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RESEARCH PAPER

Distance- and density-dependent recruitment of common ragwort is not driven by plant-soil feedbacks

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ABSTRACT

Janzen-Connell effects state that the accumulation of host-specific natural enemies near parent plants can negatively affect their offspring. Negative plant-soil feedbacks can produce patterns of seedling performance predicted by Janzen-Connell effects and influence plant populations, but their relevance in field conditions remains unclear. Here, using spatial point-pattern analysis, we examine the spatial distribution of *Jacobaea vulgaris* to assess whether distance- and density-dependent predictions of Janzen-Connell effects are evident in the field. We established 27 replicated 64 m^2 plots at two grassland sites and mapped positions of rosette-bearing and flowering *J. vulgaris* plants within each plot. To investigate temporal distribution patterns, we tracked plant positions repeatedly in three plots during a single season. Additionally, we tested whether these patterns are soil-mediated. Soil samples were collected underneath flowering plants and at a distance of 0.5 meter, and used to compare seed germination, seedling survival, and growth under controlled conditions. Furthermore, we measured *J. vulgaris* growth in soil from patches with high *J. vulgaris* densities and in soil from areas outside these patches. The density of rosette-bearing plants was lower at close distances from flowering plants than expected from null models, suggesting negative distance-dependent plant recruitment. The degree of clustering decreased over time from rosette-bearing to flowering plants, indicating density-dependent self-thinning. Seed germination was higher in soil further away from flowering *J. vulgaris* plants than in soil underneath plants at one site, but soil distance was not an overall significant factor in explaining seed germination. However, seedling mortality and biomass did not differ between soils collected at the two distances, and plants produced similar biomass in soil collected from inside and outside *J. vulgaris* patches. Our study demonstrates conspecific distance- and density-dependent plant recruitment in *J. vulgaris* in the field, but we found no evidence this depends on belowground natural enemies.

Introduction

Janzen-Connell effects state that the offspring of a plant will experience increased mortality at locations where conspecific adults have established and/or conspecifics aggregate, due to the accumulation of specialized natural enemies (e.g. seed predators, herbivores and pathogens) (Janzen, 1970; Connell, 1971). This hypothesis suggests that negative distance- and density-dependence will promote diversity in plant communities as the transfer of natural enemies will be more effective on dominant species and therefore will prevent dominant species from competitively excluding other species (Packer & Clay, 2000; Bagchi et al., 2014; Comita et al., 2014; Forrister et al., 2019). So far, Janzen-Connell effects have been confirmed in many studies primarily for tree species, but they can also play an important role in influencing plant population dynamics and plant diversity in grasslands (Mackay & Kotanen, 2008; Petermann et al., 2008). However, those propositions are often based on results from pot experiments and field monocultures, and experimental evidence for Janzen-Connell effects in natural grassland communities is rare.

Herbs often accumulate natural enemies in the soil and can influence

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later-growing plants via plant-soil feedbacks (Bever, 1994; Bever et al., 1997). Negative conspecific plant-soil feedbacks, where plants grow worse in soil of conspecifics than in soil of heterospecifics, can generate patterns of seedling performance consistent with the predictions of Janzen-Connell effects. Consequently, these effects can promote species coexistence and plant succession (Mills & Bever, 1998; Petermann et al., 2008; Fukami & Nakajima, 2013; van der Putten et al., 2013; De Long et al., 2023). Few studies have directly tested how soil-dwelling natural enemies influence predictions of Janzen-Connell effects for grassland species in the field (Liu et al., 2022). Instead, most studies conduct plant-soil feedback experiments with "home" and "away" soil collected from plants grown in pots or in field monocultures, and the results are then extrapolated to plant abundance in the field (Klironomos, 2002; Petermann et al., 2008; but see Kulmatiski & Kardol, 2008; Kos et al., 2013; Heinze et al., 2016, 2019). However, plant-soil feedback effects measured in field-collected soil from mixed plant communities may be "diluted" relative to those measured in soils conditioned by monocultures (Grenzer et al. 2021). These findings strongly emphasize the necessity of a comprehensively understanding of the role of plant-soil feedbacks in influencing plant performance and plant population dynamics.

Ecological processes such as negative density dependence can leave footprints on the spatial distribution or structure of plant species, and this is detectable by spatial point-pattern analysis (Wiegand & Moloney, 2004; Velázquez et al., 2016; Ben-Said, 2021). In a spatial analysis, a plant population is represented by a set of points within a mapped area and the organization of these points in space can be used to infer ecological processes. All else being equal, if recruitment suffers from conspecific distance-dependent mortality, we would expect to observe a lower density of young plants near adult plants compared to locations far away from adult plants (Swamy et al., 2011; Murphy et al., 2017). Moreover, if a population of growing plants experiences a progressive decline in density (known as self-thinning, Westoby, 1984), it is expected that young plants will exhibit a higher degree of clustering than adult plants (Getzin et al., 2008; Zhu et al., 2010; Das Gupta & Pinno, 2018). For many plant species, seedling recruitment in grasslands can vary strongly within a year and highly depends on environmental factors such as precipitation (Rusch & van der Maare, 1992). If seedling recruitment increases during the growth season, both the density of seedlings around adult plants and the degree of clustering among seedlings are expected to increase. Due to its elegance and power, spatial point-pattern analysis has great potential for the examination of conspecific distance- and density-dependent plant recruitment for species in dynamic mixed communities such as grasslands (De Luis et al., 2008; Zhao et al., 2020; Wang et al., 2020).

Combining analyses of seedling distribution relationships to conspecific adult plants with analyses of soil community feedbacks, enables examination of the role of soil biota in the conspecific distance- and density-dependent plant recruitment (Bever et al., 2010; Mangan et al. 2010). Negative plant-soil feedback effects are often associated with changes in the density or composition of soil organisms, and in particular with changes in microbial communities which occur more rapidly than changes in plant communities (Bezemer et al., 2006, 2018; Ke et al., 2015). Besides conditioning soil communities in pots, host-specific soil communities can also be obtained by sampling in proximity to adult plants (e.g. Kos et al., 2013; Ke et al., 2021). Therefore, rhizosphere soil from an adult plant and soil collected from an area with a high density of adult plants of the focal species are expected to harbor host-specific soil communities, which can have negatively effects on later-growing conspecific plants (negative feedback). However, such negative effects can also be attributed to other factors such as nutrient depletion rather than soil biota. To address this, field-collected soil can be added to sterilized bulk soil as inoculum or extra nutrients can be added to field-collected soil to overcome nutrient differences (Troelstra et al., 2001; Bezemer et al. 2006; Brinkman et al., 2010).

In this study, we investigate whether conspecific distance- and density-dependent plant recruitment are evident in the plant species *Jacobaea vulgaris* in two temperate grasslands in The Netherlands. Further, we examine whether the observed spatial patterns of plant recruitment are mediated by soil biota. We tested the following hypotheses:

(1) If conspecific distance-dependent plant recruitment is present in the field, the density of rosette-bearing plants near flowering *J. vulgaris* plants will be lower than expected from a random spatial distribution model.
(2) If there is conspecific density-dependent self-thinning, there will be higher spatial clustering among rosette-bearing plants than the spatial clustering between rosette-bearing plants and flowering plants, as young rosettes suffer more from density-dependent competition and/or natural enemies.
(3) Along the growing season, negative distance-based spatial patterns will become stronger, as seedling recruitment continues throughout the season and this will be inhibited nearby flowering plants.
(4) If distance-dependent effects are mediated by soil biota, plant performance will be lower in soil collected underneath flowering *J. vulgaris* plants than in soil collected away from flowering plants, this will be true in live soil and not in sterilized soil. To differentiate whether negative effects observed in underneath and away soil were due to nutrient differences in sterilized and live soil, we introduced an additional treatment with extra nutrients.
(5) If density-dependent self-thinning is soil-mediated, seedling performance will be lower in soil collected from patches with high densities of *J. vulgaris* than in soil from outside these patches.

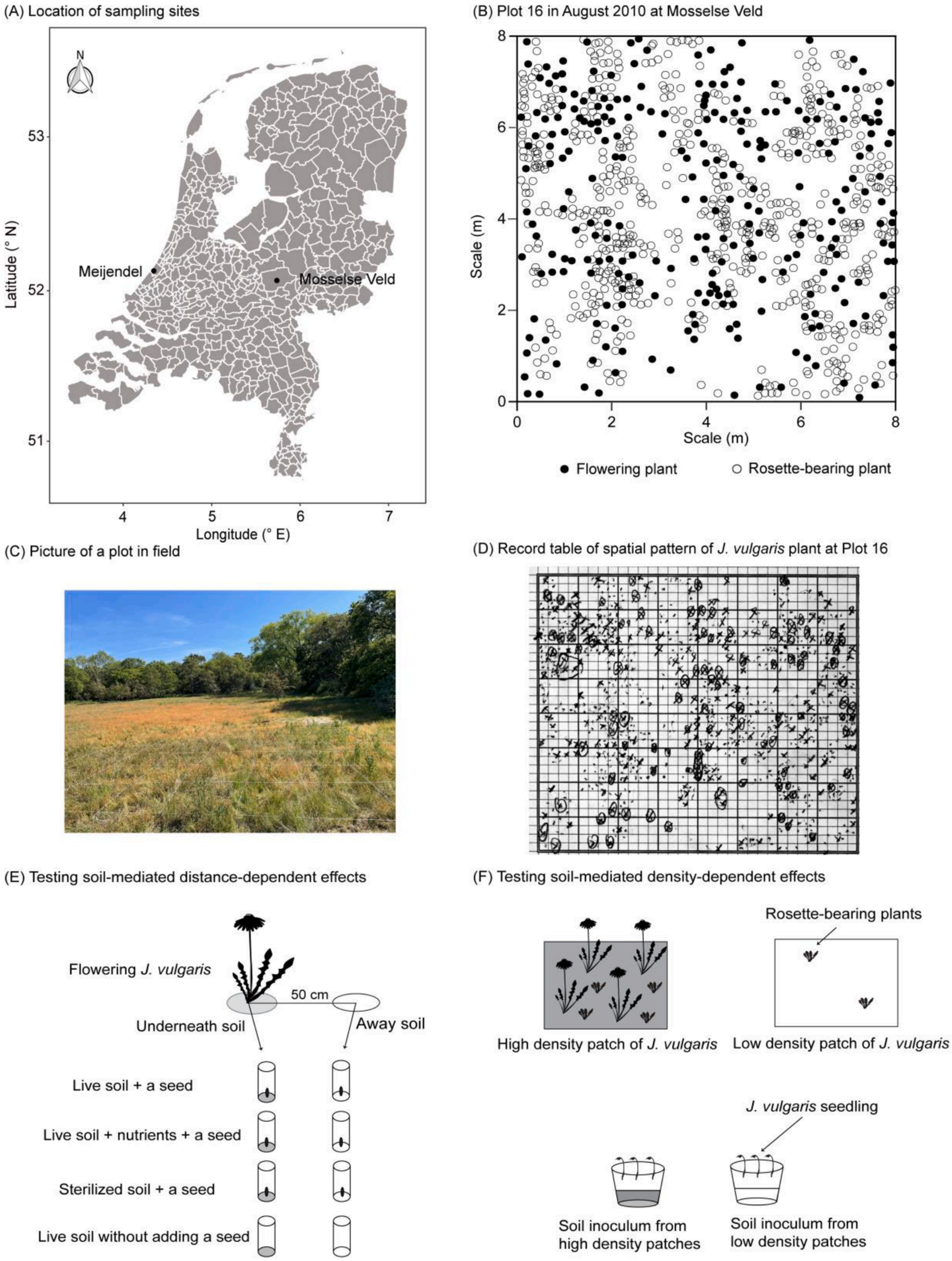
Materials and methods

Focal plant species

Jacobaea vulgaris (syn. *Senecio jacobaea* L.; Asteracaea), known as common ragwort, is a monocarpic biennial that is native in Europe (Harper & Wood, 1957). It is an early successional species that can become highly abundant in the field after disturbance (Harper & Wood, 1957; van de Voorde et al., 2012a). It has been found to suffer from strong conspecific negative plant-soil feedback, and soil biota (e.g. soil fungi) have been proposed to be important drivers of this pattern (Bezemer et al., 2006; van de Voorde et al., 2011). This species forms a rosette in the first year and flowers in the second year, even though flowering can be delayed e.g. due to herbivory (van der Meijden & van der Waals-Kooi, 1979). Plants will die after flowering and seed set (van der Meijden & van der Waals-Kooi, 1979). It can produce large numbers of seeds (up to 30,000 achenes per plant in the Meijendel, The Netherlands) (van der Meijden & van der Waals-Kooi, 1979), and seeds disperse mainly by wind and gravity (Wardle et al., 1987). Seeds can remain dormant in the soil for many years and germinate in autumn or spring (van der Meijden, 1979; van de Voorde et al., 2012a).

Study sites and spatial data recording

To investigate the spatial distributions of rosette-bearing and flowering *J. vulgaris* plants, a total of 27 plots measuring 8 m × 8 m each were established and monitored in two natural grasslands. Fifteen plots were established in a grassland of around 300 m × 100 m in Meijendel (52°07′ N, 4°20′ E) a coastal dune region (Fig. 1A) where *J. vulgaris* was present. The distance between each plot was at least 50 m at Meijendel. The population of *J. vulgaris* plants in this area exhibits significant fluctuations over years, with average cover ranging from 14 % to 0.5 % (van der Meijden, 1979). The sandy dune soil at Meijendel is known to have limited nutrient availability (Gao, 2023). Twelve plots were established in a natural grassland on a former arable field "Mosselse Veld" (where agricultural practices stopped in 1985) at the Veluwe area in the central


(A) Location of sampling sites

(B) Plot 16 in August 2010 at Mosselse Veld

(C) Picture of a plot in field

(D) Record table of spatial pattern of *J. vulgaris* plant at Plot 16

(E) Testing soil-mediated distance-dependent effects

(F) Testing soil-mediated density-dependent effects

Fig. 1. Location of the sampling sites in The Netherlands (A), spatial distribution of rosette-bearing (open dots) and flowering *J. vulgaris* plants (black dots) at plot 16 at Mosselse Veld (B), picture of a 8 m × 8 m plot at Meijendel (C), map of coordinates of rosette-bearing (dots) and flowering plants (cross) in a 8 m × 8 m plot (plot 16 at Mosselse Veld) (D), experimental design of testing for soil-mediated distance-dependent effects (E) and design of testing for soil-mediated density-dependent effects (F).

part of the Netherlands (52°04′ N, 5°44′ E) (Fig. 1A). Details about this latter site and the plots at this site are described elsewhere (Kos et al., 2013). At both sites, within a 400 m × 100 m grassland, 8 m × 8 m plots were established in areas where *J. vulgaris* was present, with a minimum distance of 80 m between plots. Plant species richness at Mosselse Veld was 15 species per m^2 on average, and the average cover of *J. vulgaris* was 6 % (Kos et al., 2013). The soil was a sandy loam soil and soil abiotic characteristics varied between plots (Kos et al., 2013). Concentrations of soil nutrients were higher at Mosselse Veld than at Meijendel (see Appendix A: Table A.1).

Data were collected in 2010, 2020 and 2021 (see Appendix A: Table A.2). Each plot was subdivided into 64 sub-plots of 1 m^2 (Fig. 1C and D) and each subplot was further divided into sixteen 0.25 m × 0.25 m cells (Fig. 1C and D). The location of each rosette and flowering

J. vulgaris was recorded on grid paper (Fig. 1D, plot 16 in August 2010 at Mosselse Veld). The abundance of *J. vulgaris* ranged from 118 to 1755 individuals/plot at Meijdendel, and from 86 to 903 individuals/plot at Mosselse Veld (see Appendix A: Table A.2). In addition, the rosette diameters of all young and flowering plants were measured and recorded in plot 1–8 in June 2020 at Meijdendel. At Meijdendel we observed that during the growth season, new seedlings of *J. vulgaris* emerged. To capture the temporal dynamics of spatial patterns of rosettes and flowering plants, we monitored three plots at Meijendel during the growth season in 2020, with plants recorded in late May, mid-July, and late August (see Appendix A: Table A.2). In the plots that were investigated in late May 2020, *J. vulgaris* plants were not yet flowering and we used the maps of flowering plants in July and August to identify the flowering plants of the first investigation.

Soil bioassay 1: testing for the soil-mediated distance-dependent effect

In June 2021, 30 flowering *J. vulgaris* plants were randomly selected at each of the two locations. The height of the flowering plants was recorded. We collected soil (3 cores, 5 cm diameter, 5 cm depth) underneath each flowering plant (*J. vulgaris* rhizosphere soil). A paired "away" soil sample was collected at 50 cm distance in a random direction and ensuring there were no other *J. vulgaris* plants nearby this away sample (Fig. 1E). In total, 120 soil samples (2 sites × 30 flowering plants × 2 soil types) were collected. Each soil sample was homogenized and gently sieved through a 1 cm sieve to remove stones and moss. Then, soil samples were stored at 4 °C until further use. To test for soil-mediated distance-dependent effects on plant growth and to examine whether this effect was related to nutrient deficiency in the soil or due to soil-borne pathogens, we conducted an experiment where plants were grown in microcosms (10 cm height, 2.5 cm diameter) filled with 10 g of soil (Fig. 1E). For each soil sample 9 microcosms were filled with soil. Three microcosms were autoclaved for 1 h to sterilize the soil (hereafter, sterilized soil). Three microcosms received 5 ml of sterilized water (hereafter, live soil), and the final set of three microcosms received 5 ml of Steiner nutrient solution which is widely used in plant growth experiments (hereafter, live soil + nutrients) (Steiner, 1968; Joosten et al., 2009; Zhang et al., 2022). The chemical composition of the Steiner nutrient solution that was used is shown in the Appendix (Appendix A: Table A.3). One surface-sterilized *J. vulgaris* seed was then placed on top of the soil in each microcosm. *J. vulgaris* seeds were collected from Meijendel in September 2020. Seeds were surface-sterilized by soaking in 5 % sodium hypochlorite for 20 min and rinsed three times with sterilized MillQ water. In total, there were 1080 microcosms (30 plants × 2 types × 2 sites × 3 treatments × 3 replicates = 1080 microcosms). Each microcosm was covered with a transparent plastic lid (2.65 cm height, 2.65 cm diameter). Microcosms were randomly placed in a tray in the climate chamber at 16/8 h light-dark regime and a 20/15 °C temperature regime and RH = 70 %. Seed germination was recorded for each microcosm every three days. 80 % of the seeds germinated within 7 days. Seedling mortality was recorded regularly. Four weeks after adding the seeds, the surviving seedlings were harvested. Roots were washed to remove soil and plants were oven-dried at 70 °C and total biomass per plant was recorded. The mean plant-soil feedback effect for each flowering *J. vulgaris* plant was then calculated with the following formula (Petermann et al., 2008; Brinkman et al., 2010; Bezemer et al., 2018): ln (plant dry mass in underneath soil / plant dry mass in away soil)

Seed bank experiment

To avoid the confounding effects of the seed bank, we also determined the seed germination of *J. vulgaris* from the seed bank in "underneath" and "away" soil collected for soil bioassay 1 (Fig. 1E). Microcosms were filled with 10 g of soil from each sample. Each microcosm received 5 mL water and was covered with a transparent plastic lid and kept under conditions as described above. Microcosms were checked regularly and germinated seedlings were identified and then gently removed with a tweezer. The number of *J. vulgaris* seedlings in each microcosm was recorded. There were three replicate microcosms for each soil sample and a total of 360 microcosms (2 sites × 30 flowering plants × 2 soil types × 3 replicates).

Soil bioassay 2: testing for the soil-mediated density-dependent effect

In March 2010, we examined whether the performance of *J. vulgaris* in soil collected from patches with high densities of *J. vulgaris* plants differed from that in soil collected outside these patches (Fig. 1F). This was done with soil samples collected from natural grassland at the Veluwe area near Mosselse Veld: Reijerscamp (52°00′ N, 5°47′ E), a former arable site where agricultural practices stopped in 2005. Sterilized *J. vulgaris* seeds were germinated in containers (10 cm × 10 cm × 4 cm) filled with a layer of sterilized glass beads submerged in water in a climate chamber at 16/8 h light-dark regime and a 20/15 °C temperature regime. Seeds were collected from Mosselse Veld in September 2009. After germination, seedlings were stored at 4 °C until further use. Soil was collected from 17 patches (Mean ± SE of the patch size: 8.53 ± 0.80 m^2) with high densities of flowering *J. vulgaris* plants over an area of 400 *m* × 400 m grassland. The distance between two patches was around 80 m. Soil samples (10 cores, 5 cm diameter, 15 cm depth) were collected in a 1-m^2 plot inside each patch and an adjacent 1-m^2 plot outside the patch. The number of *J. vulgaris* stems in each 1-m^2 plot was also recorded (see Appendix A: Table A.4). *J. vulgaris* stem density, mean height of the stems, number of rosettes (measured in May 2010), and percentages of bare soil and moss were also measured (see Appendix A: Table A.4). Each soil sample was homogenized and gently sieved (1 cm mesh size). Homogenized bulk soil, collected from the same area and sterilized with gamma radiation (> 25KGray, Isotron, Ede, The Netherlands), was mixed with the soil samples in a 1:6 ratio (live/sterilized). Pots (13 cm × 13 cm × 13 cm) were filled with l liter of the soil mixtures. Three *J. vulgaris* seedlings were planted in each pot and there were three replicate pots for each soil sample (Fig. 1F). In total 102 pots were used (17 patches × 2 density treatments × 3 replicates). Seedlings that died during the first week were replaced. The pots were placed randomly on three trolleys (3 blocks). Each block contained one replicate of the 34 samples. The position of the trolleys was regularly changed. In the greenhouse climatic conditions were controlled and light, humidity and temperature (16/8 h light-dark regime, 20/15 °C temperature regime and RH = 70 %) were equal for all pots during the experiment. Plants were watered three times per week and soil moisture content was regularly equalized during the course of the experiment by weighing pots and adding water appropriately. Eight weeks after planting, for each pot shoots were clipped and roots were rinsed with tap water to remove adhering soil. Shoot and root material was then oven dried (70 °C, 48 h), and plant dry-mass was determined.

Data analysis and statistics

Spatial pattern analysis for conspecific distance-dependent effects

The O-ring analysis was used to examine conspecific distance-dependent plant recruitment of *J. vulgaris* in the field (Wiegand & Moloney, 2004). The O-ring statistic is defined as following formula:

$$O(r) = \lambda \times g(r)$$

Where λ the point density of the pattern, and $g(r)$ is the pair correlation function, describing the mean number of points at distance *r* from the set of focal points independent of the intensity λ.

The pair correlation function is defined as:

$$g(r) = (2\pi r)^{-1} \times dK(r) / dr$$

Where $K(r)$ is the expected number of points within a circle of radius

r centered at a point, normalized by the intensity λ of the pattern (Ripley's K function). As the derivative of $K(r)$, the function $g(r)$ separates broad-scale patterns from successively cumulative short-scale patterns (Wiegand & Moloney, 2004). So that "the O-ring statistic estimates the mean number of neighboring individuals within an annulus of radius r and width w around a typical point of the pattern" (Wiegand & Moloney, 2004). In the case of our study, it can be used to quantify the density of rosette-bearing plants around flowering *J. vulgaris* at varying distances. The analyses were carried out for each of the 27 plots.

To investigate whether the distribution of flowering plants (pattern 1) restricts that of rosette-bearing plants (pattern 2), the appropriate null model is to randomize the locations of rosette-bearing plants while keeping the locations of flowering plants fixed (Velázquez et al., 2016). This is also known as antecedent conditions, in which pattern 1 influences pattern 2, but not the other way around. Because rosette-bearing plants in the 8 m × 8 m^2 plots do not follow a homogenous Poisson distribution, rosette-bearing plants were randomized following a heterogeneous Poisson process. Here, we employed a Gaussian smoothing kernel to directly estimate the intensity function λ (x, y) from the observed data (Wiegand & Moloney, 2004). We constructed the intensity function λ (x, y) using a bandwidth $R = 1.0$ m, as the median dispersal distance of *J. vulgaris* seeds is typically within 1 m (McEvoy & Cox, 1987; Wang et al., 2020). Based on a study of objects of finite size using spatial point-pattern analysis i.e. shrubs (Wiegand et al., 2006), we set the ring-width w to 0.02 m in our study. In the null models, rosette-bearing plants were spatially randomized with exactly the same density as observed in each plot. Significant departure from the null expectation (i.e., $O(r) = \lambda(r)$) was evaluated using the 5th-lowest and 5th-highest $O(r)$ statistics out of 99 simulations (95 % simulation envelopes). The aggregation of rosette-bearing plants around flowering *J. vulgaris* for each distance class (from 0 to 1 m with 0.02 m increments) was calculated. Positive values indicate positive association patterns, while negative values indicate segregation patterns. This analysis was conducted using the grid-based software Programita (Wiegand & Moloney, 2014).

To compare the aggregation of rosette-bearing plants around flowering *J. vulgaris* over different plots at the two sites, we used the method described by Graff and Aguiar (2011). The $O(r)$ values for each plot were transformed to the weighted $O(r)$ as follows:

If $O(r) >$ upper 95 % confidence limit (CL+), then the weighted $O(r) = (O(r) - \mathrm{CL}+) / \mathrm{CL}+$ (values larger than 0)

If $O(r) <$ lower 95 % confidence limit (CL−), then the weighted $O(r) = (O(r) - \mathrm{CL}-) / \mathrm{CL}-$ (values smaller than 0)

If $O(r)$ is in between the upper 95 % and the lower 95 % confidence limit (CL−), then the weighted $O(r) = 0$.

Conspecific density-dependent thinning

Density-dependent competition and accumulation of pathogens can result in mortality which thins the aggregated plants (self-thinning) and increases the distance between neighboring plants (Moeur, 1997). To infer the effect of density-dependent thinning from rosette-bearing to flowering stage, we used a case control approach (Getzin et al., 2008). The detailed information regarding this method can be found in the Appendix A. In brief, we categorized flowering plants as controls (pattern 1) and rosette-bearing plants as cases (pattern 2). Subsequently, we examined the difference between the mean number of rosette-bearing plants around a flowering *J. vulgaris* and that around a rosette-bearing plants at distance r, denoted as $g_{21}(r)$ and $g_{22}(r)$, respectively. If $g21(r) - g22(r) < 0$, this indicates that the case pattern (rosette-bearing plants) shows additional aggregation independent of the control pattern (flowering plants), reflecting a density-dependent thinning process. When $g21(r) - g22(r) < 0$, the scale r reflects the spatial scales at which density-dependent thinning takes place, and the radius with the maximum difference ($r_{\max}$) corresponds to the scale at which the strength of density dependent thinning culminates (Zhu et al., 2010). Linear regression was then used to examine the relationship between the total density of *J. vulgaris* plants (rosette-bearing plants and flowering plants) and $r_{\max}$ at plot level.

To test for any departure of the case pattern from the control pattern, we used the local random labelling method as the null model. This approach is advantageous for removing the larger-scale effects on density of rosette-bearing plants, such as environment filtering processes (Wiegand & Moloney, 2014; Velázquez et al., 2016). Specifically, we kept the locations (coordinates) of all plants as observed, but randomly assigned labels of rosette-bearing plants to locations of all plants within a 1-m scale based on the aforementioned seed dispersal range (McEvoy & Cox, 1987; Wiegand & Moloney, 2014). The difference between $g_{21}(r)$ and $g_{22}(r)$ was recalculated in each simulation. Significant departure from the null expectation (i.e. $g_{21}(r) - g_{22}(r) = 0$) with local random labelling was evaluated through the 95 % confidence intervals of randomly-generated case-control differences in 99 simulations. This analysis was conducted using the grid-based software Programita (Wiegand & Moloney, 2014). To compare the degree of self-thinning for *J. vulgaris* over plots, we used the method akin to weighted $O(r)$ as described above. To examine whether rosette-bearing plants are smaller than expected near flowering plants, we used the mark correlation function to analyze the diameter of rosette-bearing plants around flowering plants at distance r (Illian et al., 2008; Wiegand & Moloney, 2014; Velázquez et al., 2016). Detailed information regarding this method can be found in the supplementary materials.

Soil bioassay 1

Seed germination, seedling mortality and biomass: The effects of the soil type (underneath or away), soil treatment (sterilized soil, live soil, or live soil with nutrients) and their interaction on germination (yes/no) were tested using generalized linear mixed models with a binomial distribution. Analyses were done separately for the two sites. Flowering plant ID was added as a random effect. The significance of factors was assessed by comparing models with and without the factor using a Chi-squared Likelihood Ratio (LR) test and by comparing the residual deviance. Generalized linear mixed models with a binomial distribution were performed using the "glmer" function with the "lme4" package (Bates et al., 2015). The effects of soil type, soil treatment and their interaction on seedling mortality and total dry mass were tested by a two-way ANOVA with soil type and soil treatment as main factors for each site. A Tukey's post-hoc test was used for pair-wise comparisons of soil type and soil treatment. The relationship between mean plant-soil feedback effects and the height of flowering *J. vulgaris* plants was examined with linear regression using the "lm" function in R.

The difference in the number of seedlings of *J. vulgaris* that emerged from the seed bank in the underneath soil and the away soil was tested using a generalized linear mixed model with a binomial distribution and with flowering plant ID as the random effect for each site. As none of the microcosms had more than one seedling presence/absence was analyzed.

Soil bioassay 2

The effects of the origin of the soil (inside or outside the patch) on individual shoot dry mass, total shoot dry mass and total root dry mass of *J. vulgaris* were analyzed using a pairwise *t*-test with the patch treatment (inside/out) as factor. ANOVA tests were carried out with the "aov" function and post-hoc tests were performed using the "glht" function with the "multcomp" package (Hothorn et al., 2008). In all analyses, residuals were checked for homogeneity of variance using a Levene's test and normality by a Shapiro Wilk test. The Levene's test and Shapiro Wilk test were performed using the "levene_test" and "shapiro_test" functions with the "rstatix" package (Kassambara, 2022). Plant dry mass was square-root transformed to fulfil requirements of normality.

All analyses were performed using the R statistical language, version 4.0.4 (R Core Team, 2022) and the grid-based software Programita (Wiegand & Moloney, 2014).

Results

Distance-dependent spatial patterns

The neighborhood density of rosette-bearing plants around flowering plants at the two sites overall showed similar patterns but the patterns also varied between years (Fig. 2). The observed density of rosette-bearing plants near flowering plants was lower than expected from null models (a segregated pattern of rosette-bearing and flowering plants) (Fig. 2). This was evident for more than half of the plots at Meijendel in 2020 ($n = 11$, 6 out of 11) and for all plots in 2021 ($n = 4$, all 4) (Fig. 2A and C), and for more than 50 % and 75 % plots at Mosselse

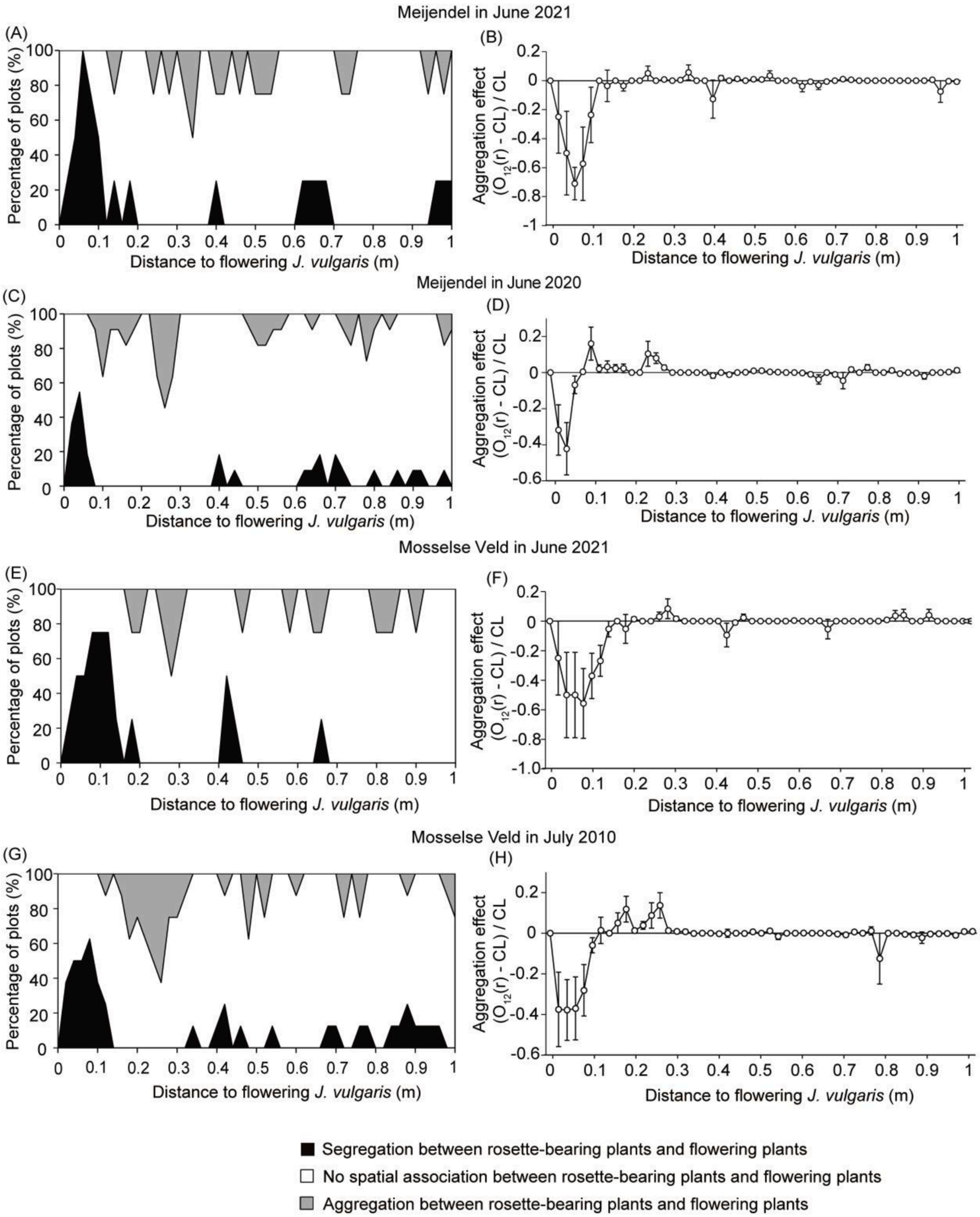


Fig. 2. Percentage of plots where the neighborhood density of rosette-bearing plants at distance r (m) to flowering *J. vulgaris* plants ($O_{12}(r)$) are lower than expected from null models, do not deviate from expected from null models or are higher than expected from null models, and mean (± SE) relative spatial association of rosette-bearing and flowering plants based on the weighted O-ring function analysis. (A) and (B) show results for June 2021 at Meijendel ($n = 4$); (C) and (D) show results for June 2020 at Meijendel ($n = 11$); (E) and (F) show results for June 2021 at Mosselse Veld ($n = 4$); (G) and (H) show results for July 2010 at Mosselse Veld is presented ($n = 8$). The antecedent condition null model (Wiegand & Moloney 2004) randomizes the locations of rosette-bearing plants (pattern 2) and keeps the locations of flowering plants fixed (pattern 1). In (A), (C), (E) and (G) the black area indicates the percentage of plots that shows segregation (values below the lower limits of the 95 % confidence interval), the white area indicates the percentage of plots with no spatial association (values in between the 95 % confidence interval), and the light grey area indicates the percentage of plots that shows aggregation (values above the higher limits of the 95 % confidence intervals). In (B), (D), (F) and (H) negative values indicate segregation, positive values indicate aggregation, and zero indicates no spatial association between rosette-bearing and flowering plants.

Veld in 2010 ($n = 8$, 4 out of 8) and 2021 ($n = 4$, 3 out of 4), respectively (Fig. 2B and D). The segregated pattern was present at 0 – 8 cm (2020) and 0 – 20 cm (2021) at Meijendel, and at 0 – 14 cm (2010) and 0 – 20 cm (2021) at Mosselse Veld (Fig. 2).

Density-dependent self-thinning

The percentage of plots that showed density-dependent thinning of rosette-bearing plants, varied between sites and years (Fig. 3). At Meijendel, in June 2021, more than half of the plots showed density-dependent thinning ($n = 4$, black area in Fig. 3A), while in June 2020 this was true for around 20 % of plots ($n = 11$; Fig. 3C). At Mosselse Veld, all plots ($n = 4$) in 2021 and 75 % ($n = 8$) of plots in 2010, showed density-dependent thinning (Fig. 4B and D). Among plots, the distance to flowering plants at which the strength of density-dependent thinning peaked was not correlated to the density of *J. vulgaris* at the plot level (see Appendix A: Fig. A.2).

During the growth season in 2020 at Meijendel, the density of rosette-bearing plants increased and the segregation pattern of rosette-bearing plants and flowering plants at fine scales increased (Fig. 4; see Appendix A: Table A.1). Specifically, there was no spatial association between rosette-bearing plants and flowering plants in late May, but there was in July and August 2020 (Fig. 4A, C and E). Along the growth season, a self-thinning pattern of rosette-bearing plants emerged (Fig. 4B, D and F). Moreover, the observed mean diameter of rosette-bearing plants nearby flowering plants did not differ from the expectation of the null model (see Appendix A: Fig. A.1).

Seed germination and seedling survival in soil bioassay 1

There was no difference in the proportion of microcosms that contained a germinating *J. vulgaris* seedling from the seed bank between underneath and away soil (Meijendel: $\chi 2 = 0.96$, $P = 0.33$; Mosselse Veld: $\chi 2 = 1.25$, $P = 0.26$; see Appendix A: Fig. A.3). The proportion of microcosms where the added seed germinated was higher in away soil than in underneath soil, but this was only true for soil samples from Mosselse Veld (Fig. 5A and B; see Appendix A: Table A.4). The proportion of microcosms with a germinated seedling varied significantly among the three soil treatments, but only at Meijendel, where it was lower in the sterilized soil than in the live soil (Fig. 5A; see Appendix A: Table A.4). There was no difference in seedling mortality between underneath soil and away soil at both sites (Fig. 5C and D; see Appendix A: Table A.4). However, mortality of the germinated seedlings at Meijendel, was higher in live soil with addition of nutrients than in sterilized soil (Fig. 5C; see Appendix A: Table A.4).

Plant biomass in the soil bioassays

Seedling biomass did not differ in underneath soil and away soil at both sites (Fig. 5E and F; see Appendix A: Table A.5). There was no difference in seedling biomass among soil treatments (Fig. 5E and F; see Appendix A: Table A.5). Flowering *J. vulgaris* plants were taller at Mosselse Veld (Mean ± SE: 75.43 ± 1.93 cm) than at Meijendel (Mean ± SE: 42.37 ± 1.75 cm) ($F_{1,\ 58} = 31.51$, $p < 0.001$), but there was no relationship between mean plant-soil feedback effects and the height of flowering *J. vulgaris* plants (see Appendix A: Fig. A.4). In the pot experiment with soil from inside and outside patches with flowering *J. vulgaris* plants, plants produced similar biomass in the soil from inside and outside of these patches (see Appendix A: Table A.6).

Discussion

The aim of this study was to examine soil-mediated Janzen-Connell effects of the herb *J. vulgaris* in natural grasslands. Four findings arise from this study. First, the density of rosette-bearing plants was overall lower at close distance from flowering plants than expected from null models. Second, there was a density-dependent self-thinning effect from rosette-bearing plants to flowering plants. This distance-based spatial pattern and the self-thinning effect appeared and became stronger during the growth season. Third, seed germination of *J. vulgaris* was lower in soil underneath flowering plants than in the away soil at Mosselse Veld, but soil distance was not an overall significant factor in explaining seed germination. Lastly, plant biomass did not differ neither in pairwise underneath and away soil, nor in soil collected from pairwise inside and outside *J. vulgaris* patches. Overall, our study provides spatially-based evidence for conspecific distance- and density-dependent plant recruitment for *J. vulgaris* in natural grasslands, but we find no evidence that this is connected to soil conditions.

With respect to the first hypothesis, the density of rosette-bearing plants was lower than expected at close distances from flowering plants, and this negative association was present in the majority of plots at both sites. This implies a conspecific distance-dependent plant recruitment for *J. vulgaris* (Barot et al., 1999; De Luis et al., 2008; Miao et al., 2018). Such distance-dependent effect is often observed within a few meters from adult trees in forests (Swamy & Terborgh, 2010; Miao et al., 2018; Malik et al., 2023). In our study, we found a negative spatial association of rosette-bearing plants and flowering plants at a much smaller distance from flowering plants. This is in line with a previous study that showed that seedlings of different herbaceous perennials exhibited a segregation pattern at centimeter scales (up to 25 cm) (De Luis et al., 2008). In the field, the flowering *J. vulgaris* plants had established at least a year earlier than rosette-bearing plants. These flowering plants could have caused abiotic (i.e. nutrient deficiency) and/or biotic changes (i.e. soil fungal communities, or allelopathic effects via chemical compounds) in the soil surrounding the plant roots and these effects can lead to negative plant-soil feedbacks (van de Voorde et al., 2012a; Kos et al., 2013, 2015). *Jacobaea vulgaris* exhibits a relatively strong negative plant-soil feedback in pot experiments (van de Voorde et al., 2011). Therefore, according to our fourth hypothesis, we predicted that young *J. vulgaris* plants would perform worse in soils collected underneath conspecific flowering plants, and that this would eventually lead to a negative spatial association between rosette-bearing plants and flowering plants in the field. Partially following our expectation, seed germination of *J. vulgaris* was lower in the underneath soil than in the away soil only at Mosselse Veld. The negative effects of underneath soil on seed germination being only detected at one site suggests that soil-dwelling natural enemies may not be the main or only reason for the observed conspecific distance-dependent plant recruitment in *J. vulgaris*. Forero et al. (2019) reported that greenhouse-derived plant-soil feedbacks were larger than and not correlated with field measured ones. Hence a strong negative plant-soil feedback in pot experiments for *J. vulgaris* does not necessarily mean that there is a strong negative plant-soil feedback in the field, and this may be an explanation for our results. In addition, recent studies suggest that plant-soil feedbacks are not operating independently but interact with multiple factors (i.e. herbivores, competition and environmental stress) under field conditions (Heinze & Joshi, 2018; Beals et al., 2020; Kardol et al., 2023). Therefore, except for soil-mediated effects, there are many other potential drivers for the observed distance-dependent effects such as herbivores, seed predators and foliar pathogens (i.e. fungal pathogens) (Forrister et al., 2019; Liu et al., 2022). In our study system, the specialized leaf-chewing caterpillars of *Tyria jacobaeae*, larvae of the cinnabar moth, feed on flowering *J. vulgaris* plants. A spillover of herbivores such as cinnabar moth larvae on neighboring conspecific rosette-bearing plants may also result in the observed the distance-dependent effect (e.g. van der Meijden, 1979).

We found neutral and negative conspecific soil effects when we measured seed germination in Meijendel and Mosselse Veld soil, respectively. This is in line with a recent study that examined the strength of plant-soil feedbacks on seed germination in conspecific versus heterospecific soils for seven plant species (Miller et al., 2019). They found that three of the plant species exhibited both neutral and

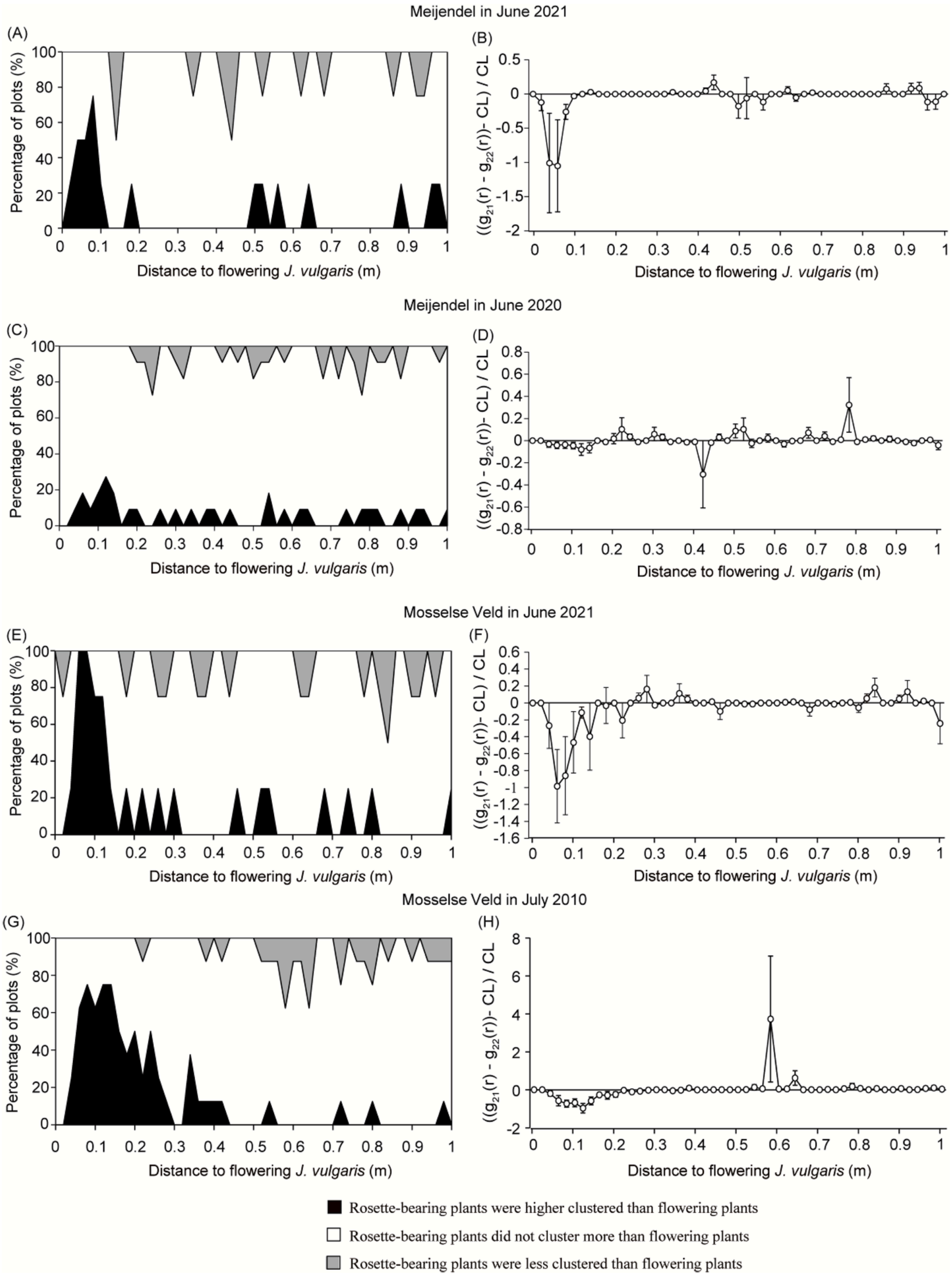


Fig. 3. Self-thinning effects at distance r (m) to flowering *J. vulgaris* plants and mean (± SE) relative self-thinning effect results from the weighted $g_{21}(r) - g_{22}(r)$ function analysis. (A) and (B) show results for June 2021 at Meijendel ($n = 4$); (C) and (D) show results for June 2020 at Meijendel ($n = 11$); (E) and (F) show results for June 2021 at Mosselse Veld ($n = 4$); (G) and (H) show results for July 2010 at Mosselse Veld ($n = 8$). We used the local random labeling null model (rosette-bearing plants were not moved more than 1 m) to access spatial correlation of rosette-bearing and flowering plants at fine scales. In (A), (C), (E), and (G) the black area indicates the percentage of plots where rosette-bearing plants were more clustered than flowering plants which represents self-thinning of plants ($g_{21}(r) < g_{22}(r)$ below the lower limits of the 95 % confidence interval), the white area indicates the percentage of plots where rosette-bearing plants did not show more clustering than flowering plants ($g_{21}(r) < g_{22}(r)$ in between the 95 % confidence interval), the light grey area indicates the percentage of plots where rosette-bearing plants were less clustered than flowering plants ($g_{21}(r) > g_{22}(r)$ above the higher limits of the 95 % confidence interval). In (B), (D), (F) and (H) negative values indicate self-thinning of plants, positive values indicate rosette-bearing plants were less clustered than flowering plants, and zero indicates rosette-bearing plants did not cluster more than flowering plants.

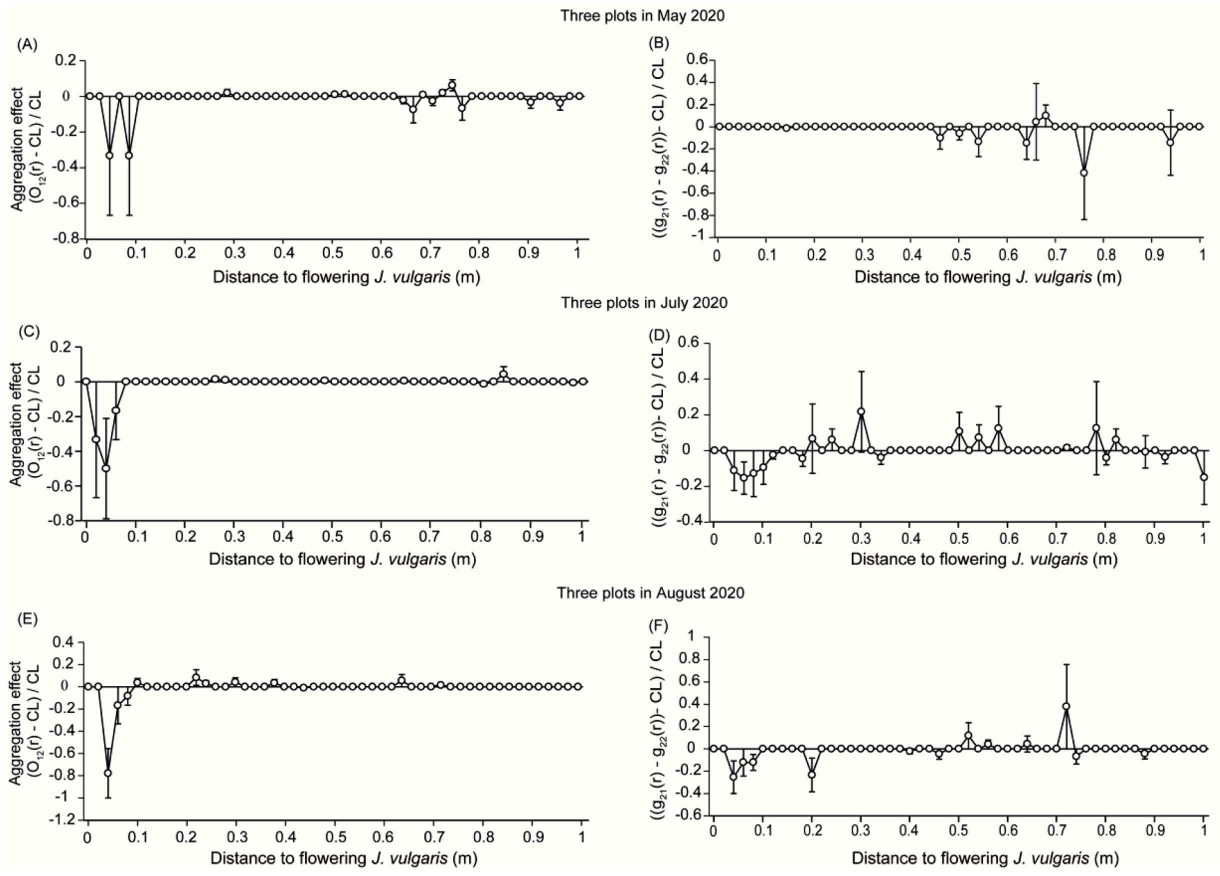


Fig. 4. Mean (± SE) relative spatial association of rosette-bearing and flowering *J. vulgaris* plants resulting from the weighted O-ring function analysis (A, C, E) and relative self-thinning effects resulting from the weighted $g_{21}(r) - g_{22}(r)$ function analysis (B, D, F) in 3 temporal plots in 2020 at Meijendel. In (A), (C) and (E), the antecedent condition null model (Wiegand & Moloney 2004) randomizes the locations of rosette-bearing plants (pattern 2) and keeps the locations of flowering plants fixed (pattern 1). Negative values indicate segregation, positive values indicate aggregation, and zero values indicate no spatial association. In (B), (D) and (F), the local random labeling null model (rosette-bearing plants were not moved more than 1 m) to access spatial correlation of rosette-bearings and flowering plants at fine scales. Negative values indicate self-thinning of plants, positive values indicate that rosette-bearing plants were less clustered than flowering plants, and zero values indicate that rosette-bearing plants did not cluster more than flowering plants.

negative plant-soil feedbacks on seed germination, depending on the identity of heterospecific soils. Germination of *J. vulgaris* can be inhibited by auto-toxic effects under laboratory conditions (van de Voorde et al., 2012b). Except for these auto-toxic effects, microbes present in the rhizosphere (i.e. root-associated bacteria) of this plant species can also suppress seed germination via volatiles (X. Liu, unpublished results). In our study, negative effects of conspecific soil on seed germination were only observed in the soils collected from Mosselse Veld. Notably, soil collected from Mosselse Veld contained a higher concentration of nutrients than soil collected from Meijendel. The presence of nutrient-rich soil can foster the growth of more detrimental soil microbial communities (Solomon et al., 2003; Wei et al., 2018). Therefore, one possible explanation is that the proportion of detrimental soil microbes may be higher in soil collected from Mosselse Veld than in the soil from Meijendel. This also aligns with the result showing a significant increase in seedling mortality after adding nutrients to the soil collected from Meijendel. Furthermore, *J. vulgaris* seeds were collected from Meijendel only in our study. A previous study has found that *Cecropia insignis* seeds collected from a foreign site experienced a lower survival than local *C. insignis* seeds when they were buried beneath the local *C. insignis* crowns (Gallery et al., 2007). We propose that *J. vulgaris* seeds from Meijendel may be adapted to soil microbes from the local soil in Meijendel, but not to soil microbes from Mosselse Veld and that this may explain the negative conspecific soil effects on seed germination at Mosselse Veld only as well. This is also consistent with our finding that the biomass of *J. vulgaris* was lower at Mosselse Veld than at Meijendel. Whether this is true needs to be examined in further studies.

Seed germination was the lowest in sterilized soil and this is somewhat unexpected and in contrast with other studies (Liu et al., 2015; Miller et al., 2019). Seed germination was also delayed in sterilized soil. The sterilized soil was somewhat compacted after being autoclaved and lower seed germination in sterilized soil may be the consequence of changes in the structure of the soil. However, it is also possible that microbes in the soil that are beneficial for germination were killed during autoclaving, and recent studies have found that soil microbes can stimulate seed germination i.e. through germination-related enzymes (Keeler & Rafferty, 2022; Cardarelli et al., 2022). Seedling mortality was not significantly different in sterilized soil and in live soil, indicating that there were limited effects of soil biota on seedling survival. However, addition of nutrients in live soil collected from Meijendel increased seedling mortality probably because detrimental soil microbial communities were stimulated in the situation with more nutrients.

In line with the second hypothesis, we observed a density-dependent self-thinning effect on *J. vulgaris* plants, as we found that rosette-bearing plants were more clustered than flowering plants. Both density-dependent competition (i.e. competition for light and space with conspecifics), herbivores and negative plant-soil feedbacks can result in this spatial pattern. In accordance with the third hypothesis, the distance-based spatial pattern and self-thinning of plants appeared and became stronger in the plots where the spatial pattern was recorded repeatedly. During the growth season, in these plots there was an increase in the density of rosette-bearing plants at the plot level, but there were even

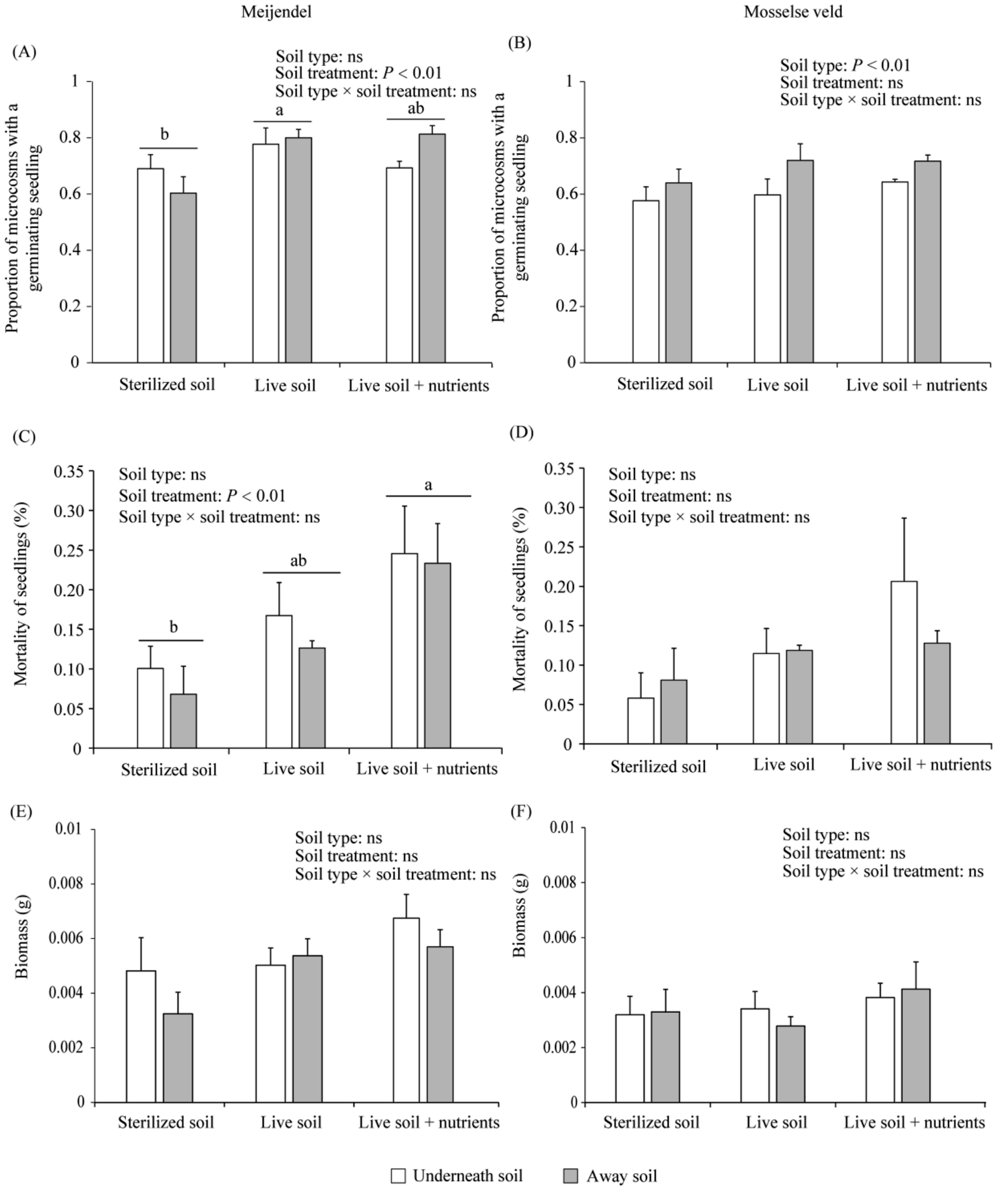


Fig. 5. Proportion of microcosms with a germinating seedling (A, B), seedling mortality (C, D) and plant biomass (E, F) in "underneath" and "away" soils for the different soil treatments (live soil, sterilized soil and live soil with nutrients). Soil was collected from Meijendel and Mosselse Veld. In (A) and (C) letters above each set of bars indicate significant differences between soil treatments ($P < 0.05$) based on a Tukey HSD post hoc test.

fewer rosette-bearing plants present at close distances to flowering plants. Further, the lack of a significant difference in seed bank density of *J. vulgaris* between underneath soil and away soil indicates that viable seeds are homogeneously distributed in the soil. Young seedlings are more fragile than older plants (Ailstock et al., 2010; Bezemer et al., 2018; Jevon et al., 2020). Therefore, the temporal variation in the spatial pattern that we observed suggests that conspecific negative effects exist and that they suppress the recruitment of *J. vulgaris* plants at fine scales. Except for the above-mentioned effects from herbivory and auto-toxicity, extracellular self-DNA i.e. via litter of flowering plants may also contribute to this (Mazzoleni et al. 2015a, 2015b).

Jacobaea vulgaris has been reported to experience a strong reduction in biomass when planted in conspecific conditioned soil in pot experiments (van de Voorde et al., 2011, 2012a). Based on these findings, we formulated the last two hypotheses, assuming that the biomass of *J. vulgaris* would be lower in soil collected from underneath flowering *J. vulgaris* plants and in soil collected from patches with high densities of *J. vulgaris*. However, contrary to our expectations, we found biomass of

J. vulgaris did not differ between field-collected "home" and "away" paired soil samples in both soil bioassays. Aligning with this, we did not observe that rosette-bearing plants were smaller than expected when they were close to flowering plants with the mark correlation function analysis. In our microcosm experiment, plants were growing in a limited amount of soil and space, and it was a short-term experiment. However, *J. vulgaris* has been found to exhibit negative plant-soil feedbacks even during the initial phase of growth (Bezemer et al., 2018; Zhang et al., 2022). Therefore, the neutral plant-soil feedback measured in field-collected soil may not be due to the experimental design. It is important to note that soil conditioning effects by monocultures in pot experiments might be more amplified than those observed in soil directly collected from field (Forero et al. 2019). This could potentially explain the absence of soil effects in our study. In a previous study at the Mosselse Veld grassland, Kos et al. (2013) reported that *J. vulgaris* exhibited the poorest growth in soil inoculated (1 part of live field soil to 6 parts of sterilized bulk soil) with soil collected from locations with high densities of *J. vulgaris*, intermediate when inoculated with soil collected from locations with low density of *J. vulgaris* and best in pots inoculated with heterospecific soil (*Calluna vulgaris* soil). In contrast, in our study, plant growth did not differ when pots were inoculated with live soil collected from inside or outside *J. vulgaris* patches from another former arable site in the same region. Previous studies have shown that disturbance can influence the outcome of plant-soil feedbacks, such as through decreased pathogen activity or increased nutrient availability (Kulmatiski & Kardol, 2008; Carvalho et al., 2010). Notably, *J. vulgaris* can establish well in disturbed soils (Cameron, 1935). Therefore, disturbance history may explain the lack of difference in biomass in the inside/outside patch soil in our study.

Conclusions

Overall, from our study we conclude that conspecific distance- and density-dependent plant recruitment are evident in this grassland species. However, we found no evidence to support the hypothesis that this is driven by belowground differences in soil biotic properties. The role of plant-soil feedbacks in influencing plant performance and establishment under natural conditions remains an important research field but more studies are needed that consider various influencing factors simultaneously (Heinze et al., 2016; Heinze & Joshi, 2018).

Data availability

All data will be available on Mendeley data upon the acceptance of the paper.

CRediT authorship contribution statement

Xiangyu Liu: Conceptualization, Methodology, Data curation, Investigation, Formal analysis, Writing – original draft. **Dong He:** Formal analysis, Writing – original draft. **Klaas Vrieling:** Data curation, Writing – original draft. **Suzanne T.E. Lommen:** Data curation, Writing – original draft. **Chenguang Gao:** Investigation, Writing – original draft. **T. Martijn Bezemer:** Conceptualization, Methodology, Data curation, Writing – original draft.

Declaration of competing interest

We declare that this work is original. We also declare that we have no conflict of interest and that our work complies with the current laws of the Netherlands.

Acknowledgments

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.baae.2024.02.003.

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