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Soil inoculation method determines the strength of plant–soil interactions

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ABSTRACT

There is increasing evidence that interactions between plants and biotic components of the soil influence plant productivity and plant community composition. Many plant–soil feedback experiments start from inoculating relatively small amounts of natural soil to sterilized bulk soil. These soil inocula may include a variety of size classes of soil biota, each having a different role in the observed soil feedback effects. In order to examine what may be the effect of various size classes of soil biota we compared inoculation with natural field soil sieved through a 1 mm mesh, a soil suspension also sieved through a 1 mm mesh, and a microbial suspension sieved through a 20 μm mesh. We tested these effects for different populations of the same plant species and for different soil origins.

Plant biomass was greatest in pots inoculated with the microbial suspension and smallest in pots inoculated with sieved soil, both in the first and second growth phase, and there was no significant population or soil origin effect. Plant-feeding nematodes were almost exclusively found in the sieved soil treatment. We show that processing the soil to obtain a microbial suspension reduces the strength of the soil effect in both the first and second growth phase. We also show that the results obtained with inoculating sieved soil and with a soil suspension are not comparable. In conclusion, when designing plant–soil feedback experiments, it is crucial to consider that soil inoculum preparation can strongly influence the observed soil effect.

1. Introduction

Plants influence the community composition of soil organisms around their roots, which, in turn, influences the plant's performance, either directly through antagonistic or mutualistic effects or through their effects on nutrient availability. This process is called plant–soil feedback (Bever et al., 1997) and is considered a key process that connects belowground and aboveground compartments in terrestrial ecosystems (Bardgett and Wardle, 2010). Despite the importance of plant–soil interactions, it remains difficult to disentangle the various roles of soil biota in this process. Plant–soil interactions can be caused by soil micro-organisms, such as bacteria, mycorrhizal or pathogenic fungi (Packer and Clay, 2000; Klironomos, 2002; Kardol et al., 2007), but also soil fauna, such as nematodes, protozoa, or collembolans can contribute to plant–soil feedbacks (De Deyn et al., 2003; Bonkowski et al., 2009). The effects of soil organisms on plant performance may depend on the size class of the organisms involved (Bradford et al., 2002), and on the co-occurrence with other groups of soil organisms (Ladygina and Hedlund, 2010).

Plant–soil feedback experiments can be set up in a variety of ways, depending on the research question, or on the type of soil studied. Frequently, in order to avoid the confounding effect of nutrient availability, sterilized (field) soil is inoculated with a non-sterilized field soil inoculum or with an aqueous suspension of the field soil (*e.g.* Troelstra et al., 2001; Klironomos, 2002; Callaway et al., 2004; Bezemer et al., 2006a,b; Kardol et al., 2006, 2007; Brinkman et al., 2010). Clearly, the experimental method that is used can affect the abundance and size classes of the organisms that will be inoculated. For example, because some soil organisms are more sensitive to soil processing or are better in recolonizing the soil than others. Kardol et al. (2007) showed that adding natural soil or an aqueous suspension of the natural soil resulted in a qualitatively similar soil effect on plant growth, but they did not compare effect sizes. As hardly any study has compared various soil inoculation methods within a single study, it remains largely unclear whether and how the method of inoculation influences plant–soil interactions.

Plant–soil interactions may also depend on the abiotic conditions of the soil (Bezemer et al., 2006b; Casper et al., 2008; Manning

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et al., 2008; Joosten et al., 2009; Harrison and Bardgett, 2010). For example, abiotic soil conditions can change the chemical composition of *Jacobaea vulgaris* plants (Hol et al., 2003; Joosten et al., 2009), which can subsequently influence soil fungal growth rate and soil fungal community composition (Hol and Van Veen, 2002; Kowalchuk et al., 2006). In addition, co-occurring plant species can change the abiotic and biotic conditions in the soil, which can then feed back to the focal plant (Aerts and Chapin, 2000; Klironomos, 2003; van de Voorde et al., 2011). Therefore, besides being influenced by the type of soil biota present, plant–soil interactions can also differ among soil origins.

In the present study, we examine how the method of inoculum preparation influences soil effects on the ruderal plant *J. vulgaris*. We study these effects using soils originating from two different fields. In a greenhouse experiment we inoculated sterilized soil with natural field soil that was sieved through a 1 mm mesh, with an aqueous soil suspension sieved through a 1 mm mesh, or with an aqueous microbial suspension sieved through a 20 μm mesh. We hypothesized that inoculation with the sieved soil and the soil suspension will have the same effect on plant performance, as they contain soil organisms of the same size fraction. In addition, we hypothesized that the effect of the microbial suspension on plant growth would be most different from the soil inoculation, as the microbial suspension is most disturbed and it does not contain soil organisms larger than 20um, which can be present in the other two inocula. To test whether the inoculum preparation method itself affects the abiotic, *e.g.* nutrients, characteristics of the inocula we also used sterilized inocula.

2. Materials and methods

We performed our study using the plant (tansy) ragwort, *J. vulgaris* ssp. *vulgaris* (synonym *Senecio jacobaea* L.). We selected this species because it is strongly affected by plant–soil interactions (Bezemer et al., 2006a; Joosten et al., 2009; van de Voorde et al., 2011). It is also one of the most abundant plant species in the study area and in the Netherlands (Bezemer et al., 2006a; van de Voorde et al., in press). We conducted the greenhouse experiments with seeds and soil that originated from two old-fields that are approximately 18 km apart, and are both situated at the Veluwe, the Netherlands (Field A (52.12 °N 5.49 °E) and Field B (52.04 °N 5.45 °E)). The characteristics of the fields and the *J. vulgaris* plants in these fields are described in van de Voorde et al. (in press) (Field A = 5A, Field B = 12) (Table S1). In short, flowering plants from Field A were significantly larger and seed weight significantly lower than of plants from Field B. Soil pH was significantly lower and Olsson P larger in Field A.

2.1. Soil and seed collection

In July 2007, soil for the inocula was collected by taking 150 soil cores of 3 cm diameter and 15 cm depth at two ex-arable old-fields. The soil samples were lumped for each field, sieved using a 5 mm mesh, and homogenized. The dry weight of a sub-sample was determined gravimetrically after 24 h at 105 °C. Ratios and weights hereafter are based on dry weights. In addition, approximately 500 kg of soil was collected from field B at 5–20 cm below the soil surface, sieved using a 0.5 cm mesh, homogenized, and sterilized by gamma irradiation at a dosage >25 KGray gamma irradiation by Isotron, Ede, the Netherlands. This served as sterilized bulk soil, to which the soil inocula were added. *J. vulgaris* seeds were collected from fields A and B from approximately 100 *J. vulgaris* plants. The seeds were surface sterilized during 1 min in 0.1% chloride solution, rinsed, and germinated on sterilized glass beads.

2.2. Inocula preparation

We prepared three types of soil inocula from both field soils: sieved soil inoculum, a soil suspension, and a microbial suspension. The soil inoculum was prepared by sieving 2 kg of the collected field soil through a 1 mm mesh. The soil and microbial suspensions were prepared by gently stirring 2 kg field soil with 1.5 L demineralised water for 2 min. The suspension was set aside for 15 min, stirred again for 2 min and left to settle for 15 min. The supernatant was then sieved. In order to prepare the soil suspension, half of the supernatant was sieved through a 1 mm mesh. The other half of the supernatant was used to prepare the microbial suspension and was sieved through sieves with mesh sizes of subsequently 1 mm, 180 μm, 75 μm, twice 45 μm, and 20 μm. Therefore, this microbial suspension does not contain micro-arthropods, nematodes, or arbuscular mycorrhizal fungi, whereas it should contain soil bacteria and fungi (Swift et al., 1979; Ames et al., 1987; Klironomos et al., 1993; Bardgett, 2005).

Pots of 0.9 L were filled with 1.2 kg of soil. In the case of the soil treatment this was a 6:1 mixture of sterilized soil and sieved field soil inoculum. In the case of the soil and microbial suspension treatments, pots were filled with 1.2 kg sterilized soil and inoculated with 75 ml suspension extracted from 100 g of field soil. Pots in the soil treatment received 75 ml of demineralised water in order to obtain equal levels of soil moisture for all treatments.

2.3. Greenhouse experiment

The greenhouse experiment consisted of two growth phases. In the first phase, *J. vulgaris* plants were grown in soils prepared as described in the previous section. Prior to planting, the pots were incubated for 3 days. Then, into each pot three one-week-old *J. vulgaris* seedlings from either population A or B were planted. Seedlings that died during the first week of the experiment were replaced, because this may have been due to the transplanting. All treatments were replicated five times, which resulted in 60 pots in total: 3 inoculum types × 2 soil origins × 2 seed origins × 5 replicates. Pots were positioned randomly in a greenhouse at 70% RH, at 16 h 21 °C (day) and 8 h 16 °C (night) and soil moisture was set at 17% based on dry weight. Natural day light was supplemented by metal halide lamps (225 μmol s^{-1} m^{-2} photosynthetically active radiation, 1 lamp per 1.5 m^2). After 10 weeks all aboveground biomass was clipped, oven-dried for five days at 70 °C and weighed. The soil and roots of each pot were divided into four equal parts. From two parts the roots were gently rinsed and nematodes were extracted from a homogenized sub-sample of these rinsed roots (see below). The remaining roots were washed, dried for five days at 70 °C, and weighed.

The soil of the two remaining parts was used in the second growth phase. The two remaining parts were homogenized and soils from the individual pots were kept separate. Large roots were removed, because they may re-sprout. All the finer roots were left in the soil, so that their rhizosphere can serve as a source of inoculum for the microbial rhizosphere community. These soils from the first phase were mixed in a 1:1-ratio with 640 g of sterilized bulk soil to balance for potential nutrient variability that may have occurred during the first growth phase. Pots were incubated for 3 days, after which three one-week-old *J. vulgaris* seedlings from either population A or B were planted, in such a way that each soil received seedlings from the same population as during the first phase. Seedlings that died during the first week of the experiment were replaced. After one week the number of seedlings per pot was randomly thinned to two seedlings per pot. Plants were grown under the same conditions as during the first phase. Six weeks after transplanting, shoots were harvested and roots were separated

from the soil and rinsed. Shoots and roots were oven-dried for five days at 70 °C, and weighed.

2.4. Soil nematodes

We determined the number of plant-feeding nematodes in the roots from each pot at the end of the first growth phase. A subsample of the roots (approximately 2 g dry root mass) was used to extract nematodes using a mistifier and an extraction time of 48 h. Plant-feeding nematodes were heat-killed and fixed (35% formaldehyde diluted to 4%), after which a minimum of 150 nematodes were identified to genus or species level, according to Bongers (1988). Numbers of nematodes were expressed per 1 g dry root material.

2.5. Inocula conditions

To test whether the three inocula types differ in abiotic conditions, during the first growth phase we also set-up pots with sterilized inocula. The three inocula (mixed between soils from the different fields) were autoclaved for 20 min at 120 °C on 3 consecutive days in order to kill of all soil biota. Sterilized bulk soil was then inoculated with the sterilized inocula as described above for the first growth phase. After inoculation, the pots were incubated for 3 days, after which three one-week-old *J. vulgaris* seedlings from either population A or B were transplanted, using 5 replicate pots per treatment. Growing and greenhouse conditions were the same as in the main plant-soil feedback experiment (see above). After 10 weeks all aboveground biomass was clipped and the roots were separated from the soil and washed. Plant biomass was dried at 70 °C for five days, and weighed.

2.6. Data analyses

Biomass data from the greenhouse experiment were analysed using three-way analysis of variance (ANOVA), with treatment (inoculum type), field origin, and seed origin as fixed factors using Genstat 12 (Payne et al., 2008). Treatments were compared using a Tukey HSD post hoc test for each growth phase separately. All data were checked for homogeneity of variances using Levene's tests ($P > 0.05$) and the assumption of normality was tested with Kolmogorov–Smirnov procedures. Biomass data were log-transformed and count data were square root-transformed prior to analysis to meet the assumptions of ANOVA. When there is no significant seed origin effect, both seed origins are shown together.

3. Results

In sterilized soil inoculated with sterilized inocula, *J. vulgaris* biomass did not differ significantly between the inoculum treatments ($F_{2,24} = 3.27$, $P = 0.07$; Fig. 1). However, plants grown from seeds collected from Field A were significantly larger than plants originating from seeds from Field B ($F_{1,24} = 29.8$, $P < 0.001$; Fig. 1).

In the greenhouse experiment with non-sterilized inocula there were no significant effects of either soil or seed origin on plant growth during both growth phases (Table 1). However, during the first growth phase, *J. vulgaris* biomass was significantly lower in pots inoculated with sieved soil than in pots inoculated with the suspensions (Fig. 2a). Plants growing in the soil suspension treatment, in turn, produced less biomass than plants growing in pots with the microbial suspensions (Fig. 2a).

In the second phase, *J. vulgaris* produced significantly less biomass in the sieved soil treatment and in the soil suspension treatment than in microbial suspension treatment (Fig. 2b). Biomass did not differ between the soil and soil suspension treatment (Fig. 2b). There was a significant positive relationship between biomass of both phases ($F_{1,59} = 11.7$, $P = 0.001$, $R^2 = 0.17$), indicating that nutrient availability was not limiting.

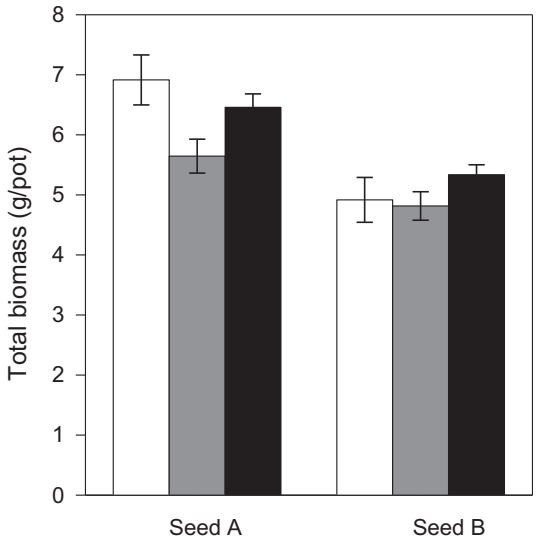

Fig. 1. Mean (±SE) total biomass of *J. vulgaris* grown in the first phase in sterilized soil inoculated with sterilized field soil (white bars), sterilized soil suspension (grey bars) or sterilized microbial suspension (black bars). Seeds originated from two fields ($n = 30$).

At the end of the first growth phase, plant-feeding nematodes belonging to the genera *Pratylenchus* and *Meloidogyne* were found in the roots. Nematode densities differed significantly between inoculation treatments (Table 1) and were largest in pots inoculated with sieved soil (Fig. 3). In most pots that were inoculated with a suspension no plant-feeding nematodes were detected.

4. Discussion

Our results clearly show that the type of inoculum can greatly influence the effect of inoculation on plant performance and the observed soil effect. In our study negative soil effects were lower in soil suspensions than in inocula with natural soil, and biomass was highest in sterile soil inoculated with a soil suspension that was sieved through a mesh of 20 μm. These results suggest that negative soil effects can be partly sieved away. This effect is robust, as it was found in two soils and in two growth phases. Hence, our results show that choices made on what soil inoculation method to use in

Table 1
Results of a three-way ANOVA for the effects of soil origin (Field A or B), inoculation treatment (soil, soil suspension or microbial suspension) and seed origin (Field A or B) on total *J. vulgaris* biomass in the first and second growth phase, and on the total number of plant-feeding nematodes (*Pratylenchus* and *Meloidogynidae*) per gram root in the first phase (In all three analyses $n = 60$).

	d.f.	Total biomass				Nematodes	
		First phase		Second phase		First phase	
		F	*P*	*F*	*P*	*F*	*P*
Soil origin (Soil)	1	1.20	0.28	1.57	0.22	1.35	0.25
Inoculum	2	29.54	<0.001	6.92	0.002	32.2	<0.001
Seed origin (Seed)	1	1.08	0.30	2.67	0.11	0.22	0.64
Soil*Inoculum	2	0.82	0.45	0.06	0.94	2.06	0.14
Soil*Seed	1	0.00	1.00	3.02	0.09	0.12	0.73
Inoculum*Seed	2	0.06	0.94	0.90	0.41	0.11	0.31
Soil*Inoculum*Seed	2	0.36	0.70	0.44	0.65	0.38	0.68
Error	48						

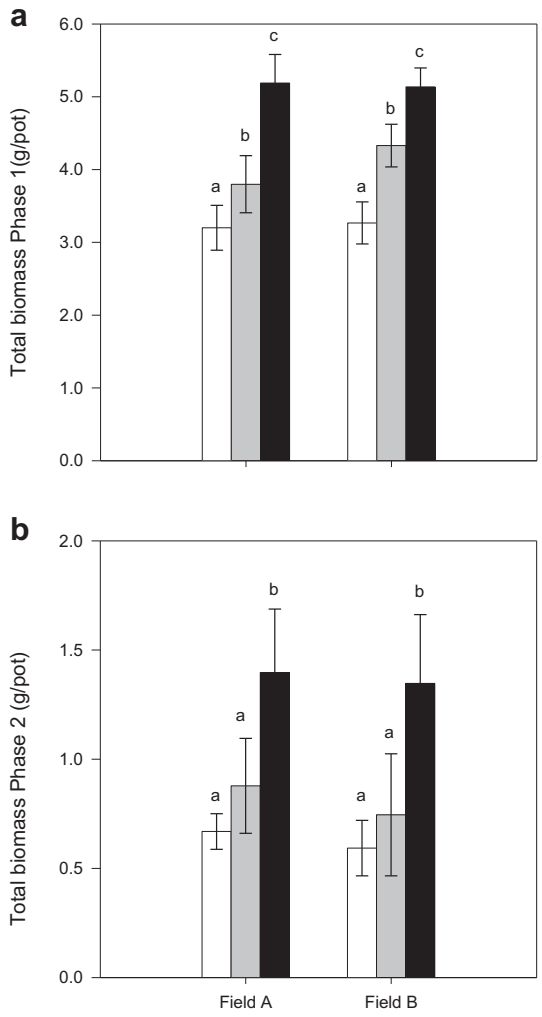

Fig. 2. Plant–soil feedback experiment. Mean (±SE) total biomass of *J. vulgaris* plants in the first phase (a), and in the second growth phase (b). *J. vulgaris* plants were grown in sterilized soil inoculated with sieved field soil (white bars), a soil suspension (grey bars) or a microbial suspension (black bars). Shown are the means of the two seed origins. Soil inocula originated from two fields. Different letters indicate significant differences ($P < 0.05$) per growth phase, based on a Tukey's HSD post hoc test (In both growth phases $n = 60$).

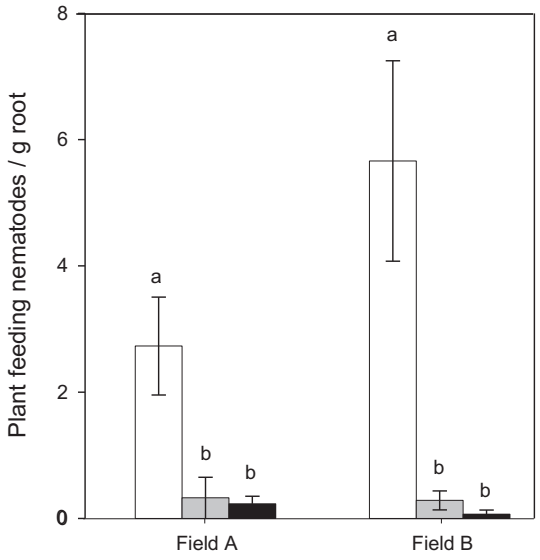

Fig. 3. Mean (±SE) total number of plant-feeding nematodes per gram of roots. *J. vulgaris* plants were grown in sterilized soil inoculated with sieved field soil (white bars), a soil suspension (grey bars) or a microbial suspension (black bars). Shown are the means of the two seed origins. Soil inocula originated from two fields. Different letters indicate significant differences ($P < 0.05$) based on a Tukey's HSD post hoc test ($n = 60$).

plant–soil interaction experiments can have large consequences on the size of the effect that the soil community has on plant performance. Below we discuss a number of possible mechanisms that may explain how the inoculation method can influence plant–soil effects.

The higher biomass production in pots inoculated only with the microbial community indicates that the main growth reducing agents were partly sieved out and thus that these agents did not pass the 20 μm sieve. The most obvious explanation is that the main growth reducing agents are larger than 20 μm. This corresponds with the observation that plants were smallest in the treatment that contained most plant-feeding nematodes. This observation points at plant-feeding nematodes as a cause of reduced *J. vulgaris* growth in pots inoculated with sieved field soil. However, there are also many pathogenic soil bacteria and fungi present in soils and it could be that some of them were filtered out by the 20 μm sieve as well. For example, because soil bacteria and fungi can be associated to larger soil aggregates (Six et al., 2004; Briar et al., 2011), which are filtered out by the 20 μm sieve. It is also possible that the differences are due to differences between soil organisms in their sensitivity to soil processing or due to a dilution effect. Recent work showed that rare soil microbes are not redundant, as they can have strong negative growth effects (Hol et al., 2010). By sieving out part of the soil (micro-)organisms from the microbial suspension, it is possible that low-abundant species are sieved out as well.

J. vulgaris biomass in the treatment inoculated with sieved soil was significantly lower than when inoculated with the soil suspension. However, both treatments did not differ when the inocula were sterilized, which suggests that nutrient availability in both inocula was similar. Even though both the sieved soil inoculum and the soil suspension were sieved through 1 mm mesh there was a clear difference in the number of plant-feeding nematodes between these treatments. Nematodes are sensitive to soil disturbance (de Rooij-van der Goes et al., 1997) and the additional sieving and stirring to create the suspensions may have reduced their number. Our results emphasize that soil suspensions should not be used as a direct substitute of soil inoculation, as their effect on plant performance is not the same.

While frequently ignored, recent studies have shown that plant–soil feedback effects can differ greatly between soils (Manning et al., 2008; Joosten et al., 2009; Harrison and Bardgett, 2010). In our study, *J. vulgaris* performance did not differ between the two soil origins for any of the three inocula. The soils that we tested originate both from old-field grasslands on the same soil substrate. Studies that reported effects of soil abiotic conditions on plant–soil interaction effects often compare soils that are more different from each other, for example, clay versus sandy loam soils (Bezemer et al., 2006b), or soils that originate from more different ecosystems, such as a dune system versus an experimental grassland (Joosten et al., 2009). However, the fact that we did find effects of the inoculation method but no soil origin effect indicates that *J. vulgaris* is more sensitive to small differences created between soil inocula from the same soil, than to differences between soils.

In the field, flowering plants collected from Field A were much larger than plants originating from Field B (van de Voorde et al., in press) and also in the experiment with sterilized inocula these plants were significantly larger. Nevertheless, in the greenhouse

experiment with non-sterilized inocula plant size did not differ between the two populations. Other studies have shown that microbial community composition and root herbivore performance can vary among genotypes or cultivars of the host plant (Kowalchuk et al., 2006; Schweitzer et al., 2008; Crutsinger et al., 2008; Johnson et al., 2010; Vandegehuchte et al., 2010) and that plant genotypes can differ in their effects on belowground processes, such as decomposition (Schweitzer et al., 2004). In an experiment with white cabbage cultivars (*Brassica oleracea* var. *capitata*) Kabouw et al. (2010) found an effect of cultivar identity on plant-feeding nematodes, but not on other parts of the soil food web. Therefore, the effects of inoculation preparation on plant-feeding nematodes in our experiment could have overruled variation between the seed origins.

5. Conclusions

We show that the method of inoculation can strongly influence the soil effect on plant performance. The strongest negative soil effect was observed when using sieved field soil. By sieving the soil to obtain a microbial suspension the negative soil effect was reduced, and this was observed during both growth phases. In addition, we show that the results obtained with inoculating sieved field soil or with an aqueous soil suspension are not comparable in terms of effect strength. Thus, when designing plant–soil feedback experiments, it is crucial to consider that soil inoculum preparation can strongly influence the effect size.

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Appendix A. Supplementary information

Table S1
Field characteristics, soil chemistry, and *J. vulgaris* characteristics of fields A and B in mean (±SE) as described in van de Voorde et al. (in press) (Field A = 5A, Field B = 12).

	Field A	Field B
Field characteristics		
Location	52.12°N 5.49°E	52.04°N 5.45°E
Soil texture	Coarse sand	Coarse sand
Soil chemistry		
Soil pH	5.4 ± 0.15 b	6.1 ± 0.07 a
Mineral N (mg/kg)	10.7 ± 1.3 a	10.6 ± 0.8 a
P (mg/kg)	121 ± 13 a	90 ± 3 b
J. vulgaris characteristics		
Abundance (%)	27.4 ± 15.0 a	10.0 ± 1.6 a
Flowering height (cm)	84 ± 6 a	63 ± 3 b
Seed weight (μg)	0.196 ± 0.01 b	0.249 ± 0.01 a
Germination (%)	54.0 ± 8.9 a	60.0 ± 3.1 a

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