Local variation in conspecific plant density influences plant-soil feedback in a natural grassland

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Abstract

Several studies have argued that under field conditions plant-soil feedback may be related to the local density of a plant species, but plant-soil feedback is often studied by comparing conspecific and heterospecific soils or by using mixed soil samples collected from different locations and plant densities. We examined whether the growth of the early successional species *Jacobaea vulgaris* in soil collected from the field is related to the local variation in plant density of this species. In a grassland restoration site, we selected eight 8x8 m plots, four with high and four with low densities of *J. vulgaris* plants. In 16 subplots in each plot we recorded the density and size of *J. vulgaris*, and characteristics of the vegetation and the soil chemistry. Soil collected from each subplot was used in a greenhouse pot-experiment to study the growth of *J. vulgaris*, both in pure field soil and in sterile soil inoculated with a small part of field soil.

In the field, flowering *J. vulgaris* plants were taller, the percentage of rosette plants was higher and seed density was larger in high- than in low-density plots. In the pot experiment, *J. vulgaris* had a negative plant-soil feedback, but biomass was also lower in soil collected from high- than from low-density plots, although only when growing in inoculated soil. Regression analyses showed that *J. vulgaris* biomass of plants growing in pure soil was related to soil nutrients, but also to *J. vulgaris* density in the field.

We conclude that in the field there is local variation in the negative plant-soil feedback of *J. vulgaris* and that this variation can be explained by the local density of *J. vulgaris*, but also by other factors such as nutrient availability.

Zusammenfassung

Verschiedene Studien haben dargelegt, dass unter Feldbedingungen das Pflanze-Boden-Feedback mit der lokalen Dichte der Pflanze in Verbindung stehen könnte, aber das Pflanze-Boden-Feedback wurde oft untersucht, indem konspezifische und heterospezifische Böden miteinander verglichen wurden oder indem gemischte Bodenproben von verschiedenen Standorten und Pflanzendichten benutzt wurden. Wir untersuchten, ob das Wachstum der frühen Sukzessionspflanze *Jacobaea vulgaris* in im Freiland gesammelten Böden mit der Variation in der lokalen Dichte dieser Art in Beziehung steht. In einem rekultivierten Grasland wählten wir acht 8 m x 8 m große Probeflächen aus, vier mit hoher und vier mit geringer Dichte von *J. vulgaris* mit jeweils 16 Unterprobeflächen. Hier registrierten wir die Dichte und Größe der *J. vulgaris*-Pflanzen, sowie Eigenschaften der Vegetation und Daten zur Bodenchemie. Boden von jeder Unterprobefläche wurde in einem Topf-Experiment im Gewächshaus verwendet, um das Wachstum von *J. vulgaris* sowohl in reiner Freilanderde als auch in steriler Erde, die mit einer geringen Menge Boden aus dem Freiland inokuliert war, zu untersuchen.

Im Freiland waren auf Flächen mit hoher Pflanzendichte die blühenden *J. vulgaris*-Pflanzen größer und der Anteil von Rosettenpflanzen sowie die Samendichte höher als auf Flächen mit geringer Pflanzendichte. Im Topf-Experiment zeigte *J. vulgaris* ein negatives Pflanze-Boden-Feedback, aber die Biomasse war ebenfalls geringer in Erde von Flächen mit dichtem Pflanzenbestand als in Erde von Flächen mit geringer Pflanzendichte - allerdings nur bei inokulierter Erde. In Regressionsanalysen zeigte die Biomasse der *J. vulgaris*-Pflanzen eine Beziehung zu den Nährstoffgehalten, wenn sie in reiner Erde wuchsen, aber auch eine Beziehung zur Pflanzendichte im Freiland.

66 Wir schließen hieraus, dass es im Freiland eine räumliche Variation des negativen
67 Pflanze-Boden-Feedbacks gibt und dass diese Variation durch die lokale Dichte von *J.*
68 *vulgaris* aber auch durch andere Faktoren wie die Nährstoffverfügbarkeit erklärt werden kann.
69
70 **Keywords:** *Calluna vulgaris*, Inoculated soil, *Jacobaea vulgaris*, Nutrients, Pure soil,
71 Ragwort, *Senecio jacobaea*, Soil pathogens, Spatial patterning.

Introduction

Plants influence the biology, chemistry and structure of the soil they grow in, which in turn, can lead to changes in the performance of plants that grow later in the soil. This process is referred to as plant-soil feedback (PSF) (Bever, Westover & Antonovics 1997; Ehrenfeld, Ravit & Elgersma 2005; Kulmatiski, Beard, Stevens & Cobbold 2008; Van der Putten, Bardgett, Bever, Bezemer, Casper et al. 2013). PSF can be mediated by abiotic soil conditions, such as availability of nutrients, as well as biotic conditions, such as presence of microorganisms (Bezemer, Lawson, Hedlund, Edwards, Brook et al. 2006b; Kulmatiski et al. 2008). Biotic PSF effects can be highly species-specific, while abiotic PSF effects are often less species-specific (Aerts & Chapin 2000; Reynolds, Packer, Bever & Clay 2003). PSF can be positive due to increased nutrient availability (Chapman, Langley, Hart & Koch 2006) or accumulation of mutualistic microorganisms such as arbuscular mycorrhizal fungi (Klironomos 2002). Negative PSFs can arise from nutrient depletion or immobilization (Ehrenfeld et al. 2005) or the build-up of pathogenic soil microorganisms (Van der Putten, Van Dijk & Peters 1993; Packer & Clay 2000; Klironomos 2002).

Several studies have argued that the PSF of a species can explain the abundance of this species in the field (Klironomos 2002; Reynolds et al. 2003; Kulmatiski et al. 2008; Mangan, Schnitzer, Herre, Mack, Valencia et al. 2010). These studies typically have examined how species-specific PSFs can explain interspecific variation, *e.g.* between dominant and rare plant species. However, several studies have also argued that for a single plant species, the strength of the PSF may be density-dependent, and hence may depend on the local density of the species in the field. Recently, a large database analysis of more than 200,000 forest plots and including 151 tree species revealed that for most species the establishment of seedlings was negatively affected by the local abundance of this species (Johnson, Beaulieu, Bever & Clay

2012). These effects are likely mediated by density-dependent effects on soil pathogens. For example, seedling mortality of the tropical tree species *Sebastiana longicuspis* increases at higher local densities of conspecifics and this is caused by soil fungal pathogens (Bell, Freckleton & Lewis 2006).

Although most of this work has been done with tree species, similar density-dependent PSF may be expected in grasslands (Bever 1994; Klironomos 2002; Petermann, Fergus, Turnbull & Schmid 2008). Recently, Van de Voorde, Van der Putten & Bezemer (2012) compared PSF of the early successional plant *Jacobaea vulgaris* in soil collected from ten fields where this species occurred in different densities, and showed that indeed PSF was negatively related to the density of this species in the field. Similarly, in a field experiment with sown and unsown plots, *J. vulgaris* grew much better in soil collected from the sown plots where it was rare or even absent, than in soil from the unsown plots where it was present at high densities in the field (Bezemer, Harvey, Kowalchuk, Korpershoek & Van der Putten 2006a). However, in the latter study not only the density of *J. vulgaris*, but also the densities of other plant species differed greatly between sown and unsown plots, and several studies have shown that there are also strong heterospecific effects on PSF (Mangan et al. 2010; Van de Voorde, Van der Putten & Bezemer 2011). So far, studies that examined PSF of *J. vulgaris* have used mixtures of soil samples taken from different locations within a field or an experimental plot. The local density of *J. vulgaris* often varies greatly within a single field (T.M. Bezemer personal observation). Whether PSF is related to the local density of *J. vulgaris* within a single field has not been tested.

Most studies test biotic PSF in sterile bulk soil inoculated with a small quantity of live field soil (e.g. Bever 1994; Kardol, Bezemer & Van der Putten 2006; Reinhart 2012). The rationale is that soil biota are introduced through the inoculum of live field soil, while the large amount of sterile bulk soil standardises soil nutrient levels across treatments (e.g.

Troelstra, Wagenaar, Smant & Peters 2001; Kardol et al. 2006). Inoculation with field soil, rather than using pure field soil, prevents confounding effects of local differences in soil nutrient availability that may exist in field soil, for example due to local disturbance of the soil. This is particularly important in bioassays where the soil effects on plant performance are tested directly without a conditioning phase (e.g. Bezemer et al. 2006a). However, using inoculated soil does not represent the natural field condition, where plants interact with biotic as well as abiotic components of the soil (Wardle, Bardgett, Klironomos, Setälä, Van der Putten et al. 2004; Wardle, Walker & Bardgett 2004) and soil resources are not homogeneously distributed (Bliss, Jones, Mitchell & Mou 2002). Furthermore, the sterile soil environment may also facilitate the rapid increase of a limited number of fast-growing species of microorganisms in all soils (de Boer, Verheggen, Gunnewiek, Kowalchuk & Van Veen 2003).

In this study we carry out a bioassay to examine whether growth of *J. vulgaris* is related to local variation in plant density within a single field. *Jacobaea vulgaris* is a poor competitor and seedlings require bare or disturbed soil to establish (McEvoy, Rudd, Cox & Huso 1993). The variation in local density of this species may therefore be related to variation in the openness of the vegetation or the seed density in the seed bank, but could also be related to the availability of soil nutrients or to PSF. A number of greenhouse studies have shown that *J. vulgaris* quickly develops a strong negative PSF (Bezemer et al. 2006a; Van de Voorde et al. 2011, 2012), probably due to the build-up of species-specific pathogenic fungi (Bezemer et al. 2006a; Van de Voorde et al. 2012). If this negative PSF plays a significant role in the spatial dynamics of this species in the field, we may expect that plants will grow less well in soil collected from locations with high *J. vulgaris* densities than from locations where the plant is not growing or is present only at low densities.

In an area of 400 x 400 m within a restoration grassland, we selected four 8x8 m plots with high density of *J. vulgaris* and four with lower density. Furthermore, we selected four

plots within an adjacent heathland that bordered the grassland but where *J. vulgaris* is absent. In 16 subplots in each plot we recorded characteristics of *J. vulgaris*, of the vegetation, and the chemistry of the soil. Soil was collected from these subplots and used in a greenhouse bioassay to examine the effects of plant density on the growth of *J. vulgaris*. Plants were grown either in pure field soil, or in sterilised bulk soil inoculated with a relatively small amount of live field soil to standardise soil nutrient levels while introducing soil biota. We hypothesised (i) that there would be a negative relationship between *J. vulgaris* density in the field and plant growth in bioassay, and (ii) that the soil-mediated density effects would be less strong when using pure field soil due to confounding effects of soil nutrients.

Materials and methods

Plant species

Ragwort, *Jacobaea vulgaris* Gaertn. spp. *vulgaris* (synonym *Senecio jacobaea* L.), is a plant species in the Asteraceae family that is native to Europe, but is invasive in other continents (Wardle 1987; Bain 1991). In its native range, it can become very abundant in disturbed areas (Van de Voorde et al. 2012). In The Netherlands, it is particularly abundant in nature restoration areas on former arable fields, and along roadsides (Bezemer et al. 2006a). *Jacobaea vulgaris* is a biannual plant that spends its first year as a rosette, and flowers during the second year, but flowering can be delayed for several years by herbivory (van der Meijden & Van der Waals-Kooi 1979; Prins, Vrieling, Klinkhamer & de Jong 1990). The plant reproduces mostly by seeds, but can also reproduce vegetatively via root or crown buds (Harper & Wood 1957; Wardle 1987).

Study area

The study was carried out at the Planken Wambuis, Veluwe, The Netherlands (52°04'N, 5°44'E). This area consists of natural grassland, heathland, forest and small patches of agricultural land. The natural areas are grazed by free roaming cattle and horses. For this study two adjacent fields were used with different vegetation types. Both fields were roughly 50 ha in size (700 m x 750 m). The first field, Mosselsche Veld, is a restoration grassland where agricultural practices were ceased in 1985. Species richness in this area was on average 15 plant species per m². Species with an average cover of 5% or more were: *Holcus lanatus* (21%), *Agrostis capillaris* (13%), *Plantago lanceolata* (13%), *Lolium perenne* (10%), *Achillea millefolium* (7%), *Jacobaea vulgaris* (6%) and *Moehringia trinervia* (5%). The adjacent field, Valenberg, is a traditional heathland that is dominated by *Calluna vulgaris* (Scotch heather) and grasses (*Molinia caerulea* and *Deschampsia flexuosa*). Both sites had a sandy loam soil (60% coarse sand, 28% fine sand, 4% silt, 8% clay). Even though the heathland borders the grassland, *J. vulgaris* was not present in the heathland. Within the grassland the density of *J. vulgaris* varied spatially.

In July 2010, within an area of 400 x 400 m of the grassland, four areas with a high density of *J. vulgaris* plants (High-density; 11.3 ± 1.4 plants m⁻²) and four with a lower density (Low-density; 2.7 ± 0.7 plants m⁻²) were selected. Within each of these eight areas a plot of 8 x 8 m was laid out. The distance between two plots was at least 80 m. These dimensions were selected so that the plot fitted well within each local high- or low-density area. Each plot was subdivided into 16 sub-plots of 2 x 2 m. In each of the 16 sub-plots, the number of rosettes and flowering *J. vulgaris* plants was recorded. In the central 0.5 x 0.5 m of each sub-plot, % cover of *J. vulgaris*, % cover of grasses, and % bare ground was estimated, and height of the tallest flowering *J. vulgaris* plant was recorded. There were also four 8 x 8 m plots in the heathland where % cover of grasses and % bare ground was recorded in the 16

subplots. *Jacobaea vulgaris* was absent in the heathland. In the central 0.5 x 0.5 m of each sub-plot, four soil cores (5 cm diameter, 15 cm depth) were collected. The soil samples from each sub-plot were pooled so that there was about 1.2 kg of soil collected from each sub-plot. In total, there were 192 soil samples (12 plots x 16 sub-plots). In the laboratory each soil sample was sieved (0.5 cm mesh size), homogenized and soil samples were then used for a seed bank experiment, a bioassay and for chemical analysis.

Seed bank experiment

To determine the density of *J. vulgaris* seeds in the seed bank in each of the 12 plots, 100 g of soil from each of four adjacent sub-plots (making up one quarter of the plot), was pooled and homogenized so that there were four replicate soil samples per plot. Plastic containers (12.5 x 12.5 cm, 13 cm height) with a layer of 2 cm (65 g) sterilised potting soil to improve drainage, were filled with 250 g pooled field soil. In total, there were 48 containers (12 plots x 4 replicate soil samples). The containers were placed randomly in a greenhouse at 70% RH and a 16 h light (21 °C) and 8 h dark (16 °C) photo regime. Natural day-light was supplemented by 400 W metal halide lamps (225 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR, 1 lamp per 1.5 m²). For a period of eight weeks, the number of *J. vulgaris* seedlings and the number of seedlings of other forbs and grasses were recorded once every two weeks. After each recording, all seedlings were removed.

Soil bioassay

In a greenhouse pot-experiment we grew *J. vulgaris* in each of the 192 soils. Pots (0.8 l) were filled with 700 g soil. For each sub-plot, there was one pot with pure field soil and one pot with a mixture of sterilised bulk soil and live field soil (6:1). Plants growing in inoculated soil were exposed to comparable levels of available nutrients. Bulk soil was collected from a

nearby restoration grassland in the Planken Wambuis area. The bulk soil was sieved (0.5 cm mesh size), homogenised and sterilised by gamma irradiation (> 25 KGray, Isotron, Ede, The Netherlands). Five additional pots were filled with 700 g sterilised bulk soil so that there were in total 389 pots (192 sub-plots x 2 soil-types + 5 control pots). *Jacobaea vulgaris* seeds were collected from a single population at the Planken Wambuis. The seeds were surface sterilised (1 min in 0.5% sodium hypochlorite solution and rinsed with water afterwards) and germinated on glass beads in a climate chamber at 20 °C. One one-week-old seedling was planted in each pot. Pots were placed randomly in the greenhouse and plants were grown under the same conditions as described above. Seedlings that died within seven days were replaced once. Plants were watered three times per week and the soil was kept at 17% moisture. Seven weeks after transplanting, the aboveground biomass of each plant was clipped and roots were carefully washed from the soil. Plant material was oven-dried (70 °C) and root and shoot dry weight of each plant was determined.

Soil chemistry

A sub-sample of 100 g soil of each of the four corner sub-plots of each plot was sieved (0.5 cm mesh size) and dried (72 h at 40 °C). In this sub-sample, pH, phosphorous (P), potassium (K^+) and magnesium (Mg^{2+}) were analysed in 1:10 (w/v) 0.01 M $CaCl_2$. Concentrations of available ammonium (NH_4^+-N) and nitrate (NO_3^--N) were determined colorimetrically in the $CaCl_2$ -extract using a Traacs 800 autoanalyzer (TechniCon Systems Inc.). Total extractable amounts of phosphorous (Olsen-P) were determined using the method of Olsen, Cole, Watanabe & Dean (1954) and colour intensity was measured at 720 nm. The % organic matter was determined as weight loss of a soil sample after ignition at 430 °C for 24 h.

Statistical analysis

247 All analyses were performed in IBM SPSS Statistics for Windows (19th edition, SPSS Inc,
248 Chicago, Illinois, USA), except when indicated otherwise. Soil chemistry, *J. vulgaris* and
249 vegetation characteristics and number of seedlings emerging from the seed bank were
250 analysed using Mixed Models with density (High- or Low-density) as a fixed factor and plot
251 identity as a random factor. In this way subplots within each plot were included in the
252 analyses but were considered as pseudoreplicates. Similar Mixed Models were used to analyse
253 the difference in soil chemistry between High-density, Low-density and *C. vulgaris* plots.
254 Prior to analysis, the number of rosettes, the number of emerged seedlings, pH, and
255 concentrations of P, K⁺, NO₃⁻ and NH₄⁺ were log-transformed; % *J. vulgaris*, % grass cover,
256 % bare soil, and % organic matter were arcsin-square root transformed to fulfil assumptions
257 of normality. Plant biomass and root/shoot ratio in the bioassay was analysed separately for
258 pure soil and for inoculated soil using Mixed Models with soil origin (High-density, Low-
259 density or *C. vulgaris*) as a fixed factor and plot identity as a random factor. Because none of
260 the plants grown in pure *C. vulgaris* soil survived, the Mixed Model with data from plants
261 grown in pure soil was carried out for High- and Low-density soils only. Individual pair-wise
262 comparisons were based on LSD tests. To test the “pure” effect of plant density in pure soil, a
263 second Mixed Model analysis was performed in which nutrient effects were included as a
264 covariable. First, a multivariate Principal Component Analysis (PCA) was performed in
265 Canoco version 4.55 (Ter Braak & Šmilauer 2002) using the soil chemistry variables as
266 species data. Second, the values of the sample scores on the first PCA axis (explaining 46% of
267 the variation) were used as a fixed covariate in the Mixed Model. The means and standard
268 errors in High- and Low-density soil predicted by the model were obtained. One-way
269 ANOVA was used to compare plant biomass in pure, inoculated and sterile soil. For this
270 analysis we used mean values per plot for pure and inoculated soil. The average of pure soil
271 was based on data from High- and Low-density plots only. A post-hoc Tukey test was used

for pair-wise comparisons. The relationship between *J. vulgaris* density in each subplot and soil chemistry, and between plant biomass in the bioassay and *J. vulgaris* density or soil chemistry in the field was tested using linear regression. Because of the experimental set-up, in which the High- and Low-density plots were selected *a priori*, linear regression analyses were performed separately for High- and Low-density plots.

Results

Field observations and seed bank experiment

The percentage of *J. vulgaris* plants that was in the rosette stage was higher in High- than in Low-density plots (Table 1). For both High- and Low-density plots, there was a positive relationship between the number of rosettes and flowering plants, but this relationship was stronger in Low-density plots (High-density: $F_{1,62} = 5.33$, $P = 0.024$, $R^2 = 0.08$; Low-density: $F_{1,62} = 24.56$, $P < 0.001$, $R^2 = 0.28$). The maximum height of flowering plants was larger in High- than in Low-density plots (Table 1), even when only the sub-plots that contained a single flowering plant in the central 0.5 x 0.5 m were included in the analysis ($F_{1,7} = 6.44$, $P = 0.040$). The number of *J. vulgaris* seedlings emerging from the seed bank was also higher in High-density plots. High- and Low-density plots did not differ in % of grass cover or bare soil (Table 1). Overall, soil abiotic characteristics did not differ between High- and Low-density plots (Table 1). However, there was a high level of heterogeneity in soil chemical characteristics within the experimental field, and soil chemistry varied greatly among the four High-density and the four Low-density plots (see Appendix A: Table 1).

In High-density plots, there was a positive relationship between the density of *J. vulgaris* plants in a subplot and pH ($F_{1,14} = 14.32$, $P = 0.002$, $R^2 = 0.51$), but a negative relationship with Olsen-P ($F_{1,14} = 6.18$, $P = 0.026$, $R^2 = 0.31$) and % organic matter ($F_{1,14} =$

7.60, $P = 0.015$, $R^2 = 0.35$). In Low-density plots, there were no significant relationships between *J. vulgaris* density and soil chemistry. There was no relation between bare soil or grass cover and *J. vulgaris* density in either High- or Low-density plots.

Soil bioassay

On average, biomass of *J. vulgaris* was highest when plants were grown in sterilised control soil, intermediate in inoculated soil, and lowest in pure field soil ($F_{2,22} = 48.31$, $P < 0.001$, Fig. 1A). Root/shoot ratios were significantly higher in pure soil than in inoculated or in sterile soil ($F_{2,22} = 20.21$, $P < 0.001$, Fig. 1B).

In pure soil, none of the *J. vulgaris* plants planted in *C. vulgaris* soil survived, and plant biomass did not differ between plants grown in soil collected from High- and Low-density plots ($F_{1,6} = 0.02$, $P = 0.904$, Fig. 1A). When the effect of soil nutrients in pure soil was removed by including it as a covariable in the analysis, plant biomass tended to be lower in High-density soil (0.71 ± 0.07 g) than in Low-density soil (0.81 ± 0.06 g), but the difference was not significant ($F_{1,6} = 4.12$, $P = 0.089$). Root/shoot ratios did not differ significantly between plants grown in pure High- and pure Low-density soil ($F_{1,6} = 4.25$, $P = 0.085$, Fig. 1B), also when the effects of soil nutrients were removed statistically ($F_{1,5} = 0.10$, $P = 0.763$).

In inoculated soil, plants grew best when soil was inoculated with *C. vulgaris* soil, significantly less well in soil inoculated with Low-density soil, and worst in soil inoculated with High-density soil ($F_{2,9} = 13.87$, $P = 0.002$, Fig. 1A). Root/shoot ratios were significantly higher in inoculated *C. vulgaris* soil than in inoculated High- or Low-density soil ($F_{2,9} = 4.90$, $P = 0.008$, Fig. 1B).

The biomass of plants grown in pure High-density soil was negatively related to the density of *J. vulgaris* plants in a subplot (Fig. 2A). In contrast, the biomass of plants grown in

pure soil collected from Low-density plots was positively related to the *J. vulgaris* density in the field (Fig. 2B). Plant biomass in pure soil collected from High-density plots was positively related to P, K⁺, NO₃⁻, Olsen-P and % organic matter (see Appendix A: Table 2). In pure soil collected from Low-density plots, plant biomass was positively related to NO₃⁻. Neither in High-density nor in Low-density inoculated soil, there was a relationship between density of *J. vulgaris* in a subplot and plant biomass in the bioassay. Plant biomass in inoculated soil was not related to soil nutrients, but for High-density plots there was a negative relationship between biomass and the pH of the inoculated soil (see Appendix A: Table 2).

Discussion

Our study shows that there is local variation in plant-soil interactions for *J. vulgaris* and that this variation is related to plant density in the field. In the bioassay, *J. vulgaris* had a negative PSF. Biomass of plants grown in inoculated soil was lower when field soil originated from plots with a high *J. vulgaris* density than when soil was collected from plots with a low density. Recently, Bezemer, Van der Putten, Martens, Van de Voorde, Mulder et al. (2013) showed that fungal pathogens such as *Phoma exigua* and *Fusarium oxysporum*, which occur at our field site as well (T.M. Bezemer, unpublished results), accumulate around the roots of *J. vulgaris*. These pathogens may subsequently inhibit the growth of the plant. Although we did not analyse the microbial soil community, our results suggest that a higher density of plants may lead to a larger build-up of soil pathogens, and that these pathogens subsequently negatively affect plant growth. This mechanism of conspecific negative density-dependence has been shown mostly for tree species (e.g. Bell et al. 2006; Johnson et al. 2012), although several studies have suggested that this may also play a role in grasslands (e.g. Bever 1994; Klironomos 2002; Petermann et al. 2008). Our results show that, indeed, this mechanism also

occurs in grasslands. Similar results have been shown when densities of *J. vulgaris* from different fields that were located several kilometres apart (Van de Voorde et al. 2012) or from different experimental plant communities (Bezemer et al. 2006a) were compared. Our study now shows that such negative density-dependent effects can also be detected on a much smaller spatial scale, *i.e.* within a single grassland area of only 400 x 400 m, where *J. vulgaris* densities varied spatially.

We studied PSF in *J. vulgaris* not only in sterile bulk soil inoculated with a small part of live field, similar to many other PSF studies (e.g. Bezemer et al. 2006a; Kardol et al. 2006; Reinhart 2012; Van de Voorde et al. 2012), but also in pure field soil to examine the strength of these plant-soil interactions under more natural conditions. When *J. vulgaris* was grown in pure field soil in the pot-experiment, biomass did not differ between soil collected from High- and Low-density plots. This is in line with our expectation that density effects mediated by soil biota are less strong in pure field soil than in inoculated soil because of the confounding effects of local differences in nutrient availability in the soil. Interestingly, when the effects of nutrients were statistically removed, plant biomass tended to be lower in pure soil from High-density plots compared to Low-density plots, although this difference was not significant. Moreover, in the regression analysis, biomass of plants grown in the greenhouse in pure soil collected from High-density plots was negatively related to the local density of *J. vulgaris* plants in the field. Hence, even though soil nutrients greatly affected plant growth in pure soil, we were still able to detect a density-dependent effect of *J. vulgaris* on plant growth. We did not find such a negative relationship between plant biomass and plant density in Low-density soils. We propose that the number of *J. vulgaris* plants in Low-density plots was too low to cause a negative PSF. It is important to note that the identity and density of other plant species may also have differed spatially in the field. Heterospecific plants can cause strong PSF effects (Mangan et al. 2010). The growth of *J. vulgaris* can also be greatly affected by soil-

mediated effects of other species (Van de Voorde et al. 2011) and the growth responses of *J. vulgaris* in the greenhouse may have been partly caused by the soil-mediated effects of other species.

It is well known that soil nutrients positively affect the growth of *J. vulgaris* (Hol, Vrieling & Van Veen 2003) and we also observed a positive relationship between plant biomass and soil nutrients in both High- and Low-density pure soil. However, soil nutrient levels did not differ significantly between the High- and Low-density plots, probably because soil nutrient levels varied greatly within and among all plots.

Jacobaea vulgaris biomass was smaller in pure field soil than in inoculated soil. This may suggest that pure soil contains higher levels of pathogenic microorganisms than inoculated soil, but may also result from the higher availability of soil nutrients in inoculated soil due to the process of sterilisation by gamma irradiation (Troelstra et al. 2001). The lower root/shoot ratio in inoculated soil supports the latter hypothesis.

None of the plants survived in pure soil collected from *C. vulgaris* plots, but this is not unexpected as *J. vulgaris* only occurs in soils with a pH above 3.95 (Harper et al. 1957). Indeed, when a small amount of soil from *C. vulgaris* plots was inoculated in sterilised grassland soil and hence soil pH was higher, plants grew very well and biomass was even similar to that of plants grown in entirely sterilised soil. This result emphasizes the important role of soil abiotic conditions for the growth of this plant species. Moreover, it suggests that soil organisms that are pathogenic to *J. vulgaris* are not present in *C. vulgaris* soil.

Although we found a negative relationship between plant density in the field and plant growth in the bioassay, *J. vulgaris* plants in the field did not yet appear to suffer from the negative density-dependent effect. Instead, flowering plants were significantly taller in High-density plots, suggesting that these plots were more suitable for *J. vulgaris* growth than Low-density plots. It is possible that, by measuring the height of only the tallest flowering plant in

the central 0.5 x 0.5 m of a sub-plot, our data is biased towards finding taller plants in plots with more plants, *i.e.* the High-density plots. However, this is not likely, as there was no relation between the number of flowering plants and the height of the tallest flowering plant (data not shown). Furthermore, when we included only the sub-plots that contained a single flowering plant in the analysis, flowering plants were still significantly taller in the High-density plots.

The density and the percentage of first-year rosette plants was higher in High- than in Low-density plots. This could be directly related to the larger number of *J. vulgaris* seedlings emerging from the seed bank, which in turn, could be related to the higher density of flowering plants that produce seeds. We do not know whether seedling establishment was negatively affected by plant density, nor whether the rosettes in High-density plots were smaller than in Low-density plots. Although the spatial patterning of *J. vulgaris* is related to variation in the openness of the vegetation (McEvoy et al. 1993), we found no difference in the percentage of bare soil or grass cover between High- and Low-density plots, nor a relation between bare soil or grass cover and plant density.

In conclusion, we show that plant-soil interactions of *J. vulgaris* vary on a local scale, *i.e.* within a single grassland, and that this variation is related to the density of plants in the vegetation. However, other factors such as soil nutrient availability and seed density in the seed bank are also important for the spatial patterning of *J. vulgaris*, and these multiple processes should be considered simultaneously. Plant-soil feedback is a rapidly developing research field, and it is increasingly acknowledged that PSFs may operate at small spatial scales (Brandt, de Kroon, Reynolds & Burns 2013). Our results show that it is important to include local spatial variation in plant-soil interactions to increase our understanding of the role of these interactions in affecting plant performance and community dynamics.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at XXXXX.

References

- Aerts, R., & Chapin, F.S. (2000). The mineral nutrition of wild plants revisited: A re-evaluation of processes and patterns. In: A.H. Fitter & D.G. Raffaelli (Eds.), *Advances in Ecological Research, Vol 30* (pp. 1-67). San Diego: Elsevier Academic Press Inc.
- Bain, J.F. (1991). The biology of Canadian weeds 96: *Senecio jacobaea* L. *Canadian Journal of Plant Science*, 71, 127-140.
- Bell, T., Freckleton, R.P., & Lewis, O.T. (2006). Plant pathogens drive density-dependent seedling mortality in a tropical tree. *Ecology Letters*, 9, 569-574.
- Bever, J.D. (1994). Feedback between plants and their soil communities in an old field community. *Ecology*, 75, 1965-1977.

446 Bever, J.D., Westover, K.M., & Antonovics, J. (1997). Incorporating the soil community into
 447 plant population dynamics: the utility of the feedback approach. *Journal of Ecology*, 85,
 448 561-573.

449 Bezemer, T.M., Harvey, J.A., Kowalchuk, G.A., Korpershoek, H., & Van der Putten, W.H.
 450 (2006a). Interplay between *Senecio jacobaea* and plant, soil, and aboveground insect
 451 community composition. *Ecology*, 87, 2002-2013.

452 Bezemer, T.M., Lawson, C.S., Hedlund, K., Edwards, A.R., Brook, A.J., Igual, J.M.,
 453 Mortimer, S.R., & Van der Putten, W.H. (2006b). Plant species and functional group
 454 effects on abiotic and microbial soil properties and plant-soil feedback responses in two
 455 grasslands. *Journal of Ecology*, 94, 893-904.

456 Bezemer, T.M., Van der Putten, W.H., Martens, H., Van de Voorde, T.F.J., Mulder, P.P.J., &
 457 Kostenko, O. (2013). Above- and below-ground herbivory effects on below-ground plant-
 458 fungus interactions and plant-soil feedback responses. *Journal of Ecology*, 101, 325-333.

459 Bliss, K.M., Jones, R.H., Mitchell, R.J., & Mou, P.P. (2002). Are competitive interactions
 460 influenced by spatial nutrient heterogeneity and root foraging behavior? *New Phytologist*,
 461 154, 409-417.

462 Brandt, A.J., de Kroon, H., Reynolds, H.L., & Burns, J.H. (2013). Soil heterogeneity
 463 generated by plant-soil feedbacks has implications for species recruitment and coexistence.
 464 *Journal of Ecology*, 101, 277-286.

465 Chapman, S.K., Langley, J.A., Hart, S.C., & Koch, G.W. (2006). Plants actively control
 466 nitrogen cycling: uncorking the microbial bottleneck. *New Phytologist*, 169, 27-34.

467 de Boer, W., Verheggen, P., Gunnewiek, P., Kowalchuk, G.A., & Van Veen, J.A. (2003).
 468 Microbial community composition affects soil fungistasis. *Applied and Environmental*
 469 *Microbiology*, 69, 835-844.

470 Ehrenfeld, J.G., Ravit, B., & Elgersma, K. (2005). Feedback in the plant-soil system. *Annual*
 471 *Review of Environment and Resources*, 30, 75-115.

472 Harper, J.L., & Wood, W.A. (1957). *Senecio Jacobaea* L. *Journal of Ecology*, 45, 617-637.

473 Hol, W.H.G., Vrieling, K., & Van Veen, J.A. (2003). Nutrients decrease pyrrolizidine
 474 alkaloid concentrations in *Senecio jacobaea*. *New Phytologist*, 158, 175-181.

475 Johnson, D.J., Beaulieu, W.T., Bever, J.D., & Clay, K. (2012). Conspecific negative density
 476 dependence and forest diversity. *Science*, 336, 904-907.

477 Kardol, P., Bezemer, T.M., & Van der Putten, W.H. (2006). Temporal variation in plant-soil
 478 feedback controls succession. *Ecology Letters*, 9, 1080-1088.

479 Klironomos, J.N. (2002). Feedback with soil biota contributes to plant rarity and invasiveness
 480 in communities. *Nature*, 417, 67-70.

481 Kulmatiski, A., Beard, K.H., Stevens, J.R., & Cobbold, S.M. (2008). Plant-soil feedbacks: a
 482 meta-analytical review. *Ecology Letters*, 11, 980-992.

483 Mangan, S.A., Schnitzer, S.A., Herre, E.A., Mack, K.M.L., Valencia, M.C., Sanchez, E.I., &
 484 Bever, J.D. (2010). Negative plant-soil feedback predicts tree-species relative abundance in
 485 a tropical forest. *Nature*, 466, 752-U710.

486 McEvoy, P.B., Rudd, N.T., Cox, C.S., & Huso, M. (1993). Disturbance, competition, and
 487 herbivory effects on ragwort *Senecio jacobaea* populations. *Ecological Monographs*, 63,
 488 55-75.

489 Olsen, S.R., Cole, C.V., Watanabe, F.S., & Dean, L.A. (1954). Estimation of available
 490 phosphorus in soils by extraction with sodium bicarbonate. *USDA circular*, 139, 1-19.

491 Packer, A., & Clay, K. (2000). Soil pathogens and spatial patterns of seedling mortality in a
 492 temperate tree. *Nature*, 404, 278-281.

493 Petermann, J. S., Fergus, A. J. F., Turnbull, L. A. & Schmid, B. (2008). Janzen-Connell
 494 effects are widespread and strong enough to maintain diversity in grasslands. *Ecology*, 89,
 495 2399-2406.

496 Prins, A.H., Vrieling, K., Klinkhamer, P.G.L., & de Jong, T.J. (1990). Flowering behavior of
 497 *Senecio jacobaea*: Effects of nutrient availability and size-dependant vernalization. *Oikos*,
 498 59, 248-252.

499 Reinhart, K.O. (2012). The organization of plant communities: negative plant-soil feedbacks
 500 and semiarid grasslands. *Ecology*, 93, 2377-2385.

501 Reynolds, H.L., Packer, A., Bever, J.D., & Clay, K. (2003). Grassroots ecology: Plant-
 502 microbe-soil interactions as drivers of plant community structure and dynamics. *Ecology*,
 503 84, 2281-2291.

504 Ter Braak, C.J.F., & Šmilauer, P. (2002). *CANOCO Reference Manual and CanoDraw for*
 505 *Windows user's Guide. Software for Canonical Community Ordination, Version 4.5.* .
 506 Microcomputer Power, Ithaca, New York.

507 Troelstra, S.R., Wagenaar, R., Smant, W., & Peters, B.A.M. (2001). Interpretation of
 508 bioassays in the study of interactions between soil organisms and plants: involvement of
 509 nutrient factors. *New Phytologist*, 150, 697-706.

510 Van de Voorde, T.F.J., Van der Putten, W.H., & Bezemer, T.M. (2011). Intra- and
 511 interspecific plant-soil interactions, soil legacies and priority effects during old-field
 512 succession. *Journal of Ecology*, 99, 945-953.

513 Van de Voorde, T.F.J., Van der Putten, W.H., & Bezemer, T.M. (2012). The importance of
 514 plant-soil interactions, soil nutrients, and plant life history traits for the temporal dynamics
 515 of *Jacobaea vulgaris* in a chronosequence of old-fields. *Oikos*, 121, 1251-1262.

516 Van der Meijden, E., & Van der Waals-Kooi, R.E. (1979). Population ecology of *Senecio*
 517 *jacobaea* in a sand dune system. *Journal of Ecology*, 67, 131-153.

518 Van der Putten, W.H., Bardgett, R.D., Bever, J.D., Bezemer, T.M., Casper, B.B., Fukami, T.,
 519 Kardol, P., Klironomos, J.N., Kulmatiski, A., Schweitzer, J.A., Suding, K.N., Van de
 520 Voorde, T.F.J., & Wardle, D.A. (2013). Plant–soil feedbacks: the past, the present and
 521 future challenges. *Journal of Ecology*, *101*, 265-276.

522 Van der Putten, W.H., Van Dijk, C., & Peters, B.A.M. (1993). Plant-specific soil-borne
 523 diseases contribute to succession in foredune vegetation. *Nature*, *362*, 53-56.

524 Wardle, D.A. (1987). The ecology of ragwort (*Senecio jacobaea* L) - a review. *New Zealand*
 525 *Journal of Ecology*, *10*, 67-76.

526 Wardle, D.A., Bardgett, R.D., Klironomos, J.N., Setälä, H., Van der Putten, W.H., & Wall,
 527 D.H. (2004). Ecological linkages between aboveground and belowground biota. *Science*,
 528 *304*, 1629-1633.

529 Wardle, D.A., Walker, L.R., & Bardgett, R.D. (2004). Ecosystem properties and forest
 530 decline in contrasting long-term chronosequences. *Science*, *305*, 509-513.

Table 1. Field vegetation characteristics, the number of seedlings per container (cont.)
germinating from the seed bank and soil chemistry (mean $\pm$ SE) in field plots containing
High- and Low-densities of *J. vulgaris* plants

Characteristic	High-density	Low-density	F^a	P
Vegetation				
<i>J. vulgaris</i> plants (no. m ⁻²)	11.3 $\pm$ 1.4	2.7 $\pm$ 0.7	$F_{1,6} = 31.09$	0.001
Rosettes (no. m ⁻²)	6.6 $\pm$ 1.3	1.1 $\pm$ 0.3	$F_{1,6} = 29.23$	0.002
Flowering plants (no. m ⁻²)	4.6 $\pm$ 0.4	1.7 $\pm$ 0.3	$F_{1,6} = 31.71$	0.001
% rosettes	55.1 $\pm$ 5.1	29.5 $\pm$ 4.5	$F_{1,6} = 12.77$	0.012
Max. <i>J. vulgaris</i> height (cm)	60.6 $\pm$ 4.5	42.6 $\pm$ 2.7	$F_{1,8} = 8.82$	0.019
<i>J. vulgaris</i> cover (%)	11.6 $\pm$ 1.5	4.7 $\pm$ 1.1	$F_{1,6} = 19.78$	0.004
Grass cover (%)	60.4 $\pm$ 3.2	64.2 $\pm$ 3.8	$F_{1,6} = 0.48$	0.517
Bare soil (%)	28.7 $\pm$ 2.2	28.2 $\pm$ 3.6	$F_{1,6} < 0.01$	0.998
Seed bank				
<i>J. vulgaris</i> seedlings (no. per cont.) ^b	1.0 $\pm$ 0.2	0.1 $\pm$ 0.1	$F_{1,30} = 19.94$	< 0.001
Other forb seedlings (no. per cont.)	16.2 $\pm$ 2.6	14.8 $\pm$ 2.4	$F_{1,6} = 0.19$	0.677
Grass seedlings (no. per cont.)	2.9 $\pm$ 1.4	3.6 $\pm$ 0.8	$F_{1,6} = 0.48$	0.513
Soil chemistry				
pH CaCl ₂	4.60 $\pm$ 0.04	4.57 $\pm$ 0.03	$F_{1,6} = 0.24$	0.642
P CaCl ₂ (mg kg ⁻¹)	1.53 $\pm$ 0.24	1.46 $\pm$ 0.33	$F_{1,6} = 0.03$	0.867
K ⁺ CaCl ₂ (mg kg ⁻¹)	50.74 $\pm$ 6.85	32.51 $\pm$ 5.33	$F_{1,6} = 5.75$	0.054
Mg ²⁺ CaCl ₂ (mg kg ⁻¹)	43.49 $\pm$ 1.82	36.06 $\pm$ 3.22	$F_{1,6} = 4.05$	0.091
NH ₄ ⁺ CaCl ₂ (mg kg ⁻¹)	3.25 $\pm$ 0.31	3.02 $\pm$ 0.27	$F_{1,6} = 0.32$	0.594
NO ₃ ⁻ CaCl ₂ (mg kg ⁻¹)	3.07 $\pm$ 0.81	4.60 $\pm$ 1.10	$F_{1,6} = 1.33$	0.293
Olsen-P (mg kg ⁻¹)	82.30 $\pm$ 6.01	84.56 $\pm$ 5.89	$F_{1,6} = 0.07$	0.796
Organic Matter (%)	4.63 $\pm$ 0.33	3.98 $\pm$ 0.43	$F_{1,6} = 1.50$	0.267

Means are shown based on the average value of each plot. Statistical analyses were performed by Mixed Models.

^a Shown in subscript after the F -value are the numerator-df and the denominator-df of the Mixed Model, rounded off to integers.

^b Statistical analysis performed by one-way ANOVA instead of Mixed Model

Figure legends

Figure 1. Mean (A) total plant biomass and (B) root/shoot ratio ($\pm$ SE, based on the average values per plot) of *Jacobaea vulgaris* plants in the bioassay grown in pure field soil ('Pure soil'), in sterilised bulk soil inoculated with live field soil ('Inoculated soil') or in sterile soil. The field soil was collected from four plots with high *J. vulgaris* density ('High-density plots'), four with low *J. vulgaris* density ('Low-density plots') or from four plots dominated by *Calluna vulgaris* from which *J. vulgaris* was absent ('*C. vulgaris* plots'). *Jacobaea vulgaris* did not grow in pure *C. vulgaris* soil. Significant differences are indicated by different letters, with capital letters denoting pair-wise differences among the three soil types (pure soil, inoculated soil and sterile soil) based on one-way ANOVA, and lowercase letters denoting pair-wise differences among the three soil origins (High-density, Low-density and *C. vulgaris* plots) based on Mixed Models ($P < 0.05$).

Figure 2. Relationship between *Jacobaea vulgaris* density in the field and its growth in the greenhouse in field-collected soil. (A) Relationship between the number of plants per m^2 and *J. vulgaris* biomass in pure field soil collected from plots with high *J. vulgaris* density ($F_{1,61} = 10.83$, $P = 0.002$, $R^2 = 0.15$). (B) Relationship between the number of plants per m^2 and *J. vulgaris* biomass in pure field soil collected from plots with low *J. vulgaris* density ($F_{1,61} = 7.90$, $P = 0.007$, $R^2 = 0.11$). The line is the estimated relationship based on linear regression analysis.