in the highest frequency and local cover category were considered as dominant species. The thresholds used to group the species in abundance classes were adapted from the ones presented by [18] in order to provide a relevant reference in the local context. The effect of weed management intensity on lettuce biomass and crop parameters (SPAD values, height, and circumference) was tested using linear mixed effect models ("lmer" function, "lme4" package) [19], followed by an analysis of variance of the best model (function "Anova", "car" package) [20]. All models were validated using the Kolmogorov–Smirnov test to detect significant deviation and outliers with the "simulateresiduals" function in the "DHARMa" package [21]. When a resulting factor was significant, post-hoc Tukey test was performed with the function "emmeans" in the package "emmeans" [22]. The critical period of weed interference interval was obtained by fitting four parameter log-logistic models to the relative yield of each plot compared to the weed-free control in two subsets of the yield dataset. In this way, a curve was calculated for the weedy gradient, and one for the weed-free gradient [14]. The models and graph were produced using the "drm" function in the "drc" package [23]. Cumulative growing degree days (GDD) were used on the x axis to best represent crop growth within the two different growing seasons, and they were calculated by performing the cumulative sum of the average daily temperatures from crop transplanting until cop harvest with the base temperature set at 4 °C [24]. GDD values were then averaged over both years.

Finally, the "TR8" package [25] was used to retrieve the functional traits of species for a subset of plots (the weedy control, the minimum necessary weeding regime, and the treatments directly prior and following it) using the weed community at harvest to analyze weed composition at its maximum expression. TR8 retrieves information from a variety of ecological databases; the ones used in this case were "Biolflor" [26], "Catminat" [27], "Ecoflora" [28], and "LEDA" [29]. The selected traits represented flower color, type of insect supported, growth form, Grime strategy, and life cycle. The data were then used to analyze the relationships between environmental characteristics and species traits, combining a fourth corner analysis, which produces an unweighted Pearson correlation matrix ("fourthcorner" function, "ade4" package), and an RLQ analysis to plot the data ("rlq" function in the "ade4" package) [30]. After obtaining the relative yield of each weeding regime determined by the CPWI curve, a subset of weeding regimes was used to perform the RLQ analysis. The necessary weeding regime represents the minimum number of weed-free days needed to achieve an acceptable crop yield. This was chosen as a reference point to analyze the potential agroecosystem service delivery of the weed community. Plots that received an additional weeding operation were chosen to determine how excessive weeding affects the weed community. Similarly, plots left weedy after transplanting were taken into consideration to understand the effect of insufficient weed management on the weed community. The weedy control was also included, assuming that the weed community in this treatment expresses the maximum service-provisioning potential. All statistical analyses were performed using R (v. 4.1.0, R core Team 2021).

3. Results

3.1. Effect of Weeding Regime on Lettuce Yield

Weeding regime affected lettuce yield in both the weedy and weed-free gradients ($p = 0.005$ and $p = 0.0003$, respectively). Year had no significant effect.

Regarding the weedy treatments, lettuce yield in the weedy control was significantly lower compared to lettuce yield in the weed-free control, and in the treatments that were left weedy in the first 10 days and 20 days after transplanting ($p < 0.05$) (Table 1). Regarding the weed-free plots, lettuce yield was significantly lower in the weedy control compared to all plots that remained weed-free plots for at least the first 10 days ($p < 0.05$) (Table 1). Due to the variability in lettuce yield, no significant differences were found between treatments that kept the plots weed-free during the first 10, 20, 30, or 40 days or even until harvest.

Table 1. Effect of increasing duration of the weed-free period (top) and decreasing duration of the weedy period (bottom) on lettuce yield (g/m^2) averaged over both years of the critical period of weed interference trial. Values represent arithmetic means and standard errors of lettuce fresh marketable biomass in each weeding regime. Letters represent significant differences in lettuce yield between weeding regimes (Tukey HSD post-hoc test, $p < 0.05$).

Weeding Regime	Marketable Lettuce Mean Fresh Biomass (g/m^2)
Weedy control	29.99 ± 7.19 ^{a}
Weedy 0-40 DAT	155.20 ± 22.89 ^{ab}
Weedy 0-30 DAT	143.14 ± 15.31 ^{ab}
Weedy 0-20 DAT	262.26 ± 20.88 ^{b}
Weedy 0-10 DAT	218.15 ± 20.94 ^{b}
Weed-free control	245.84 ± 26.08 ^{b}
Weedy control	29.99 ± 7.19 ^{a}
Weed-free 0-10 DAT	171.49 ± 17.54 ^{ab}
Weed-free 0-20 DAT	267.40 ± 19.20 ^{b}
Weed-free 0-30 DAT	227.22 ± 20.65 ^{b}
Weed-free 0-40 DAT	278.12 ± 23.06 ^{b}

Weed-free control	245.84 ± 26.08 [b]

3.2. Effect of Weeding Regime on Lettuce Yield Components

Regarding lettuce chlorophyll content (SPAD values), there was an additive effect of weeding regime ($p < 0.0001$) and sampling date ($p < 0.0001$) for the plots of the weed-free gradient. Lettuce plants in the weedy control registered significantly lower SPAD values compared to all other weed-free weeding regimes ($p < 0.05$) throughout the entire cropping season (Table 2). For the plots of the weedy gradient, a significant interaction was found between weeding regime and year ($p = 0.002$), with SPAD values being higher in 2019 compared to 2020 (Table 3). No effect of sampling date was found, meaning that differences between treatments in lettuce SPAD values were consistent throughout the growing season. In 2019, lettuce plants in the weedy control registered lower SPAD values compared to all other weedy regimes and the weed-free control ($p < 0.05$). In 2020, lettuce plants in the treatments that were weedy during the first 40 days (W40) registered lower SPAD values compared to plants in the weed-free control, W10 and W20 ($p = 0.006$, $p = 0.04$, $p < 0.0001$). Plants in W20 had higher SPAD values compared to plants in W30 and the weedy control as well ($p = 0.02$).

Height in both the weedy and weed-free plots was affected only by the sampling date ($p < 0.0001$). Whereas lettuce circumference in the weed-free plots was affected by weeding regime ($p < 0.0001$) and sampling date ($p < 0.0001$), but no significant interaction was found. Lettuce circumference in the weedy control was significantly lower compared to all weed-free plots ($p < 0.05$), and lettuce circumference of plants in plots that remained weed-free during the first 10 days (C10) was significantly lower compared to that of plants in the weed-free control ($p = 0.007$) (Table 2). Even though no significant differences were detected between C10 and the other weed-free regimes, plots that were left weed-free for more than ten days after crop transplanting were able to develop a higher circumference than plants that were subjected to weed competition from the beginning or from just 10 days after the start of the cropping season. In the weedy plots, a significant interaction effect was found between treatment and sampling date ($p = 0.008$) (Table 3). Starting from day 35, plants in the weed-free control, W10 and W20, were significantly larger than plants in W30 ($p < 0.0001$, $p = 0.0008$, $p = 0.008$) and the weedy control ($p = 0.0002$, $p = 0.005$, $p = 0.04$). Right before harvest, plants in the weed-free control were significantly larger than plants in W30, W40, and the weedy control ($p = 0.002$, $p = 0.0003$, $p < 0.0001$). Plants in W10 and W20 were larger than plants in W40 ($p = 0.03$, $p = 0.01$) and the weedy control ($p < 0.0001$).

Table 2. Effect of an increased duration of the weed-free period on lettuce SPAD values and mean lettuce head circumference (cm) averaged over both years of the critical period of weed interference trial. Values represent arithmetic means and standard errors of lettuce SPAD values and circumference in each plot of the weed-free gradient. Letters represent significant differences between weeding regimes (Tukey HSD post-hoc test, $p < 0.05$).

Weeding Regime	Lettuce Chlorophyll Content (SPAD Values)	Lettuce Circumference (cm)
Weed-free control	29.47 ± 0.48 [b]	59.85 ± 1.44 [c]
Weed-free 0–10 DAT	29.10± 0.55 [b]	53.83 ± 1.19 [b]
Weed-free 0–20 DAT	30.12 ± 0.56 [b]	55.16 ± 1.33 [bc]
Weed-free 0–30 DAT	28.91 ± 0.68 [b]	57.69 ± 1.47 [bc]
Weed-free 0–40 DAT	28.95 ± 0.63 [b]	57.92 ± 1.50 [bc]
Weedy control	26.19 ± 0.44 [a]	46.36 ± 1.28 [a]

Table 3. Effect of an increased duration of the weedy period on lettuce chlorophyll content (SPAD values) for both years, averaged over sampling dates, and lettuce head circumference (cm) for each sampling date, averaged over both years of the critical period of weed interference trial. Values represent arithmetic means and standard errors of lettuce SPAD values and circumference in each plot of the weedy gradient. Letters represent significant differences between weeding regimes (Tukey HSD post-hoc test, $p < 0.05$).

Weeding Regime	Lettuce Chlorophyll Content (SPAD Values)		Lettuce Circumference (cm)		
	2019	2020	25 DAT	35 DAT	45 DAT
Weed-free control	31.17 ± 0.74 [b]	27.73 ± 0.49 [bc]	53.08 ± 2.6 [n.s.]	60.80 ± 2.16 [c]	65.86 ± 2.18 [c]
Weedy 0-10 DAT	31.48 ± 0.96 [b]	27.16 ± 0.38 [bc]	48.63 ± 2.68 [n.s.]	58.10 ± 2.62 [c]	61.57 ± 2.20 [bc]
Weedy 0-20 DAT	30.89 ± 0.78 [b]	28.92 ± 0.63 [c]	52.38 ± 2.29 [n.s.]	56.08 ± 2.56 [bc]	62.43 ± 2.50 [bc]
Weedy 0-30 DAT	31.93 ± 1.04 [b]	25.71 ± 0.62 [ab]	46.68 ± 2.75 [n.s.]	45.14 ± 2.15 [a]	53.68 ± 2.22 [ab]
Weedy 0-40 DAT	29.66 ± 1.19 [b]	24.34 ± 0.52 [a]	50.63± 2.14 [n.s.]	53.47 ± 2.58 [abc]	52.10 ± 2.15 [a]
Weedy control	26.82 ± 0.72 [a]	25.57 ± 0.52 [ab]	46.97 ± 2.23 [n.s.]	46.8 ± 1.97 [ab]	45.30 ± 2.48 [a]

3.3. Effect of Different Weed Groups on Crop Yield, Growth, and Stress Components

Table 4 summarizes the effect of individual weed groups (grasses, broadleaved species, *Cyperaceae*) on lettuce, and it shows that grass biomass had a significant negative effect on lettuce dry biomass, yield, and lettuce head circumference. Broadleaved species biomass also had a significant negative effect on crop yield and circumference. A positive correlation between lettuce height and broadleaved biomass was detected, indicating higher lettuce plants in plots with a higher broadleaved weed biomass. Although broadleaved species grew much taller than the grasses, the latter produced a higher overall biomass and had a higher impact on crop yield and growth.

Table 4. Correlations between weed biomass and cover per weed group and for the total weed community measured at lettuce harvest and lettuce biomass, stress, and growth components. Data have been averaged over the two years. Asterisks represent the p-values (* $0.01 < p < 0.05$; ** $0.001 < p < 0.01$; *** $p < 0.0001$).

			Lettuce Stress/Growth Parameters				
			Dry Total Biomass	Fresh Marketable Biomass	Chlorophyll Content (SPAD Values)	Plant Height	Head Circumference
Weeds	Biomass	Grasses	−0.52 ***	−0.37 **	−0.14	−0.03	−0.39 **
		Cyperus spp.	0.12	−0.01	−0.18	0.27 *	−0.05
		Broadleaved	−0.24	−0.45 **	−0.12	0.30 *	−0.56 ***
		Total	−0.46 **	−0.55 ***	−0.19	0.24	−0.64 ***
	Cover	Grasses	−0.52 ***	−0.33 *	−0.19	−0.06	−0.37 **
		Cyperus spp.	0.15	0.09	−0.21	0.25	0.03
		Broadleaved	−0.27 *	−0.47 **	−0.10	0.14	−0.57 ***
		Total	−0.49 ***	−0.50 ***	−0.25	0.12	−0.61 ***

3.4. Critical Period of Weed Interference

Based on the statistical analyses of lettuce yield, data for the CPWI curve was averaged over both years. According to the CPWI curve (Figure 2), lettuce yield was below the economic threshold of acceptable yield loss for the whole growing season in the weedy treatments. (Acceptable yield loss was set at 5% as set by Knezevic et al. [31]. The models used to determine the beginning and end of the CPWI show that weeds should not be tolerated in romaine lettuce from around 130 GDD (ca. 8 DAT, for both years) up to 321 GDD (19 DAT in 2019 and 22 DAT in 2020.) After that time, lettuce heads begin to grow exponentially and cover the inter-row spaces, diminishing weed development.

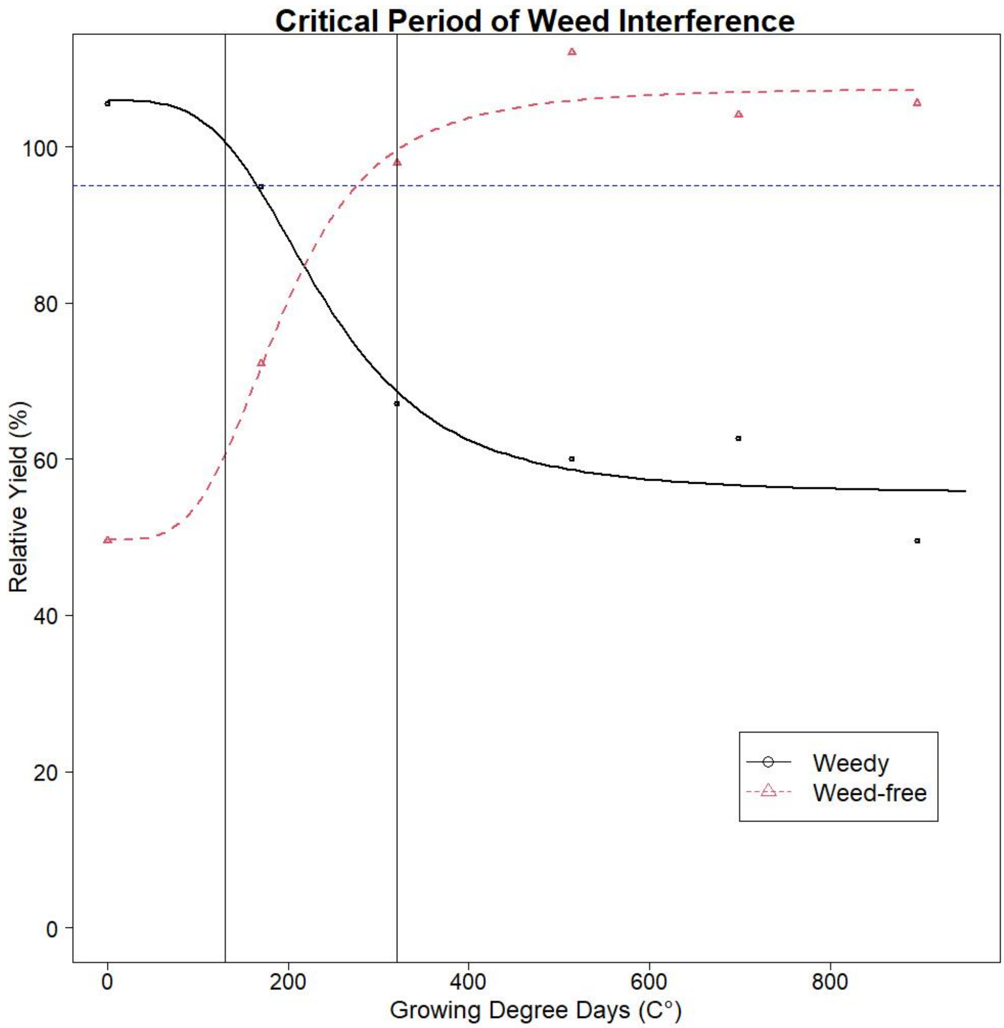

Figure 2. Relative lettuce yield (%) with respect to the weed-free plots of the increasingly weedy (black curve) and the increasingly weed-free (red curve) plots. Data have been averaged over blocks and years. The blue horizontal line represents the threshold of acceptable yield loss set at 5%. The vertical lines represent the beginning and end of the critical period of weed interference expressed in terms of growing degree days (GDD). One data point has been taken out since it was an outlier from a damaged plot.

3.5. Weed Community

We recorded 31 weed species in 2019 and 30 weed species in 2020. Figure 3 shows the development of the total weed cover in the weedy control plots in both 2019 and 2020. The graph shows that despite the warmer weather in 2019, and thus a higher GDD accumulation, the weed cover increased slightly quicker in 2020. Weed cover at lettuce harvest was comparable among the two years. The dominant species were *Amaranthus retroflexus*, *Chenopodium album*, *Cynodon dactylon*, *Cyperus* spp., *Digitaria sanguinalis,* and *Solanum nigrum* (in 2020). *Cynodon dactylon* and *Cyperus* spp. had the highest overall frequency and were present in 87.2% and 85.6% of the plots, respectively, in 2019. In 2020, *C. dactylon* and *Cyperus* spp. were present in 82.8% and 83.4% of the plots, respectively. Tables showing the classification of the weed species by local cover and frequency of observation together with a full list of the species found in each year are provided in the Supplementary Materials (Table S1, Table S2, Table S3).

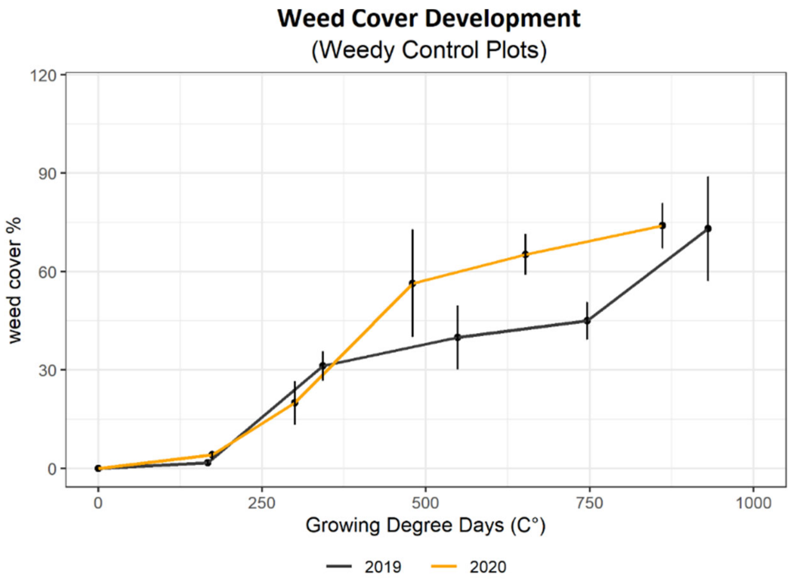

Figure 3. Weed cover (%) development in the weedy control plots in 2019 and 2020 during the critical period of weed interference trial. Black bars represent the standard error of the five sampled weedy plots at each sampling time (10, 20, 30, 40, and 50 DAT). Days from transplanting are indicated as cumulative growing degree days.

3.6. Biological Pest Control Provisioning Potential

Even though species that support beneficial insects were also found in the treatments left weed-free for 30 days (C30) and 20 days (C20), the majority of diversity was found in the plots left weed-free during the first 10 days (C10) and in the weedy control plots (Figure 4). This indicates that if weed management is reduced to the minimum (C20), the weed community that is left at harvest cannot guarantee a potential service provisioning. The fourth corner analysis confirmed that there was no effect of weeding regime or year on any of the weed traits when the appropriate adjustment method for the *p*-values was applied.

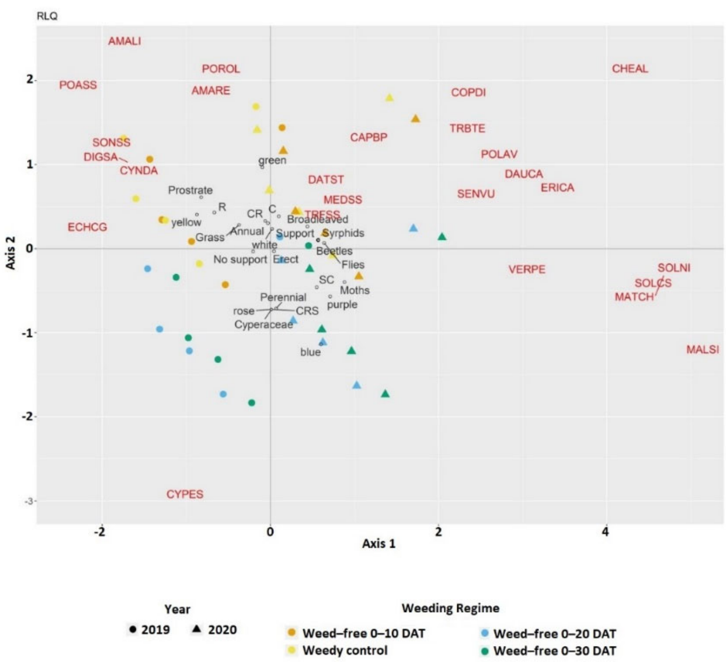

Figure 4. RLQ plot showing species traits associated with the selected weeding regimes of the critical period of weed interference trial. No significant correlation was found between weed traits and

weeding regimes. EPPO codes of weed species are explained in table S3. 'S', 'R', 'C', and the combinations thereof refer to the stress-tolerant, ruderal, and competitive life history traits of the weed species.

4. Discussion

The findings of this research confirm results from previous studies showing that romaine lettuce grown in open-field conditions can tolerate weeds with no significant impact on crop yield if weeding is performed within the first 20 days after crop transplanting. The aim of this study was to take this conclusion one step further and examine the functional traits of the weed communities that develop under the various weeding regimes, to determine if they can potentially support beneficial insects for conservation biological control. The analysis of the functional traits of the weed community that developed after 20 days of weeding shows that the weed community is not particularly rich in species that are known to support beneficial insects. On the other hand, the weed community that developed in weedy plots or plots that are weeded only for the first 10 days was more species rich and contained more species that are known to support beneficial insects.

Giancotti et al. [3] reported a yield loss of up to 25% if weeding was not performed within 21 days after crop emergence. This was also confirmed by Parry and Shrestha [16], who recorded 58% marketable yield loss if early weeding was not performed. Even though the authors found that a 35-day weed-free period significantly reduced weed seed return, this additional weeding pass (with a hand hoe) added about 87 h ha^{-1} $person^{-1}$, with no significant increase in crop yield or quality. Our results showed quite clear effects of the extreme weeding regimes on lettuce yield, chlorophyll content, and head circumference, but as shown in other studies, the effects on the intermediate weeding regimes were less clear (Tables 1, 2 and 3). This is quite normal in open-field trials that aim to reproduce the common crop growing conditions. We located the trial in a field with a rather homogenous weed community, but some variability is natural to all weed communities. The fact that the field had a homogenous weed community composition emerges clearly from the Supplementary Tables S1, S2, and S3, demonstrating that the weed species and abundances between the two years were very similar even though the plots were located in a different part of the same field to avoid a legacy effect of the first year's experiment and weeding regimes. Even these slight differences in weed composition may cause variability in crop yield. To overcome this problem, we replicated the trial in five blocks, instead of the common three or four blocks, increasing the statistical power.

In 2019, both the crop and weeds developed faster due to a rapid increase in temperatures shortly after lettuce transplanting. In 2020, temperatures were generally cooler, and the weed biomass in the plots that were left weedy for the first 20 days was not enough to impact crop yield, basically behaving like the weed-free counterpart. Despite this, at around 400 growing degree days (ca. 25 days after transplanting, for both years), temperatures began to rise quickly, and weed biomass rapidly increased, suggesting that even if plots seemed to have an acceptable weed infestation during the first 20 days, it is crucial to perform weeding in the early growth stage (up to 400 degree days) because the weed biomass is able to quickly develop and reduce lettuce yield in the second part of the growth cycle. This is important with respect to both grass and dicot biomass, which, as shown in the results (Table 4), were negatively correlated with lettuce yield.

While the weeding regime had no impact on plant height, plants in plots that were left weed-free for more than 10 days after crop transplanting developed a significantly larger circumference than the weedy plots. This confirms results from earlier studies on lettuce [32,33]. Lettuce head circumference is an important parameter for the evaluation of the crop's market value, and our results further confirm that lettuce plants grown in plots that were weed-free around 400 GDD were able to grow significantly larger than the ones in the weedy plots. For future research, it may be interesting to gather data on leaf number, as done by Casadei et al. [34] in a lettuce and pigweed (*Amaranthus retroflexus L.*) competition experiment.

Regarding relative leaf chlorophyll content (expressed as SPAD values), the differences between years is mainly due to an early senescence of lettuce plants in 2019, or a higher stress during the last 20 days of the growing season due to the high temperatures. The weedy control showed the lowest SPAD values in both years, but the range of values is quite narrow, and no significant correlation with total weed biomass was found. Contrary to these findings, Galon et al. [35] found clearer differences in a competition experiment between lettuce and ryegrass. The authors concluded that lower chlorophyll indices were likely caused by weed height, a parameter that we did not measure, rather than by weed biomass, due to the shadow effect created when weeds overgrow lettuce. Indeed, we found a positive correlation between broadleaved weed biomass and lettuce height (Table 4), likely due to the presence of *Chenopodium album* plants, since most of the other weeds generally grew lower than the crop. On the other hand, grasses reduced lettuce biomass but they did not stimulate lettuce height like the broadleaved species, while *Cyperus* spp. did not affect lettuce biomass, but lettuce was higher with increasing *Cyperus* spp. biomass and cover.

The results of this study show that the potential to provide weed-mediated biological pest control services cannot be guaranteed without compromising lettuce yield. Even though plots with an early weed-free regime (C20 and C30) were associated with species that can support beneficial insects, such as *Matricaria chamomilla* or *Malva sylvestris*, the dominant species in these plots was *Cyperus spp*. (Figure 4), which was able to establish itself quickly after the end of the critical weed-free period. In the weedy control and C10 plots, most of the beneficial weeds present were either prostrate (e.g., *Medicago spp.*, *Portulaca oleracea, Trifolium spp*.) or generally short (e.g., *Capsella bursa-pastoris, Senecio vulgaris*), but they had a low frequency and cover. The dominant weeds in our trial were *Amaranthus retroflexus* and *Chenopodium album*. Although *C. album* has been listed as a beneficial weed in supporting farmland birds [36,37], the traits related to height and growth rate suggest that these species should not be left unmanaged in a lettuce field. Furthermore, *C. album* has been found to support many polyphagous insects, and both weeds are considered secondary hosts to several crop pests [38–40]. When considering the attractiveness of weed flowers based on their color, most of the potentially interesting weed species associated with disturbed areas, such as *Portulaca oleracea* or *Polygonum aviculare* [5], did not flower during the lettuce growth cycle. Since lettuce can be grown in several cycles throughout the year, it is possible that weed communities that germinate in early spring may have a different capacity to support beneficial insects, and the community may be less competitive towards the crop compared to the weeds species that germinated during our trial, which was performed in late spring. The relationship between crop transplanting time and weed community composition should be further investigated. The critical weed-free period should also be calculated for different cropping seasons.

Our results show that weeding can be reduced in the last part of the lettuce growth cycle without compromising the marketable yield, and this may provide a reduction in the cost of weeding. From our results, we cannot conclude that the reduced weeding intensity will result in the development of a more diverse weed community with less competitive species, as has been shown for arable crops [12,13,41]. Our results indicate that there will be a selection for fast-growing species with a short life cycle, such as *Cyperus* spp. The question arises if it would be more efficient to develop vegetable cropping systems in a permanent living mulch of sown beneficial species, such as prostrate legumes. If species with optimal traits are selected, this may optimize the provisioning of services such as conservation biological control, support to crop pollinators, and increased soil fertility, while reducing the competition with the crop [42]. Another approach to increase ecosystem service provisioning in vegetable systems is to focus on land-sparing approaches, such as the management of field margins aimed at the conservation of beneficial spontaneous plant species [43], or the establishment of sown flower mixtures for specific beneficial insects, as has been successfully applied in open-field tomato production [43,44].

5. Conclusions

Previous studies have demonstrated that weed control in open-field lettuce can be reduced around 20–25 days after transplanting (DAT). In this study, we confirmed that plots that are kept weed-free from 130 GDD (8 DAT in both years) up to 321 GDD (19–22 DAT) did not suffer a significant yield loss. The innovative objective of our study was to analyze the weed communities that develop under various weeding regimes and determine their potential to support conservation biological control. Grasses, broadleaved weeds, and *Cyperus* spp. have a different effect on lettuce yield and growth parameters. Increasing *Cyperus* spp. biomass and cover did not reduce lettuce yield or head circumference, but it stimulated lettuce height. Grasses reduced lettuce yield and head circumference but did not affect height. Broadleaved weeds affected lettuce as grasses, but they stimulated plant height, likely due to *Chenopodium album* plants overgrowing lettuce. Fourth corner and RLQ analyses showed that the weed community developing in the weedy control and in plots weeded only during the first 10 days were different from the weed community in the plots weeded during the first 20 and 30 days. The former group contained more broadleaved weeds with a potential beneficial effect on conservation biological control, such as *Medicago spp., Portulaca oleracea, Trifolium spp*., while the latter had a higher abundance of *Cyperus* spp. Therefore, the results of our study suggest that, in our experimental conditions, tolerating weeds for potential ecosystem service provisioning is not likely to be successful. If we still want to embrace the idea of land sharing to increase ecosystem service provisioning in vegetable-cropping systems, future research should focus on the effects of the timing of the cropping cycle on the weed community composition, and in parallel, the use of sown living mulches could be studied. The opportunities to adopt a land-sparing approach to support pest control in lettuce are discussed since successful solutions for field margin management have been shown for other open-field vegetable crops. This study further shows that reduced weed control in vegetable crops may not be as successful as in arable crops to improve ecosystem service provisioning, probably due to the ephemeral characteristic of these systems. It further suggests the importance of weed functional trait analyses for ecosystem service provisioning to detect the most successful solutions for agroecological weed management in various cropping systems.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8090787/s1, Table S1: Classification of weed species by frequency of observation and local cover. Critical period of weed interference trial on romaine lettuce in 2019 (San Piero a Grado, Pisa); Table S2: Classification of weed species by frequency of observation and local cover. Critical period of weed interference trial on romaine lettuce in 2020 (San Piero a Grado, Pisa); Table S3: Full list of species found during cover samplings in each experimental year. Table shows species' botanical name, EPPO code, and frequency of observation at each sampling date in the weedy plots (n = 25).

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Data Availability Statement: The data presented in this study are openly available in Zenodo at DOI: 10.5281/zenodo.7032197. If interested in the R scripts used for data analysis, please contact the corresponding author.

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