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Embracing the complexity of plant-microbe-insect interactions under a changing climate for sustainable agriculture

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Abstract:	Using beneficial soil bacteria to promote plant growth and reduce pests is a promising direction for sustainable agriculture. However, we need to understand the ecological basis of these interactions in order to identify those with the greatest potential to have an impact in the field. To do this, we need to embrace the complexity of multifactorial experiments to observe the strength of the benefits across variable environments. I briefly review the recent literature on plant-microbe-insect interactions across changing environments, focusing on those using multiple factors. I finish by exploring ecological research approaches and multifactorial experimental designs that can be used to simplify the study of plant-microbe-insect interactions.
Author Comments:	

Embracing the complexity of plant-microbe-insect interactions under a changing climate for sustainable agriculture

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Abstract

Using beneficial soil bacteria to promote plant growth and reduce pests is a promising direction for sustainable agriculture. However, we need to understand the ecological basis of these interactions in order to identify those with the greatest potential to have an impact in the field. To do this, we need to embrace the complexity of multifactorial experiments to observe the strength of benefits across variable environments. I briefly review the recent literature on plant-microbe-insect interactions across changing environments, focusing on those using multiple factors. I finish by exploring ecological research approaches and multifactorial experimental designs that can be used to simplify the study of plant-microbe-insect interactions.

Introduction

Plant-growth-promoting bacteria (PGPB) associated with plant roots have been well-studied for their ability to improve plant nutrition, bioavailability of insoluble minerals, and root system architecture [1-3]. These root-associated microbes can also benefit the plants through enhanced resistance to insect pests in some cases while simultaneously promoting plant growth [4,5], leading to all-round better plant health (termed biocontrol-PGPB [6]). While the potential of using such microbes in sustainable agriculture (as augmentative biological control agents [6,7]) is readily acknowledged in recent reviews [for example, 8,9-11] we lack empirical research on the mechanisms of effect that separate plant growth effects from those of biological control [6]. Additionally, studies often focus on pairwise interactions without considering how they might be altered by higher-order interactions, e.g. with the changing abiotic environment [reviewed by 12,13,14]. A microbe that provides beneficial services under all environments with limited change to the strength of effect would be most suited for commercialisation [15]. Despite many studies still inferring ecosystem effects based on extrapolation from multiple pairwise effect studies [for a methodological review of this approach see 16], there is a growing number of studies that consider the effect of multiple factors on plant growth and pest resistance. One advantage of multifactorial experiments is the ability to examine indirect effects including those that occur through interaction modifications where the effect of one species on another depends on another factor (Fig. 1) [17]. In plant-microbe-insect systems, the changing climate can have direct effects on the individual species but can also alter how these interact with one another, e.g. by changing how the plant responds to a microbe or to insect attack. A further example is if earthworms increase the abundance of beneficial rhizobacteria as well as nutrient availability around the plant roots, these can both increase plant resistance and tolerance to insect attack but via different interactions (i.e rhizobacteria-plant and plant-insect) (Fig. 1). Whether or not these different effects work independently (additive) or interactively (multiplicative) can be determined by using a factorial experimental design.

I briefly review the state of knowledge of this area, with particular focus on studies from the previous two years that use multiple experimental factors to determine the effect of rhizobacteria on reducing insect pests. The primary variables of interest in these studies are plant growth/yield, the population growth (suppression) of pest insects and recruitment of natural enemies, while the main experimental factors include inoculation by rhizobacteria species/strain, plant species/variety, and climate (Fig. 1). I will also focus on interactions involving rhizobacteria, however mycorrhizal or endophytic fungi can also benefit plant resistance to insects [recently reviewed by 18,19].

Plant-microbe-insect interactions across a changing climate

The effect of rhizobacteria on leaf-feeding insects occurs indirectly via the plant (Fig. 1) by inducing plant defences via the jasmonic acid (JA) or salicylate acid (SA) pathways, or altering plant volatiles that attract natural enemies of the pest insects, e.g. parasitoid wasps. Pulido, et al. [20] used volatile assays to show that parasitoid wasps were more attracted to plants inoculated by two rhizobacteria (*Bradyrhizobium japonicum* and *Delftia acidovorans*), and that these bacteria could restore suppression of these volatiles induced by a plant virus. del Rosario Cappellari, et al. [21] showed that *Bacillus amyloliquefaciens* GB03 and *Pseudomonas putida* SJ04 both increased shoot volatile and phenolic compounds on peppermint plants (*Mentha piperita*), as well as JA and SA phytohormones, to similar levels as induced by feeding caterpillars. This can prime the plant against future caterpillar attack, reducing the time-lag between herbivore arrival and plant defence activation or predator recruitment. Another study also found that *Bacillus amyloliquefaciens* GB03 altered plant volatiles that attracted predatory earwigs (*Doru luteipes*), with a synergistic effect potentially benefiting pest control [22]. However, in these studies the link back to insect population growth was only inferred but not directly tested (although it is an expectation based on ecological knowledge). A negative effect on caterpillar growth was shown in a study on *Arabidopsis*, where differential induction of JA/SA genes by two *Pseudomonas* spp. rhizobacteria strains reduced caterpillar growth [23]. *Pseudomonas* strain CH267 induced greater JA-dependent defences (stronger defence against chewing insects, such as caterpillars) while WCS417 disrupted the JA/SA antagonism priming plants in both pathways (defence against chewing and sucking insects, such as aphids, as well as for pathogens). For sap-feeding insects, combinations of rhizobacteria reduced aphid population growth on wheat (*Bacillus* sp. and *Pseudomonas* sp.; Naeem, et al. [5]) and canola (a commercial product, Roshdafza by Biorun Iran, containing *Pseudomonas fluorescens*, *Azotobacter chroococcum*; and *Azospirillum brasilense*; Nasab, et al. [24]) and *Pseudomonas* spp. were found to reduce whitefly survival on tomato [25].

The most studied climate factors in the recent literature on plant-microbe-insect interactions has been elevated carbon dioxide (eCO_2) or elevated ground-level ozone (eO_3) level. Higher CO_2 is generally associated with increased absolute plant growth, but it also reduces plant nutrition, alters plant physiology, and alters the growth rate of insects and pathogens [26-28]. Ground-level ozone is a known stressor that reduces plant growth and increases plant susceptibility to pests and disease [29]. While many recent studies either consider the effect of elevated CO_2/O_3 on modifying soil microbial communities [30-32] or on altering plant-insect interactions [33-40], a number of studies considered all these factors together. For example, Zytynska, et al. [4] found that inoculating barley with *Acidovorax radicis* N35 increased plant growth while simultaneously reducing aphid densities, with greatest effect in a high-stress eO_3 environment, but reduced effects under eCO_2. This is interesting since the benefits were stronger when the plant 'needed help' under stress of eO_3, but not under eCO_2 when the plants were potentially able to make better use of other resources. Aphids and other sap-feeding insects also appear to be able to compensate for reduced plant nutrition under eCO_2, through increased ingestion of phloem or manipulation of local nutrient provisioning [4,33,35,41,42]. Another multifactorial experiment by Li, et al. [43] found that both eCO_2 and rhizobacteria inoculation (*Azospirillum brasilense* and *Azotobacter chroococcum*) reduced caterpillar (*Mythimna separata*) fitness on maize, with fitness effects on adult longevity and fecundity induced by the rhizobacteria only observed under eCO_2. These results suggest that biocontrol effects of rhizobacteria could be increased in the future climate for chewing-insects but decreased for sap-feeding insects. However, we need more multifactorial experiments assessing these effects over multiple plant, insect and rhizobacteria species before any generalisations or predictions can be made.

As an alternative to inoculating individual bacterial strains, Pineda, et al. [44] showed that conditioning soil with certain grass or forb species created soil microbiomes that were able to reduce the growth rate of thrips, but not spider mites, on chrysanthemum. While host-plant species or genotype is a known strong factor determining rhizobacterial community, other soil dwelling species such as earthworms can also have significant effects on microbial community composition [recently reviewed by 45]. These interactions can also influence the ability of the microbiome to suppress pests, for example Zytynska, et al. [4] found that while earthworms increased aphid densities, the inoculated rhizobacteria *A. radicis* N35 was able to limit this increase in an ambient and eCO_2 environment. When investigating the impact of earthworms on rhizosphere activity in sugarcane, Braga, et al. [46] identified 70 microbial functions that differed due to earthworm treatment. Under earthworm presence, plants recruited more rhizobacteria that were able to use the 'earthworm-induced' N_2O, to the benefit of the plant. Metatranscriptomic analysis of agricultural soils with a history of pesticides and heavy metals identified high abundance of stress-related genes in the bacterial community [47], and is a promising method for further investigation into functional traits underlying plant-microbe-insect interactions.

Research approaches and multifactorial experimental design

I will now explore some ways in which we can approach the study of multispecies interactions and how we can maximise the outputs without running unnecessarily extravagant experiments. While the focus is on plant-microbe-insect interactions across a changing abiotic environment, much of what is discussed can be transferred into other areas. Working with diverse species requires effective time management as well as interdisciplinary technical skills, including preparation of sufficient microbial inoculant, growing healthy plants, and maintaining stock insect populations at correct densities; keeping them all alive with no cross-contamination for the duration of the experiment can also be a rather daunting prospect. With additional replication required across climate treatments, these experiments can often be viewed as formidable, ambitious, and too complex to provide reliable results. I argue that such complex experiments are needed to produce invaluable information on the ecological consequences of these interactions, but this value will be enhanced through smaller follow-up experiments that focus on understanding the underlying mechanisms.

The natural world is complex, but there are many approaches we can use to understand it. Traditionally, ecological studies can be classed into two major types: (1) observational studies that take place in the field and rely on detecting associations between variables that fit reasonable expectations, and (2) experimental studies that are designed to control most variables apart from those of interest in the hypothesis. A combination of these approaches is often followed in ecology, with observational results leading to hypothesis-driven experiments, or in the other direction hypothesis-driven experiments being transferred to the field [48]. The more controlled an experiment, the more we can infer the mechanisms driving the effects, but this simplifies the system and ignores potential interactions that would occur in a natural system. We can increase the number of factors to understand effects across variable environments making experimental systems somewhat more realistic by simulating ecosystems (albeit still simplified) [16,49,50]. However, with every included factor the size and cost of the experiment increases. An additional 2-level factor doubles the number of experimental units, while adding abiotic factors such as temperature or carbon dioxide requires extra infrastructure.

The design of optimal multifactorial experiments is a long-standing discussion [51], particularly in industrial research where high-throughput testing of many factors is needed. Many ecological experiments aim to use a fully-factorial design, meaning that every possible combination of treatment factors is included. This is a very powerful approach for statistical analysis of higher-order interactions, but this design can quickly lead to very large experiments. Incomplete factorial designs can be used where some treatment combinations are ignored and can help to reduce experimental sizes if the consequences are considered beforehand. For example, in plant-microbe-insect systems, hypotheses focused on identifying effects on insect growth could omit control plants without insects if plant effects are already established. To ensure the microbial inoculation is doing what is expected, a limited number of ‘no insect’ replicates could be used (i.e. as technical controls). Such approaches reduce replicate numbers, and therefore space and cost of the experiment, while still providing sufficient information to test the focal hypotheses. While all experiments should be designed based on a hypothesis, following an incomplete design approach requires clearly defined hypotheses from the very beginning since omitting important treatment combinations will limit the interpretation of the results. Unequal allocation of replicates to different treatment combinations is also possible without sacrificing statistical power [52]. Many clinical designs aim to minimise the number of treated human or animal patients but maximise the number of control patients to boost statistical power [53]. Similar approaches could be used when deciding on replicate number for ecological experiments; however, knowledge of expected variation and power is needed to optimise this.

Running a set of smaller experiments to test multiple hypotheses at the same time can also maximise cost and space efficiency, especially when they share control treatments. This can be especially helpful for experiments that include different levels of experimental units, e.g. changing the environment at a chamber level (eCO_2, eO_3, temperature) and at the pot level (water availability, nutrients, microbial inoculation, insect/plant genotype). The chamber level replication will be set based on the infrastructure, with often too few replicated chambers available, while the pot level replication will be based on the space inside the chambers. While the number of chambers is difficult to change it is possible to optimise the space and avoid pseudoreplication or chamber effects by ‘chamber swapping’ where plants are circulated among the available chambers and environment adjusted [for an analysis of this approach see 50]. Within a climate chamber, use of a complete randomised block design will reduce confounding factors that might occur due to pot location and inherently provides a matched pairs design for analysis. Use of matched pairs analyses can help to account for variation across blocked replicates, by calculating the relative effect between treated vs untreated pots and analysing this over the other multiple factors. We used such an approach in Zytynska, et al. [4]

reducing a 5-way interaction model (bacteria, plant genotype, earthworms, eCO2, eO3) to a slightly more interpretable 4-way interaction focused on how the other factors altered the effect of the bacteria on the aphid. A combination of reducing unnecessary treatment combinations, optimising the allocation of replicates, and chamber swapping can all be combined to produce data with good statistical power on which to test the proposed hypotheses.

Another approach for larger designs is to stagger the experimental set-up over temporal blocks [54]. For example, a set of three (minimum) replications of each individual treatment combination can be run within each temporal block, producing a triplicate of data for each block run. The inherent replication within and across temporal blocks benefits analysis of the consistency of the interaction effects, just like replicating the experiment multiple times. Another advantage is that the data can be analysed after each temporal block, and a post-hoc power analysis used to determine the ideal number of replicates needed; although, an a priori power analysis should be performed to inform on the likely optimal number of replicates. Additional advantages include the ability to include further replicates if one block fails, moving away from the ‘all-or-nothing’ of a single time-point experiment - useful when relying on multiple species to be ‘experimentally-ready’ at a given time. Such an approach is also beneficial when space is limited due to using controlled climate chambers, and replication across chambers is required [54].

Conclusion

The study of plant-microbe-insect interactions is promising for fundamental understanding of the ecology, but also for the applied potential in agriculture. Building on the strength of knowledge from pairwise interaction experiments, we now need to increase the complexity to understand how these interactions behave in a more diverse environment. This is important to transfer the knowledge to the field, where there is an already established soil microbiome, soil macrofauna community, and environmental conditions are highly changeable. Ideally, we will identify microbes that are beneficial across multiple crops and environments, and that persist in field soils. Additionally, the more we study these interactions, the more we uncover the molecular mechanisms involved in pest suppression and these can be used for breeding new plant varieties. Finally, to achieve the great potential for this area we must be ambitious and brave by embracing the complexity of ecological systems into our experimental work.

Acknowledgements

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as

*** of special interest**

**** of outstanding interest**

[1] Trivedi P, Leach JE, Tringe SG, Sa T, Singh BK: **Plant–microbiome interactions: from community assembly to plant health**. *Nature Reviews Microbiology* 2020.

[2] Backer R, Rokem JS, Ilangumaran G, Lamont J, Praslickova D, Ricci E, Subramanian S, Smith DL: **Plant growth-promoting rhizobacteria: context, mechanisms of action, and roadmap to commercialization of biostimulants for sustainable agriculture**. *Frontiers in plant science* 2018, **9**.

[3] Etesami H, Adl SM: **Plant growth-promoting rhizobacteria (PGPR) and their action mechanisms in availability of nutrients to plants**. In *Phyto-Microbiome in Stress Regulation*. Edited by: Springer; 2020:147-203.

[4] Zytynska SE, Eicher M, Rothballer M, Weisser WW: **Microbial-Mediated Plant Growth Promotion and Pest Suppression Varies Under Climate Change**. *Frontiers in Plant Science* 2020, **11**:1385.

**** Multifactorial study showing that the rhizobacterium *Acidovorax radicis* N35 simutaneously promotes plant growth and suppresses aphid population across barley cultivars, with/without earthworms, and across elevated CO_2 and O_3 environments. Stronger pest suppression was found under ambient and O_3 environments, and the rhizobacteria could mitigate some of the earthworm-mediated increase in aphid densities.**

[5] Naeem M, Aslam Z, Khaliq A, Ahmed JN, Nawaz A, Hussain M: **Plant growth promoting rhizobacteria reduce aphid population and enhance the productivity of bread wheat**. *Brazilian Journal of Microbiology* 2018, **49**:9-14.

**** Empirial experimental study showing variability across *Bacillus* sp. and *Pseudomonas* sp. strains for growth-promotion effects on straw and grain yield of wheat and pest suppression of aphids, with a combination providing stronger pest suppression.**

[6] Heimpel GE, Mills NJ: *Biological Control*: Cambridge University Press; 2017.

[7] Stenberg JA, Sundh I, Becher PG, Björkman C, Dubey M, Egan PA, Friberg H, Gil JF, Jensen DF, Jonsson M, et al.: **When is it biological control? A framework of definitions, mechanisms, and classifications**. *Journal of Pest Science* 2021.

[8] Gadhave KR, Gange AC: **Interactions Involving Rhizobacteria and Foliar-Feeding Insects**. In *Aboveground–Belowground Community Ecology*. Edited by: Springer; 2018:117-133.

[9] Noman A, Aqeel M, Qasim M, Haider I, Lou YG: **Plant-insect-microbe interaction: A love triangle between enemies in ecosystem**. *Science of the Total Environment* 2020, **699**.

[10] Bach EM, Ramirez KS, Fraser TD, Wall DH: **Soil Biodiversity Integrates Solutions for a Sustainable Future**. *Sustainability* 2020, **12**.

[11] Neher DA, Barbercheck ME: **Soil Microarthropods and Soil Health: Intersection of Decomposition and Pest Suppression in Agroecosystems**. *Insects* 2019, **10**.

[12] Giron D, Dubreuil G, Bennett A, Dedeine F, Dicke M, Dyer LA, Erb M, Harris MO, Huguet E, Kaloshian I, et al.: **Promises and challenges in insect-plant interactions**. *Entomologia Experimentalis Et Applicata* 2018, **166**:319-343.

[13] Pellissier L, Rasmann S: **The functional decoupling of processes in alpine ecosystems under climate change**. *Current Opinion in Insect Science* 2018, **29**:126-132.

[14] Castex V, Beniston M, Calanca P, Fleury D, Moreau J: **Pest management under climate change: The importance of understanding tritrophic relations**. *Science of the Total Environment* 2018, **616**:397-407.

[15] Backer R, Rokem JS, Ilangumaran G, Lamont J, Praslickova D, Ricci E, Subramanian S, Smith DL: **Plant growth-promoting rhizobacteria: context, mechanisms of action, and roadmap to commercialization of biostimulants for sustainable agriculture**. *Frontiers in plant science* 2018, **9**:1473.

[16] Carrara F, Giometto A, Seymour M, Rinaldo A, Altermatt F: **Inferring species interactions in ecological communities: a comparison of methods at different levels of complexity**. *Methods in Ecology and Evolution* 2015, **6**:895-906.

[17] Wootton JT: **The nature and consequences of indirect effects in ecological communities**. *Annu Rev Ecol Syst* 1994, **25**:443-466.

[18] Mantzoukas S, Eliopoulos PA: **Endophytic Entomopathogenic Fungi: A Valuable Biological Control Tool against Plant Pests**. *Applied Sciences* 2020, **10**:360.

[19] Jaber LR, Ownley BH: **Can we use entomopathogenic fungi as endophytes for dual biological control of insect pests and plant pathogens?** *Biological Control* 2018, **116**:36-45.

[20] Pulido H, Mauck KE, De Moraes CM, Mescher MC: **Combined effects of mutualistic rhizobacteria counteract virus-induced suppression of indirect plant defences in soya bean**. *Proceedings of the Royal Society B* 2019, **286**:20190211.

**** Multi-factorial study showing that *Bradyrhizobium japonicum* and *Delftia acidovorans* rhizobacteria can alter plant volatiles that enhance plant attractiveness to parasitoid wasps on soybean. Further, that viral infection reduces these volatiles but the bacteria can restore this important fucntion for biocontrol.**

[21] del Rosario Cappellari L, Chiappero J, Palermo TB, Giordano W, Banchio E: **Impact of Soil Rhizobacteria Inoculation and Leaf-Chewing Insect Herbivory on Mentha piperita Leaf Secondary Metabolites**. *Journal of Chemical Ecology* 2020, **46**:619-630.

*** Showed that *Bacillus amyloliquefaciens* GB03 and *Pseudomonas putida* SJ04 both increased shoot volatile and phenolic compounds on peppermint plants (*Mentha piperita*), as well as JA and SA phytohormones, to similar levels as induced by feeding caterpillars.**

[22] Bell K, Naranjo-Guevara N, Santos RCd, Meadow R, Bento J: **Predatory Earwigs are Attracted by Herbivore-Induced Plant Volatiles Linked with Plant Growth-Promoting Rhizobacteria**. *Insects* 2020, **11**:271.

*** Found that *Bacillus amyloliquefaciens* GB03 altered plant volatiles that attracted predatory earwigs (*Doru luteipes*), with a synergistic effect potentially benefiting pest control on arugula/rocket (*Eruca sativa*).**

[23] Haney CH, Wiesmann CL, Shapiro LR, Melnyk RA, O'Sullivan LR, Khorasani S, Xiao L, Han J, Bush J, Carrillo JJMe: **Rhizosphere-associated Pseudomonas induce systemic resistance to herbivores at the cost of susceptibility to bacterial pathogens**. 2018, **27**:1833-1847.

[24] Nasab RS, Yali MP, Bozorg-Amirkalaee M: **Effects of humic acid and plant growth-promoting rhizobacteria (PGPR) on induced resistance of canola to Brevicoryne brassicae L**. *Bulletin of Entomological Research* 2018:1-11.

[25] Qessaoui R, Amarraque A, Lahmyed H, Ajerrar A, Mayad EH, Chebli B, Walters AS, Bouharroud R: **Inoculation of tomato plants with rhizobacteria suppresses development of whitefly Bemisia tabaci (GENNADIUS)(HEMIPTERA: ALEYRODIDAE): Agro-ecological application**. *Plos one* 2020, **15**:e0231496.

*** Identified three Pseudomonas isolates (Q172B, Q110B and Q036B) from tomato that increase adult and larval whitefly mortality on tomato, with comparison across laboratory and glasshouse experiments**

[26] Sun Y, Guo H, Ge F: **Plant–Aphid Interactions Under Elevated CO2: Some Cues from Aphid Feeding Behavior**. *Frontiers in Plant Science* 2016, **7**:502.

[27] Zhou Y, Van Leeuwen SK, Pieterse CM, Bakker PA, Van Wees SC: **Effect of atmospheric CO 2 on plant defense against leaf and root pathogens of Arabidopsis**. *European Journal of Plant Pathology* 2019, **154**:31-42.

[28] Robinson EA, Ryan GD, Newman JA: **A meta-analytical review of the effects of elevated CO2 on plant–arthropod interactions highlights the importance of interacting environmental and biological variables**. *New Phytologist* 2012, **194**:321-336.

[29] Agathokleous E, Feng Z, Oksanen E, Sicard P, Wang Q, Saitanis CJ, Araminiene V, Blande JD, Hayes F, Calatayud V, et al.: **Ozone affects plant, insect, and soil microbial communities: A threat to terrestrial ecosystems and biodiversity**. *Science Advances* 2020, **6**:eabc1176.

[30] Sayer EJ, Oliver AE, Fridley JD, Askew AP, Mills RTE, Grime JP: **Links between soil microbial communities and plant traits in a species-rich grassland under long-term climate change**. *Ecology and Evolution* 2017, **7**:855-862.

[31] Wang P, Marsh EL, Ainsworth EA, Leakey ADB, Sheflin AM, Schachtman DP: **Shifts in microbial communities in soil, rhizosphere and roots of two major crop systems under elevated CO2 and O3**. *Scientific Reports* 2017, **7**:15019.

[32] Changey F, Bagard M, Souleymane M, Lerch T: **Cascading effects of elevated ozone on wheat rhizosphere microbial communities depend on temperature and cultivar sensitivity**. *Environmental pollution* 2018, **242**:113-125.

[33] Kremer JMM, Nooten SS, Cook JM, Ryalls JMW, Barton CVM, Johnson SN: **Elevated atmospheric carbon dioxide concentrations promote ant tending of aphids**. *Journal of Animal Ecology* 2018, **87**:1475-1483.

[34] Lu CK, Qi JF, Hettenhausen C, Lei YT, Zhang JX, Zhang M, Zhang CP, Song J, Li J, Cao GY, et al.: **Elevated CO2 differentially affects tobacco and rice defense against lepidopteran larvae via the jasmonic acid signaling pathway**. *Journal of Integrative Plant Biology* 2018, **60**:412-431.

[35] Penalver-Cruz A, Garzo E, Prieto-Ruiz I, Diaz-Carro M, Winters A, Moreno A, Fereres A: **Feeding behavior, life history, and virus transmission ability of Bemisia tabaci Mediterranean species (Hemiptera: Aleyrodidae) under elevated CO2**. *Insect Science* 2020, **27**:558-570.

[36] Qian L, He SQ, Liu XW, Huang ZJ, Chen FJ, Gui FR: **Effect of elevated CO2 on the interaction between invasive thrips, Frankliniella occidentalis, and its host kidney bean, Phaseolus vulgaris**. *Pest Management Science* 2018, **74**:2773-2782.

[37] Reineke A, Selim M: **Elevated atmospheric CO2 concentrations alter grapevine (Vitis vinifera) systemic transcriptional response to European grapevine moth (Lobesia botrana) herbivory**. *Scientific Reports* 2019, **9**.

[38] Wang CY, Fei MH, Meng L, Harvey JA, Li BP: **Effects of elevated CO2 and temperature on survival and wing dimorphism of two species of rice planthoppers (Hemiptera: Delphacidae) under interaction**. *Pest Management Science* 2020, **76**:2087-2094.

[39] Zhang Q, Dai WT, Wang XH, Li JX: **Elevated CO2 concentration affects the defense of tobacco and melon against lepidopteran larvae through the jasmonic acid signaling pathway**. *Scientific Reports* 2020, **10**.

[40] Zhang YF, Dai Y, Wan GJ, Liu B, Xing GN, Chen FJ: **Effects of Elevated CO2 on Plant Chemistry, Growth, Yield of Resistant Soybean, and Feeding of a Target Lepidoptera Pest, Spodoptera litura (Lepidoptera: Noctuidae)**. *Environmental Entomology* 2018, **47**:848-856.

[41] Jakobs R, Schweiger R, Müller C: **Aphid infestation leads to plant part-specific changes in phloem sap chemistry, which may indicate niche construction**. *New Phytologist* 2019, **221**:503-514.

[42] Florencio-Ortiz V, Sellés-Marchart S, Zubcoff-Vallejo J, Jander G, Casas JL: **Changes in the free amino acid composition of Capsicum annuum (pepper) leaves in response to Myzus persicae (green peach aphid) infestation. A comparison with water stress**. *PloS one* 2018, **13**:e0198093.

[43] Li Z, Parajulee MN, Chen FJ: **Influence of elevated CO2 on development and food utilization of armyworm Mythimna separata fed on transgenic Bt maize infected by nitrogen-fixing bacteria**. *Peerj* 2018, **6**.

**** Multifactorial experimental design testing plant-microbe-insect interactions across ambient and elevated CO_2 environments. found that both eCO_2 and rhizobacteria inoculation (*Azospirillum brasilense* and *Azotobacter chroococcum*) reduced caterpillar (*Mythimna***

***separata*) fitness on maize, with fitness effects on adult longevity and fecundity induced by the rhizobacteria only observed under eCO$_2$.**

[44] Pineda A, Kaplan I, Hannula SE, Ghanem W, Bezemer TM: **Conditioning the soil microbiome through plant–soil feedbacks suppresses an aboveground insect pest**. *New Phytologist* 2020, **226**:595-608.

*** Highlight an alternative approach, by conditioning soils to be pest suppressive. Found that certain grass or forb species created soil microbiomes that were able to reduce the growth rate of thrips, but not spider mites, on chrysanthemum.**

[45] Medina-Sauza RM, Álvarez-Jiménez M, Delhal A, Reverchon F, Blouin M, Guerrero-Analco JA, Cerdán CR, Guevara R, Villain L, Barois I: **Earthworms building up soil microbiota, a review**. *Frontiers in Environmental Science* 2019, **7**:81.

[46] Braga LP, Yoshiura CA, Borges CD, Horn MA, Brown GG, Drake HL, Tsai SM: **Disentangling the influence of earthworms in sugarcane rhizosphere**. *Scientific Reports* 2016, **6**:38923.

[47] Sharma R, Sharma PK: **Metatranscriptome sequencing and analysis of agriculture soil provided significant insights about the microbial community structure and function**. *Ecological Genetics and Genomics* 2018, **6**:9-15.

[48] Clobert J, Chanzy A, Le Galliard J-F, Chabbi A, Greiveldinger L, Caquet T, Loreau M, Mougin C, Pichot C, Roy J, et al.: **How to Integrate Experimental Research Approaches in Ecological and Environmental Studies: AnaEE France as an Example**. *Frontiers in Ecology and Evolution* 2018, **6**.

[49] Levine JM, Bascompte J, Adler PB, Allesina S: **Beyond pairwise mechanisms of species coexistence in complex communities**. *Nature* 2017, **546**:56.

[50] Johnson SN, Gherlenda AN, Frew A, Ryalls JM: **The importance of testing multiple environmental factors in legume–insect research: replication, reviewers, and rebuttal**. *Frontiers in Plant Science* 2016, **7**:489.

[51] Plackett RL, Burman JP: **The design of optimum multifactorial experiments**. *Biometrika* 1946, **33**:305-325.

[52] Liu X: **Statistical power and optimum sample allocation ratio for treatment and control having unequal costs per unit of randomization**. *Journal of Educational and Behavioral Statistics* 2003, **28**:231-248.

[53] Rosenberger WF, Lachin JM: *Randomization in clinical trials: theory and practice*: John Wiley & Sons; 2015.

[54] Casler MD: **Blocking principles for biological experiments**. *Applied statistics in agricultural, biological, and environmental sciences* 2018:53-72.

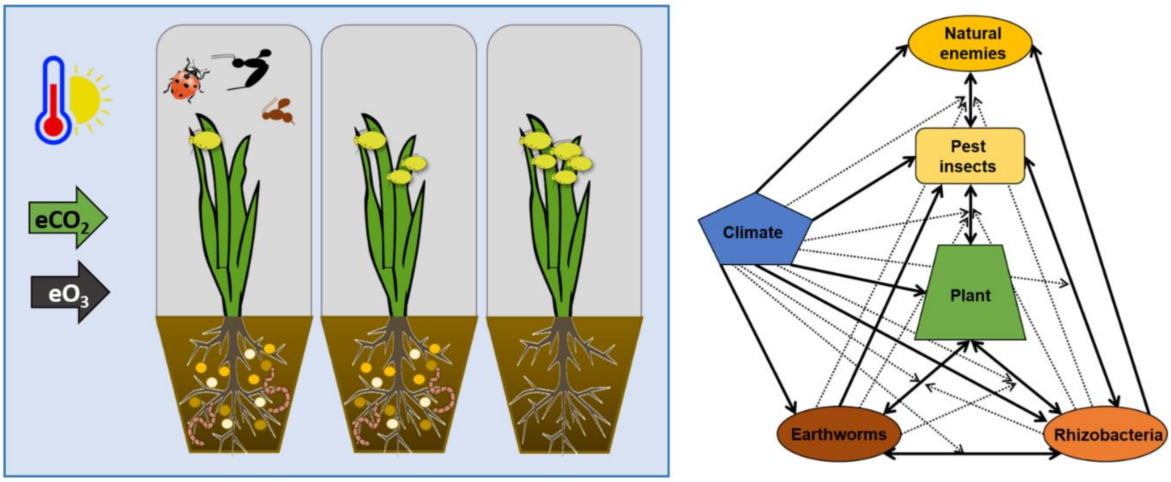

Figure 1. Unravelling plant-microbe-insect interactions across a changing environment. Many plant-microbe-insect interactions are performed in mesocosms, often covering individually-potted plants with fine-mesh or air-permeable cellophane to avoid transfer of microbes or insects to other plants. This allows for high replication of a set of treatment combinations (with pot as the experimental unit) across different climate environments (where chamber would be the level of replication). When analysed appropriately these experiments can identify direct (bold arrows) and indirect (including interaction modifications, dotted line arrows) interactions among the species involved.