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Exogenous application of plant defense hormones alters the effects of live soils on plant performance

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Abstract

The overall effect of a live soil inoculum collected from nature on plant biomass is often negative. One hypothesis to explain this phenomenon is that the overall net pathogenic effect of soil microbial communities reduces plant performance. Induced plant defenses triggered by the application of the plant hormones jasmonic acid (JA) and salicylic acid (SA) may help to mitigate this pathogenic effect of live soil. However, little is known about how such hormonal application to the plant affects the soil and how this, in turn, impacts plant growth. We grew four plant species in sterilized and inoculated live soil and exposed their leaves to two hormonal treatments (JA and SA). Two species (*Jacobaea vulgaris* and *Cirsium vulgare*) were negatively affected by soil inoculation. In these two species foliar application of SA increased biomass in live soil but not in sterilized soil. Two other species (*Trifolium repens* and *Daucus carota*) were not affected by soil inoculum and for these two species foliar application of SA reduced plant biomass in both the sterilized and live soil. Application of JA reduced plant biomass in both soils for all species. We subsequently carried out a multiple generation experiment for one of the plant species, *J. vulgaris*. In each generation, the live soil was a mixture of 10% soil from the previous generation and 90% sterilized soil and the same hormonal treatments were applied. The negative effects of live soil on plant biomass were similar in all four generations, and this negative effect was mitigated by the application of SA. Our research suggests that the application of SA can mitigate the negative effects of live soil on plant growth. Although the inoculum of soil containing a natural live soil microbial community had a strong negative effect on the growth of *J. vulgaris*, we found no evidence for an increase or decrease in negative plant-soil feedback in either the control or the SA treated plants. Also plant performance did not decrease consistently with succeeding generations.

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Keywords: Plant-soil interactions; Plant-soil feedback; Induced resistance; Rhizosphere soil; Salicylic acid; Jasmonic acid

Introduction

The interactions between plants and soil microorganisms have long been recognized for their importance in terrestrial

ecological systems (Bever, 1994; Seipel, Ishaq & Menalled, 2019; Van der Heijden, Bardgett & van Straalen, 2008). Although the effects may vary depending on the plant species and the soils tested, in the majority of cases the soil microbial community has a negative effect on plant growth (Kulmatiski, Beard, Stevens & Cobbold, 2008). Plants also affect the composition of the soil microbial community,

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which, in turn, will impact plant growth. This process is called plant-soil feedback (Bever, Westover & Antonovics, 1997; van Breemen & Finzi, 1998). Most plant species exhibit negative conspecific soil feedbacks. This means that they grow worse in soil, in which the same species has been grown than in soil where other species have grown (Kulmatiski et al., 2008). From natural situations and agriculture, it is well-known that soil can become less suitable for a species if this species is grown in the same soil for multiple generations. This negative effect is thought to be caused by soil pathogens or root herbivores, allelopathy, nutrient immobilization or nutrient depletion (Miki, 2012). In some cases, plants also cause positive plant-soil feedbacks and these can be mediated by plants promoting rhizobacteria, mycorrhizal fungi or other unknown mechanisms (Revillini, Gehring & Johnson, 2016; van der Putten, 2017).

Plant-induced resistance has been regarded as a promising defense strategy against pathogens or herbivores (Haney & Ausubel, 2015; Lebeis et al., 2015; Yang, Ahammed, Wu, Fan, & Zhou, 2015). In nature, plants are exposed to complex selection pressures, involving both abiotic and biotic stresses. Plants are under constant attack by a myriad of pathogens and pests and have to compete with neighboring plants. As a result, plants have evolved a wide range of responses to cope with biotic stresses. The abilities of plants to respond to different biotic stresses are regulated through sophisticated interacting hormonal signaling networks (Arnaud & Hwang, 2015; Bezemer & van Dam, 2005; Fujita et al., 2006). Phytohormones are a group of natural plant compounds with low molecular weights. Salicylic acid (SA), jasmonic acid (JA) and its methylated form MeJA, ethylene (ET), abscisic acid (ABA), auxin, cytokinins (CKs), gibberellins and brassinosteroids are commonly studied phytohormones. Plant hormones regulate many developmental and signaling networks. Although most hormones have been implicated to be involved in defense pathways, the key regulator against pathogens and pests, are the phytohormones JA and SA (Bari & Jones, 2009). Experimental evidence indicates that application of SA to plant leaves, activates systemic acquired resistance (SAR) against pathogens (Mandal, Mallick & Mitra, 2009; Raymond & Farmer, 1998). JA, in turn, activates induced defenses against herbivores and necrotrophic pathogens (Nahar, Kyndt, de Vleeschauwer, Höfte & Gheysen, 2011). Although to some extent, the SA or JA-induced hormonal signaling pathway could interact with other phytohormones, such as CKs, ET, ABA and auxins, they do show clear effects on the plant's defense system when applied as single hormones (Berens et al., 2019; Fujita et al., 2006; Yang et al., 2015).

A still uncharted territory is how plant hormone-activated signaling pathways impact soil microbial communities and how these, in turn, affect plant growth. Here we restricted ourselves to two prime hormones involved in activating defense pathways, SA and JA. We aimed to quantify the effect of SA- or JA-induced resistance to the soil microbial

communities that affect plant biomass. If the negative effect of the live soil (sterilized soil inoculated with a live soil microbial community) on plant biomass is caused by an overall pathogenic effect, we expect that activating SA signaling in the plant by exogenous application mitigates the negative effects. As a result, we expect that the effects of SA application on plant biomass differ between plants in sterile soil and in live soil. Exogenous application of JA typically induces resistance against herbivores and necrotrophic pathogens (Antico, Colon, Banks & Ramonell, 2012; Carvalhais, Schenk & Dennis, 2017; Palmer, Shang & Fu, 2017; Pieterse, Leon-Reyes, van der Ent & van Wees, 2009; Van Dam & Oomen, 2008). JA signaling can exhibit negative crosstalk with SA signaling (Leon-Reyes et al., 2010). The responses of plants, after activating hormonal defense pathways, to an inoculum containing a live soil microbial community are, as yet, not well studied and understood. Moreover, the evidence for the existence of such effects is contradictory. Activation of JA and SA signaling pathways did not affect the resident soil microflora in several studies (Berendsen, Pieterse & Bakker, 2012; Doornbos, van Loon & Bakker, 2012; Rashid & Chung, 2017), but a more recent study showed that SA modulates colonization of the root microbiome by specific bacterial taxa (Lebeis et al., 2015).

If the induction of signaling pathways in the plant leads to changes in the composition of the soil microbial community, its effect is likely to extend over time or plant generations. Potentially this could lead to the selection of more beneficial soil microbial communities either by suppressing pathogens or by promoting beneficial microbes. As far as we are aware, the effects of plant hormones through plants on soils containing a live microbial community over multiple generations have not been studied so far, despite its potential to select for more beneficial soils containing plant growth-promoting microbial communities in agriculture.

In a preliminary experiment, we found strong evidence for negative effects of soil that consisted of a mixture of 90% sterilized soil and 10% live soil on the growth of common ragwort (*Jacobaea vulgaris*), compared to sterilized soil. After treating plants with SA, this negative effect diminished. Based on these findings, we grew four different plant species individually in both sterilized soil and live soil. For *J. vulgaris*, the species that showed the strongest negative effect towards the live soil, and for which this negative effect was mitigated by foliar application of SA, we grew plants for three more generations. For each generation, sterilized soil was inoculated with live soil from the previous generation from the same treatment. We addressed four questions: (1) Do the effects of live soil on plant biomass differ among plant species? (2) Does the foliar application of JA and SA alter the effects of the live soil on plant biomass for those species that were negatively affected by the live soil? (3) Does the negative effect of live soil change in four successive generations of *J. vulgaris* for control plants and plants treated with SA or JA.

Materials and methods

Plant material and seed germination

Jacobaea vulgaris (common ragwort), *Cirsium vulgare* (bull thistle), *Trifolium repens* (white clover) and *Daucus carota* (wild carrot) were chosen because they are common native species in the dune area where we collected soil. We collected seeds at the dunes for *J. vulgaris*, *C. vulgare*, and *D. carota*. *T. repens* seeds were bought from Cruydt-Hoeck, a seed company that sells seeds of wild plant species (Nijberkoop, The Netherlands). Prior to seed germination, all seeds were shaken for 2 min in 70% ethanol, then washed with sterilized water, put for 12 min in 2% bleach, and finally rinsed four times with sterilized water to minimize influences of seed-borne microbes.

Soil material

The soil was collected at Meijndel, a calcareous sandy area from a coastal dune area north of The Hague, The Netherlands (52 °11 N, 4 ° 31 'E). The topsoil was collected to a depth of 15 cm after removing the grassland vegetation and the organic layer of the surface. The soil was sieved using a 5 mm mesh, homogenized with a concrete mixer, and then stored in 20-liter plastic bags (Nasco Whirl-Pak Sample Bag). Bags were either sterilized by 35-K Gray gamma-irradiation (Synergy Health Company, Ede, The Netherlands) or kept at 4 C for inoculation.

Plant growth and foliar application of hormones

Surface-sterilized seeds of the four species (*J. vulgaris*, *C. vulgare*, *T. repens* and *D. carota*) were germinated in sterile Petri dishes on filter paper. After one week, 60 seedlings per species were planted individually in 500 ml pots containing either sterilized soil or inoculated live soil. The live soil consisted of a mixture of 90% sterilized soil and 10% live soil. Nutrient availability often increases after sterilization of the soil, and we therefore inoculated the sterilized soil rather than using pure live soil, to enable comparison of the two types of soil. Sterilized soil and live soil were kept in bags and left in the climate room for 14 days to enable the establishment of microbial communities in the inoculated soil before potting. Before potting, the soil in each bag was mixed. After planting the seedlings, pots were randomly distributed over a climate room (relative humidity 70%, light 16 h at 20 C, dark 8 h at 20 C). Plants were watered regularly with Milli-Q water. Five ml Steiner nutrient solution was added per plant on day 7. Ten ml Steiner nutrient solution was added on day 13, and 20 ml Steiner nutrient solution was added on days 19, 28, 37 and 42. The Steiner nutrient solution (Steiner, 1979) was prepared from seven different stock solutions (106.2 g $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 29.3 g

KNO_3 , 13.6 g KH_2PO_4 , 49.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 25.2 g K_2SO_4 , 2.24 g KOH and 3.29 g Fe-EDTA added to 1 liter demineralized water, and a stock solution with micro elements (a mixed solution of 0.181 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.286 g H_3BO_3 , 0.022 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0078 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 0.0126 g $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ added to 1 liter demineralized water). Ten ml of each stock solution was diluted in 1 liter of demineralized water before use.

The pots for each species were divided over six treatments: two soil treatments (sterilized soil and live soil) and three hormonal treatments (JA, SA and control (only solvent)). Each treatment was replicated 10 times. The experiment, therefore, consisted of 240 pots (4 species \times 2 soil treatments \times 3 (2 hormonal treatments and control) \times 10 replicates). The plant hormones JA and SA were applied through foliar application three times a week for four consecutive weeks. The first application was given when plants were 14 days old. Either 0.75 ± 0.05 ml of 100 μM JA or SA was sprayed on the leaves while carefully avoiding spill-over to the soil. The hormonal concentrations were chosen based on a previous research with *J. vulgaris* (Wei, Vrieling, Kim, Mulder & Klinkhamer, 2021). One week later the treatment was repeated with 1.50 ± 0.05 ml of 100 μM JA or SA. In the next week, the treatment was repeated with 2.25 ± 0.05 ml of 100 μM JA or SA. The JA-solution was prepared by adding 105.135 μl JA stock solution to Milli-Q water until a final volume of 500 ml. The JA stock solution was prepared by adding 500 mg JA to 5 ml ethanol. JA was purchased from Cayman Chemical Company (product number: 88,300). SA solution (purchased from Sigma-Aldrich, $\geq 99.0\%$) was made by dissolving 6.9055 mg in 69.055 μl of ethanol to which Milli-Q water was added until a final volume of 500 ml. Control plants were sprayed with sterile water with the same solvent (85 μl ethanol in 500 ml Milli-Q water).

Harvesting plants and soil samples

Fifty-four days after planting, all plants were harvested, except for *C. vulgare*. *C. vulgare* plants were considerably larger than the other species and were therefore harvested after 45 days to prevent pot size limiting growth. Plants were gently removed from the pots. Shoots were separated from roots with a scissor just above the root crown, and roots were rinsed with water and then put into paper bags. Harvested plant parts were oven-dried at 60 C for approximately one week. The dry weight of roots and shoots was determined to the nearest 0.1 mg. The rhizosphere soil was harvested individually from each pot by gently shaking the roots and soil three times to remove the loosely adhering soil, after which rhizosphere soil samples were collected onto a sterile filter paper by removing the remnant soil from the roots with a fine sterilized brush. Finally, all the labeled soil samples were transferred to a 4 C room and stored for the multiple generation experiment.

Multi-generation experiment

J. vulgaris was chosen for the multiple generation selection experiment to examine if the observed effect on plant biomass of the first generation would increase further over later generations. The soil for the multi-generational *J. vulgaris* experiment was derived from the pots from the previous generation of *J. vulgaris* plants. For *J. vulgaris* we grew the plants from each of the six treatments (sterilized and live soils, two hormone treatments and control) for another three generations under the same conditions as described for the first generation. The only difference being that each time, the soil inoculum was derived from the previous generation from the same treatment, 100% sterilized soil was used as control. A schematic drawing of the experiment is presented in Fig. 1. Fourteen days after mixing the sterilized and live soil, a single *J. vulgaris* seedling was planted into each pot. All replicate rhizosphere soils from a single treatment were mixed before inoculation to avoid a selection of particular microbial species in individual pots. All treatments were carried out as described above.

Statistical analysis

Data were first checked for homogeneity of variance (Levene's test) and normal distribution (Shapiro Wilks) of errors and data were transformed when necessary. To test whether the effect of the live soil was different among the four species we performed a three-way ANOVA on the total data set of the first experiment with soil (sterilized and live, 2 levels), hormones (3 levels) and species (4 levels) as fixed factors. Live-soil responsiveness was used as a dependent variable and was arcsine square-root transformed prior to analysis (Rowe, Brown & Claassen, 2007). Live-soil responsiveness was calculated as 100 times the dry mass of a plant divided by the average dry mass of the control plants from the same species in the sterilized soil. In this way, the average dry mass of the control plants in the sterilized soil was set to 100 for each of the four species. By doing so we removed species-specific size differences to make the data more comparable among species. This analysis showed a significant soil × species interaction (results section). On the basis of this we divided the data set in two groups. One group for the

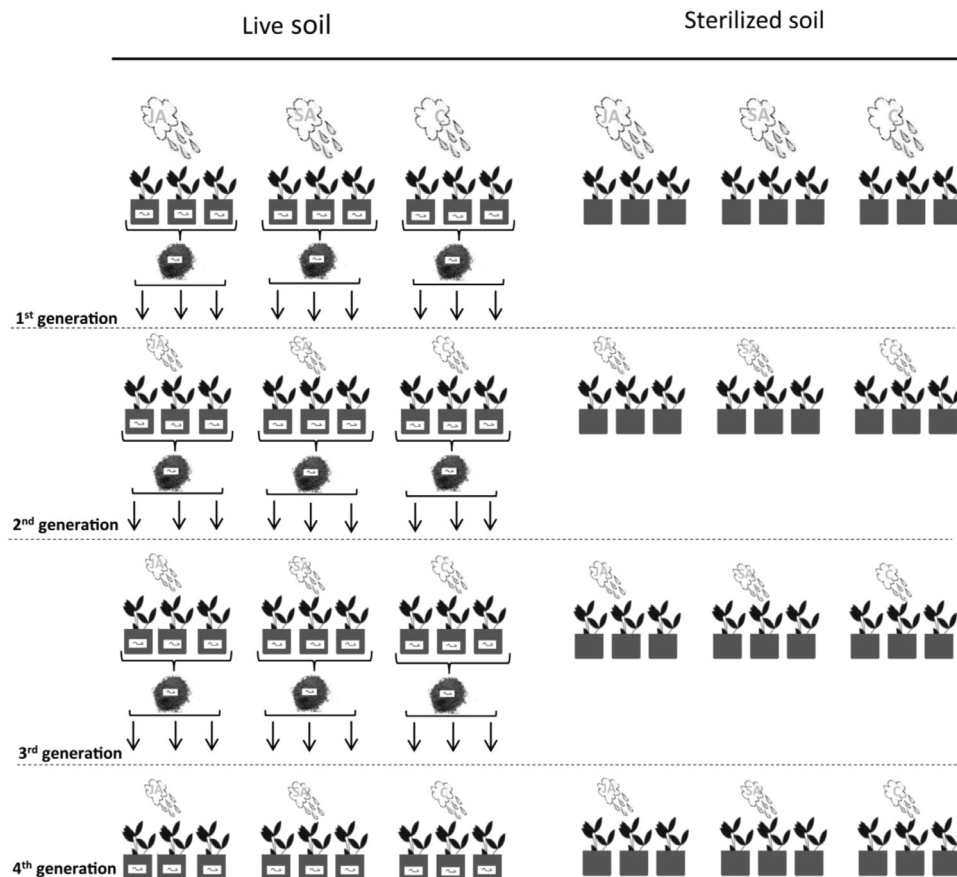


Fig. 1. Experimental design of the multigenerational experiment with *J. vulgaris*. Soil used for the 1st generation was a mixture of 90% sterilized soil and 10% live soil both collected from the dunes. Soil used for the 2nd, 3rd and 4th generations was a mixture of 10% rhizosphere soil collected from the previous generation from the same treatment and 90% sterilized soil collected from the dunes. In each generation plants were grown for six weeks and all seeds were derived from the same batch of seeds. In each generation we tested two hormonal treatments in inoculated and 100% sterilized soil. JA denotes foliar application of jasmonic acid, SA denotes foliar application of salicylic acid and C denotes control. In each treatment 10 replicates were used even though only three are depicted.

two species that were negatively affected by the live soil and one group for the two species that were not. We did this because we expected the effect of the hormonal treatments to be only present for the species that were affected by live soil. To answer the question if the effect of the live soil was affected by foliar application of hormones we performed four three-way ANOVAs (for the two groups of species and the two hormonal treatments) with plant mass as dependent variable and species (2 levels), soil (2 levels) and hormonal treatment (2 levels) as fixed factors. Usually, the negative effects of live soil on plant biomass are stronger in the roots than the shoots, thus we also carried out three-way ANOVA analysis for shoot-root ratios of the four plant species.

To answer the question whether the negative effect of live soil changes in four successive generations of *J. vulgaris* for control plants and plants treated with SA or JA we used a three-way ANOVA with soil (2 levels), generation (4 levels) and hormones (3 levels) as fixed factors, and log-transformed plant dry mass or shoot-root ratio as dependent variables. We furthermore compared the effects of the two hormones separately using a three-way ANOVA with log-transformed plant dry mass as a dependent variable and soil (2 levels), hormone (2 levels) and generation (4 levels) as fixed factors. Differences between treatments were tested with a Tukey post-hoc test.

We used a linear regression model to estimate the effects of SA and JA on the growth of *J. vulgaris* over four consecutive generations in both sterilized and live soil. In the regression model, the dry mass of plants of the SA or JA treatments divided the dry mass of control plants was the

dependent variable and generation was the independent variable. Since we could not pair the pots (SA or JA/control) and we only had 10 replicates for each treatment, we used a Monte-Carlo simulation to test if the linear regression model differed from $y = 1$. Each time we randomly paired one plant of the hormone treatment and one plant of the control to calculate the ratio of the dry mass of treated and dry mass of control. Then we repeated this procedure 1000 times, to obtain 1000 ratios of each generation per soil. Then we took the mean of 1000 ratios per generation to fit linear regression models for the two soils, respectively. To test whether the linear regressions in sterilized and live soils differed from $y = 1$, we calculated the 95% confidence intervals (CFI) of the slopes for both soils. We also tested whether the two linear regression models differed between sterilized and live soils with ANCOVA (analysis of covariance) analysis. All analyses were performed in IBM SPSS Statistics 25.

Results

Do the effects of live soil on plant biomass differ among plant species?

For *J. vulgaris* and *C. vulgare*, biomass in live soils was about half that in sterilized soils. This negative effect of the live soil was present irrespective of the hormonal treatment. For the other two species (*T. repens* and *D. carota*) biomass was not significantly different in live and sterilized soils (Fig. 2, Table 1). The difference in response to live soils

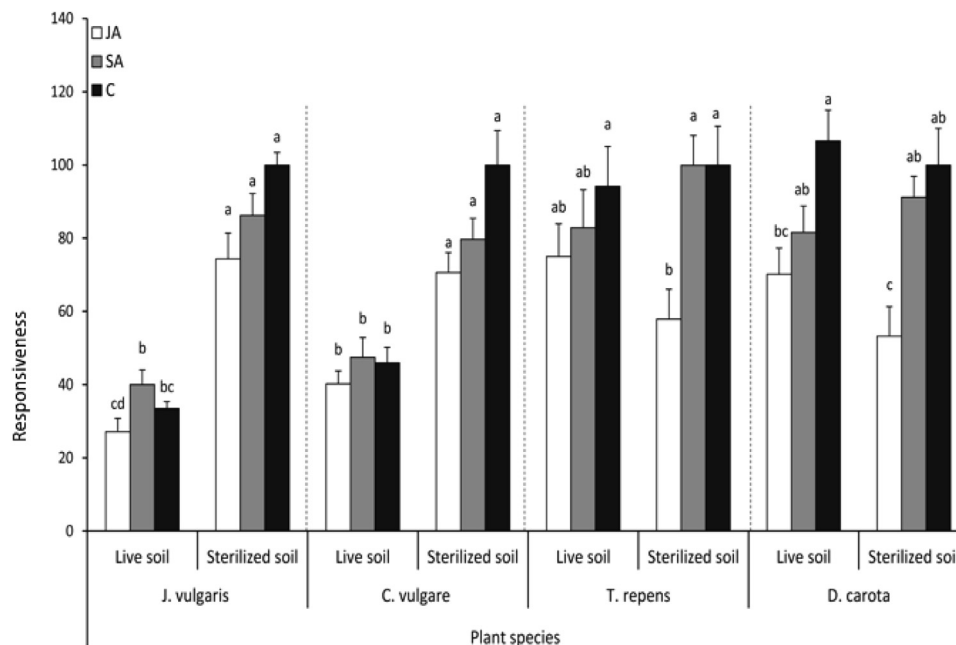


Fig. 2. Mean (+ SE) responsiveness (%) of *J. vulgaris*, *C. vulgare*, *T. repens* and *D. carota* plants treated with JA and SA in sterilized soil and live soil. Responsiveness is dry mass of a treated plant divided by the average dry mass of the control plants in sterilized soil *100. C represents control. Note: within species different letters above bars indicate significant differences between treatments based on a Tukey post-hoc test for each single species.

Table 1. Three-way ANOVA of arcsine square-root transformed responsiveness of *J. vulgaris*, *C. vulgare*, *T. repens* and *D. carota* in live and sterilized soil for plants treated with JA or SA and for control plants. *df* = degrees of freedom.

Source of variation	<i>df</i>	F-value	<i>P</i>
Species	3, 239	53.67	***
Soil	1, 239	147.78	***
Hormone	2, 239	27.17	***
Species × Soil	3, 239	59.81	***
Species × Hormone	6, 239	0.45	ns
Soil × Hormone	2, 239	4.75	**
SP × S × H	6, 239	1.48	ns

** *P* < 0.01,.

*** *P* < 0.001, ns not significant.

among the four species is reflected by the highly significant interaction term (species × soil) in the ANOVA (Table 1).

Does the foliar application of JA and SA alter the effects of live soil on plant biomass for those species that were negatively affected by live soil?

Salicylic acid

For the two species (*J. vulgaris* and *C. vulgare*) that were negatively affected by the live soil, foliar application of SA reduced the biomass of plants grown in the sterilized soil while it increased the biomass of plants grown in the live soil (Fig. 2). As a result, the main effect of SA in the ANOVA was not significant (Table 2). Although by itself

the differences between the SA treatment and the control were not significant (Fig. 2), the effect of the SA treatment, as we hypothesized, depended strongly on soil type as is reflected by the significant soil × hormone interaction term in the ANOVA (Table 2). For the two species (*T. repens*, *D. carota*) that were not negatively affected by the live soil, foliar application of SA reduced plant biomass in both soils, although this effect was not significant (Fig. 2, Table 2).

Jasmonic acid

Foliar application of JA decreased plant mass in all plant species in both sterilized and live soils. For the two species that were negatively affected by live soil the negative effect of JA was stronger in sterilized soil than in live soil (Fig. 2). This difference in response between plants grown in the two soils was significant as reflected by the soil × hormone interaction term in the ANOVA (Table 2). For the two species that did not grow less well in the live soils, such a difference in the response to JA application in the two soils was not found (Table 2).

The shoot-root ratio of plants differed among species, soils and hormone treatments (see Appendix A: Table S1). Except for *T. repens*, JA application increased the shoot-root ratio. We found no significant effects of SA application on the shoot-root ratio. The effects of hormone application on the shoot-root ratio varied among species and soils. In all species, the shoot-root ratio was, on average, higher in live soils (See Appendix A: Fig. S1).

Table 2. Three-way ANOVAs of arcsine square-root transformed responsiveness for species that grew less well in live soil compared to sterilized soil (upper part) and for species that were not negatively affected by the live soil (lower part). Left: hormonal treatment is foliar application of SA. Right: hormonal treatment is foliar application of JA. Species, soil (live or sterilized), and hormone treatment were used as fixed factors. *df* = degrees of freedom.

Species respond to soil effect	Source of variation	SA treatment			JA treatment		
		<i>Df</i>	F-value	<i>P</i>	<i>df</i>	F-value	<i>P</i>
Yes (<i>J. vulgaris</i> <i>C. vulgare</i>)	Species	1, 79	10.00	*	1, 79	5.25	*
	Soil	1, 79	190.26	**	1, 79	191.88	***
	Hormone	1, 79	2.87	ns	1, 79	21.04	***
	Species × Soil	1, 79	11.17	**	1, 79	12.57	**
	Species × Hormone	1, 79	0.35	ns	1, 79	0.08	ns
	Soil × Hormone	1, 79	8.20	**	1, 79	8.49	**
	SP × S × H	1, 79	0.05	ns	1, 79	0.00	ns
No (<i>T. repens</i> <i>D. carota</i>)	Species	1, 79	7.56	**	1, 79	5.74	*
	Soil	1, 79	0.92	ns	1, 79	1.97	ns
	Hormone	1, 79	0.35	ns	1, 79	32.94	***
	Species × Soil	1, 79	0.48	ns	1, 79	0.36	ns
	Species × Hormone	1, 79	1.10	ns	1, 79	1.86	ns
	Soil × Hormone	1, 79	1.21	ns	1, 79	1.58	ns
	SP × S × H	1, 79	0.08	ns	1, 79	1.44	ns

* *P* < 0.05,

** *P* < 0.01,

*** *P* < 0.001, ns not significant.

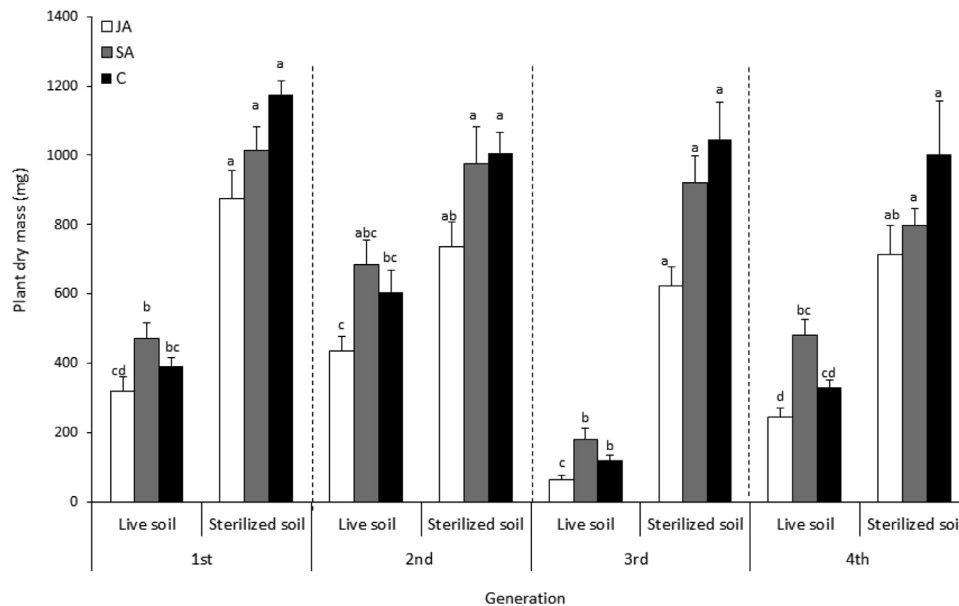


Fig. 3. Mean (+ SE) plant dry mass of *J. vulgaris* during four successive generations treated with JA and SA in sterilized soil and live soil. C represents control. For each generation soil from the previous generation and originating from the same treatment was used as an inoculum. Within each generation, different letters above bars indicate significant differences between groups based on a Tukey post-hoc test.

Does the negative effect of live soil change in four successive generations of *J. vulgaris* for control plants and plants treated with SA or JA?

The effect of the live soil across generations

As in generation 1, in all three subsequent generations plants grew less well in live soil than in sterilized soil. Although the strength of this effect varied among generations there was no clear trend across subsequent generations (Fig. 3, Table 3).

The effect of foliar application of SA across generations

Again as in generation 1, in all three subsequent generations foliar application of SA reduced plant biomass in sterilized soil and increased plant biomass in live soils (Fig. 3, Table 4). Although within generations and soils these differences were not significant, plants responded clearly differently to the SA treatment in the two soils as is reflected by the significant soil \times hormone interaction term in the ANOVA (Table 4). The effect of foliar SA application did not differ among generations as was reflected by the non-significant interaction term in the ANOVA (Table 4). To examine if the effect of hormone application in live and sterilized soil showed a trend over generations in more detail, we regressed the ratio between the dry mass of SA-treated and control plants in both sterilized and live soils against generations (Fig. 4). This ratio was higher than 1 for all

generations in live soils while it was close to 1 in sterilized soils. This difference between the two soils was significant (ANCOVA $df = (1, 7)$, $F = 20.18$, $P < 0.01$, Fig. 4A). The slopes of the regressions for both sterilized and live soils did not significantly differ from 0 (for sterilized soil the lower and upper 95% CIs are -0.15 and 0.19 ; for live soil the lower and upper 95% CIs are -0.13 and 0.33). The latter results indicate that there is no significant trend in the effect of foliar application of SA over generations.

The effect of foliar application of JA across generations

As in generation 1, in all three subsequent generations foliar application of JA reduced plant biomass in both sterilized and live soil (Fig. 3, Table 4). This reduction was less strong in live soil, as is reflected by the significant soil \times hormone interaction term in the ANOVA (Table 4). The effect of foliar JA application did not differ among generations as was reflected by the non-significant interaction term in the ANOVA (Table 4). To examine if the effect of JA application in live and sterilized soils showed a trend over generations in more detail, we regressed the ratio between the dry mass of JA-treated and control plants in both sterilized and live soils against generations (Fig. 4B). This ratio was lower than 1 for all generations in both live soils and sterilized soils. The ratios did not differ between the two soils (ANCOVA $df = (1, 7)$, $F = 0.01$, $P > 0.05$, Fig. 4B). The latter result is somewhat surprising given the significant interaction we found between the effects of JA application and soil type in Table 4. The slopes of the regressions for both

Table 3. Three-way ANOVA of log-transformed plant dry mass of *J. vulgaris* during four generations in live and sterilized soils after JA, SA treatments and control. *df* = degrees of freedom.

Source of variation	<i>df</i>	F-value	<i>P</i>
Soil	1, 250	569.88	***
Hormone	2, 250	39.83	***
Generation	3, 250	68.36	***
Soil × Hormone	2, 250	8.17	***
Soil × Generation	3, 250	57.96	***
Hormone × Generation	6, 250	1.88	ns
<i>S × H × G</i>	6, 250	0.68	ns

*** *P* < 0.001, ns not significant.

sterilized and live soils did not significantly differ from 0 (for sterilized soil the 95% CFI is −0.31 to 0.21; for live soil the 95% CFI is −1.8 to 0.24). The latter results indicate that there is no significant trend in the effect of foliar application of JA over generations.

The shoot-root ratio of *J. vulgaris* differed among generations and was affected by soil and hormone treatments (See Appendix A: Table S2). While the effects of hormone application on the shoot-root ratio did not vary among generations, the effects of the hormone treatments differed among soils. In general, in both sterilized and live soils, application of JA increased shoot-root ratios relative to the control and the SA treatments except for the third generation in live soil (See Appendix A: Fig. S2). Application of SA did not affect

Table 4. Three-way ANOVAs of plant dry mass of *J. vulgaris* during four generations in live and sterilized soils with soil (live and sterilized soils), generation, hormone (control and SA, or control and JA) as fixed factors. *df* = degrees of freedom.

Source of variation	SA treatment			JA treatment		
	<i>df</i>	F-value	<i>P</i>	<i>df</i>	F-value	<i>P</i>
Soil	1, 164	241.79	***	1, 170	307.05	***
Hormone	1, 164	0.28	ns	1, 170	39.11	***
Generation	3, 164	8.98	***	3, 170	11.07	***
Soil × Hormone	1, 164	8.75	**	1, 170	11.36	**
Soil × Generation	3, 164	7.98	***	3, 170	7.12	***
Hormone × Generation	3, 164	0.17	ns	3, 170	0.14	ns
<i>S × H × G</i>	3, 164	0.50	ns	3, 170	0.70	ns

** *P* < 0.01,.

*** *P* < 0.001, ns not significant.

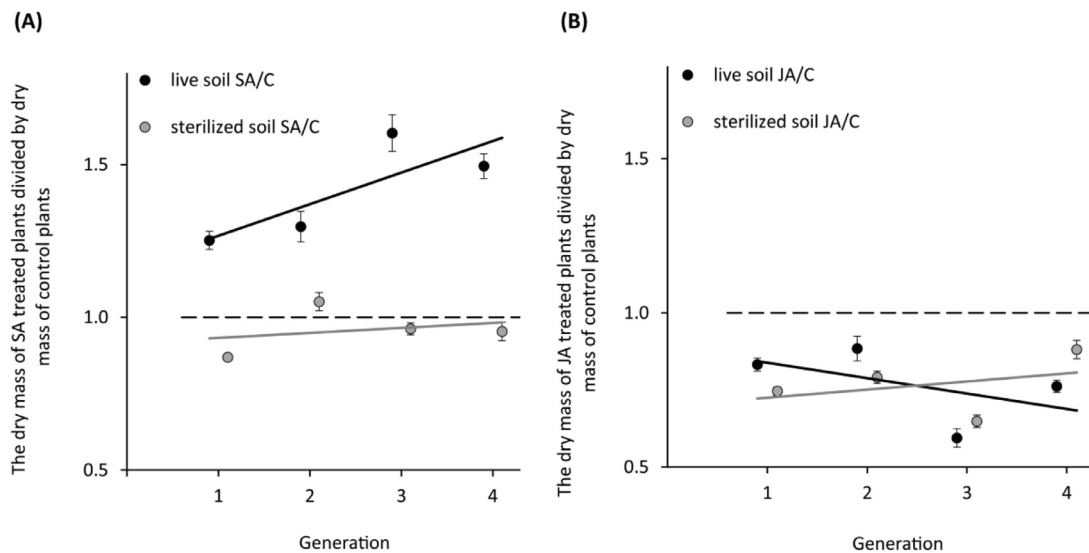


Fig. 4. The ratio of dry mass of hormone-treated *J. vulgaris* plants divided by control plants in both sterilized and live soil for four generations. (A) SA-treated plants (B) JA-treated plants. C represents control. The dashed line indicates *y* = 1. The data points are the average of 1000 ratios of dry mass of SA or JA and dry mass of control for each generation, the error bars represent the 95% confidence intervals of 1000 ratios for each generation (see material and methods for details).

the shoot-root ratio across generations in the sterilized and live soils.

Discussion

In this study, we examined how exogenous application of the plant signaling hormones SA and JA interacts with the effects of inoculation of soil on plant biomass of different plant species and how those effects altered plant performance during multiple consecutive generations. In two of the four species, plant biomass was lower in live soil than in sterilized soil. We found that foliar application of SA mitigated the negative effects of the live soil on plant performance for these species. We then grew *J. vulgaris* for three additional generations and found that SA application mitigated the negative effect of live soil on plant biomass in all four generations, and that this overall effect was significant.

The response of plant species to live soil is species-specific

Our results show that the effect of live soil on plant biomass strongly varied among plant species, although all plants received the same soil inoculum containing a natural soil microbial community and the growth conditions were identical for all four species. Species-specific effects have also been found in other plant-soil feedback experiments, which showed that *J. vulgaris* and *C. vulgare* responded negatively to soil conditioning by conspecifics, while this is not clear for *T. repens* and *D. carota* (Harrison & Bardgett, 2010; Joosten, Mulder, Klinkhamer & van Veen, 2009; Klironomos, 2002; Wang et al., 2019). Together these results show that the responses of plant species to live soil are highly species-specific. Other studies have suggested that net positive or negative plant-soil feedback effects are related to the capacity of plants to cope with biotic or abiotic stresses, to influence soil nutrients, or to the way they impact soil microbial communities (Bezemer et al., 2006; De Long et al., 2019; Eisenhauer et al., 2011; Van der Heijden et al., 2008). The soil microbial community present in live soil might have pathogenic effects on plant growth. This is in line with previous studies that indicate that soil sterilization enhanced plant biomass by killing soil-borne pathogens in crops (Li et al., 2019).

The response of plant species to SA application is species-specific

The effect of SA application also varied among plant species. Interestingly, a positive effect of SA on plant biomass in live soils occurred in *J. vulgaris* and *C. vulgare*, *J. vulgaris* and *C. vulgare* responded negatively to exposure to the

live soil. These results strongly suggest that the negative impact of the live soil on plant biomass is driven by pathogens. *T. repens* and *D. carota* were unresponsive to the soil with a live soil inoculum. The difference in response between the four species can have different non-exclusive causes. The pathogenic effect of the live soils itself may differ among plant species due to specificity of the soil microbial species in the live soil inoculum, or due to inherent plant characteristics. We started with our hormone application when seedlings were 14 days old. In retrospect, we should have started earlier. The negative effects of the live soil on plant biomass are most apparent during the first few weeks of plant growth (Bezemer, Jing, Bakx-Schotman & Bijleveld, 2018). If we would have applied the exogenous SA earlier, effects may therefore have been stronger because plants in our study may have outgrown the negative effects of the live soil e.g. by upregulating their defense system (Métraux, 2001; Netherway, Bengtsson, Krab, & Bahram, 2020; Vernooij et al., 1994). In addition, in this paper, we used only one concentration of the phytohormones. Plant species may have a different sensitivity to the foliar application of these hormones, and the species that did not show a response may have responded to higher concentrations. It is important to note that, in this paper, we performed experiments with two phytohormones. Other plant hormones like auxins and cytokinins have been reported to play a role in fighting off the potentially pathogenic bacteria in the live soil via changing physiological and morphological features of plants (Clarke, Burritt, Jameson & Guy, 2000; Hamill, 1993). They may interact with JA or SA signaling pathways, however, this is still not fully understood. Applying combinations of different phytohormones would present a next logical step. To find a clear effect of SA and JA on plant biomass against the pathogenic effect caused by live soils is the base for carrying out more extensive experiments. For example, in further tests, different plant hormones and their crosstalk effects could be tested.

Foliar application of SA mitigates the negative live soil effect on *J. vulgaris*

Importantly, application of SA mitigated the negative effects of the live soil on the growth of *J. vulgaris* in all four generations. Sterilization of the soil resulted in higher plant biomass, indicating an overall pathogenic effect due to soil-borne pathogens, and SA-induced resistance may help to mitigate this pathogenic effect caused by soil pathogens. Activation of SA-dependent signaling pathways lead to the expression of pathogenesis-related proteins (PRP) contributing to resistance, by limiting pathogen growth, the access of pathogens to water and nutrients in the plant, or by changing the composition of the cell wall of the plant (Glazebrook, 2005; Heil & Bostock, 2002; O'Donnell, Jones, Antoine, Ciardi & Klee, 2001; Spoel, Johnson & Dong,

2007). All this can result in higher plant mass in SA-treated plants than in control plants in live soil. In addition, activation of the SA pathway regulates a myriad of enzymes, for example, peroxidase (POD), polyphenol oxidase (PPO), superoxide dismutase (SOD). Those enzymes play an important role in plant SA-induced defense against biotic stresses caused by pathogens (Achuo, Audenaert, Meziane & Höfte, 2004; Bakker, Chaparro, Manter & Vivanco, 2015; War, Paulraj, War & Ignacimuthu, 2011). The effects of SA in the first generation were similar to those observed in the second or later generations indicating that SA application did not select for less pathogenic or more beneficial soil microbial communities over time. The soil microbial community present in live soil might have pathogenic effects on plant biomass and this is related to the accumulation of pathogens across generations (Li, Ding, Zhang & Wang, 2014), however, we did not find a significant decrease in biomass over generations. In part, this may be an artefact of the experimental set-up. For each generation, we used an inoculum, which means that we placed a subset of the microbial community in a sterile background. This may have led to selection for microbes with characteristics in each of the four generations. However, we urge not to over-emphasize the conclusion that application of SA results in a change in the effects of live soils on plant biomass over generations. Future studies should also include a comparison between the growth of SA-treated plants and control plants grown in soils that are conditioned by either SA-treated plants and control plants in a full factorial design.

Foliar application of hormones affected plants

JA-induced defenses are activated in response to herbivore attack, the infection of necrotrophic organisms or nematodes (Carvalhais et al., 2017; Nahar et al., 2011; Palmer et al., 2017; Pieterse et al., 2009; Van Dam & Oomen, 2008). In our study, the foliar JA application led to a significant negative effect on plant biomass. This exemplifies that hormonal signaling is costly for plants (Baldwin, 1998; Vos, Pieterse & van Wees, 2013).

So far, the positive role of activation of the SA pathway in plant species against different microbial pathogens has been reported in several species. For example, Achuo et al. (2004), showed that activation of the SA-signaling pathway is effective against *Oidium neolycopersici* in tomato which causes powdery mildew disease, and against *Botrytis cinerea* in tobacco which causes grey mould disease. Moreover, SA-induced resistance can help plants against herbivory by e.g. aphids, slugs and snails (Kästner et al., 2014). In this study, we show that activation of SA to plant leaves significantly increased plant biomass. This highlights the potential role of using hormonal application to control microbial pathogens or herbivores in agriculture to improve crop production.

Conclusions

Overall, our study suggests that negative effects in live soil on plant biomass can be mitigated with foliar applications of SA. Sterilization benefited plant biomass for two of the four species we investigated, suggesting the microbial community in live soils contains pathogens. For *J. vulgaris*, the plant species that responded most strongly to SA application, we did not observe an increasingly stronger effect on plant biomass over further plant generations, but instead, the effect was stable over time. To better understand what caused the positive effect of SA application on plant biomass in live soil, the changes in the diversity and functional role of the soil microbial community in live soil should be examined in future studies.

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Declaration of Competing Interest

None

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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.2021.07.011](https://doi.org/10.1016/j.baae.2021.07.011).

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