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Deciphering multitrophic interactions for aphid control on okra in Cameroon

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To my parents

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Summary

It is well established that intensive agricultural measures such as pesticide application reduce environmental quality and are therefore not sustainable. With crop pests being one of the dominant causes of crop productivity loss, it is crucial to develop environment friendly pest control measures. Numerous interspecific interactions occur in agroecosystems, which in turn can significantly affect pest abundances and plant yield. Understanding multitrophic interactions in agroecosystems may help in development of more resilient pest control measures. Okra is an economically important vegetable in Cameroon, often grown by small-scale farmers in intercropped fields. Many different okra plant varieties are grown in Cameroon. The main pest species of okra include aphids, leaf beetles and whiteflies. Natural predators such as spiders and syrphids larvae are also observed okra. In addition, ants are commonly found attending the cotton aphid on okra for their honeydew and are also observed carrying okra produced pearl bodies back to their nests. Multiple interspecific interactions can occur in such diverse okra-associated invertebrate community. My thesis aims to understand these interactions and their effect on okra yield.

During my thesis field and controlled experiments were conducted in Germany and in Cameroon. The first chapter examined the role of additional crops on the okra-associated multitrophic interactions by intercropping okra with bean and maize at different plant densities (high and low). Crop identity and plant density were found to significantly affect okra pests, their predators and okra yield. Leaf beetles were the only pests that affected okra yield and their abundance reduced at high plant density. Overall, okra grown with bean at high plant density was found to be the most productive and profitable combination. Additional crop species did affect the okra-associated invertebrates therefore, to decipher the okra-specific interactions; I focused only on the monocultures of okra in the second chapter. The field experiment showed that ants did not protect aphids in the field and syrphid larvae predators significantly reduced aphid numbers. Moreover, aphids were not found to reduce okra yield. Instead, ant attraction by aphids was beneficial for okra plants as ants reduced chewing herbivore damage. In the controlled experiment *Pheidole* ants even reduced aphids, but their reduction of aphids varied across different okra varieties; this was potentially mediated by okra pearl bodies. Therefore, these ant-aphid-plant interactions were further explored in the third chapter where I used 4-5 okra varieties and tested: (a) ant preference of okra pearl bodies over aphid honeydew, (b) effect of aphid presence on pearl body production and, (c) effect of pearl body removal on pearl body production. Only ants of genus *Pheidole* were found to favour okra pearl bodies over aphid honeydew. Additionally, pearl body production was higher, and was not affected by aphids when these were artificially removed. Interestingly, aphids were found to increase plant growth. Further, pearl body production and presence of *Pheidole* ants also varied across okra varieties. Thus, plant variety did mediate the ant-aphid interactions on okra. Belowground biota may also affect these interactions. To test the effect of plant genetic variation on the aboveground-belowground interactions, a controlled experiment using an earthworm-plant-aphid system was conducted. Effect of belowground earthworms on aboveground black bean aphid was mediated by plant genetic variation.

Overall, my thesis provides evidence that species interactions vary with several factors such as plant variety, crop identity, plant density, presence of other species such as ants and belowground organisms. I suggest that farmers practicing intercropping can grow okra-bean at high densities to derive high yield and low pest numbers. Farmers growing okra in monocultures can enhance syrphid larvae abundance. Further, ant presence in okra fields should be enhanced, e.g. by growing varieties with pearl bodies favourable to ants. Finally,

more studies are needed to link aboveground-belowground interactions in agriculture as belowground biota can strongly affect plant yield. Understanding multitrophic interactions can help to suggest various pest control measures to farmers and provide vast opportunities to develop more resilient agroecosystems.

Zusammenfassung

Es ist allgemein bekannt, dass die derzeitigen intensiven landwirtschaftlichen Maßnahmen, wie die Anwendung von Pestiziden, die Umwelt schädigen und somit nicht nachhaltig sind. Da Pflanzenschädlinge eine der Hauptursachen für Einbußen beim Ernteertrags von Nutzpflanzen sind, ist es wichtig, umveltverträgliche Maßnahmen zur Schädlingsbekämpfung zu entwickeln. In Agrarökosystemen existieren zahlreiche interspezifische Interaktionen, die das Vorkommen von Schädlingen und den Pflanzenertrag signifikant beeinflussen können. Ein besseres Verständnis der multitrophischen Interaktionen in agrarökologischen Systemen kann dabei helfen, belastbarere Schädlingsbekämpfungsmaßnahmen zu entwickeln. Okra ist ein wirtschaftlich bedeutendes Gemüse in Kamerun, das oft in Mischkultur von Kleinbauern angebaut wird. In Kamerun gibt es verschiedene Okra-Sorten, welche von Schädlingen wie Blattläusen, Blattkäfern und weißen Fliegen befallen werden. Daneben werden natürliche Feinde, wie Spinnen, Schwebfliegenlarven, auf Okrapflanzen beobachtet. Außerdem befinden sich häufig Ameisen auf den Pflanzen. Sie „melken“ die Baumwoll-Blattläuse (*Aphis gossypii*), um deren Honigtau zu ernten, und tragen sogenannte, von den Okrapflanzen produzierte Perldrüsen in ihre Nester. Zwischen den vielen unterschiedlichen wirbellosen Besiedlern der Okrapflanzen finden zahlreiche verschiedene Interaktionen statt. Das Ziel meiner Arbeit ist es, diese Interaktionen und deren Einfluss auf den Okra-Ertrag zu verstehen.

Während meiner Doktorarbeit wurden Experimente im Freiland und in Gewächshäusern in Deutschland und Kamerun durchgeführt. Im ersten Kapitel untersuche ich die Rolle von zusätzlichen Feldfrüchten (Nutzpflanzenarten) durch das Anlegen einer Mischkultur aus Okra, Bohnen und Mais bei verschiedener Pflanzendichte (hoch und niedrig), auf die okra-assoziierten, multitrophischen Interaktionen. Pflanzenart und Pflanzendichte beeinflussten Okra-Schädlinge, ihre natürlichen Feinde und den Okra-Ertrag erheblich. Unter den

Schädlingen beeinflussten nur die Blattkäfer den Okra-Ertrag und ihre Abundanz verringerte sich bei hoher Pflanzendichte. Insgesamt erwies sich die Kombination aus Okra und Bohnen bei hoher Pflanzendichte als die produktivste und profitabelste Variante. Zusätzliche Pflanzenarten hatten einen Einfluss auf die Okra-assoziierten Invertebraten. Deshalb fokussiere ich im zweiten Kapitel meiner Arbeit nur auf Okra-Monokulturen. Die Freilandexperimente zeigten, dass Ameisen die Blattläuse im offenen Feld nicht beschützen und Schwebfliegen-Larven als natürliche Prädatoren die Anzahl der Blattläuse erheblich reduzierten. Ferner zeigte sich, dass Blattläuse den Okra-Ertrag im Freiland nicht verringerten, sondern dass Anlocken von Ameisen durch Blattläuse den Schaden durch kauende Pflanzenfresser (z.B. Blattkäfer) sogar reduzierte. Unter kontrollierten Bedingungen verringerten Ameisen der Gattung *Pheidole* sogar die Anzahl an Blattläusen, das Ausmaß dieser Reduktion war jedoch abhängig von der Okra-Sorte. Da diese Ameisen-Blattlaus-Pflanzen Interaktion im Zusammenhang mit den Okra-Perldrüsen stehen könnte, wird diese in meinem dritten Kapitel näher untersucht. Hierfür verwendete ich 4-5 Okra-Sorten und testete (a) die Bevorzugung von Okra-Perldrüsen gegenüber Blattlaus-Honigtau durch Ameisen (b) die Auswirkung der Blattlaus-Präsenz auf die Perldrüsen-Produktion und (c) die Auswirkung der Entfernung von Perldrüsen auf die Perldrüsen-Produktion. Nur Ameisen der Gattung *Pheidole* bevorzugten Okra Perldrüsen gegenüber Honigtau von Blattläusen. Darüber hinaus war die Perldrüsen-Produktion höher und wurde nicht durch Blattläuse beinflusst, wenn die Perldrüsen künstlich entfernt wurden. Interessanterweise haben Blattläuse das Pflanzenwachstum erhöht. Die Perldrüsen-Produktion und das Vorhandensein von Pheidole-Ameisen variierten zwischen den Okra-Sorten. Die Pflanzensorte hatte also einen Einfluss auf die Ameisen-Blattläuse-Interaktion bei Okra. Unterirdische Organismen könnten diese Wechselwirkungen ebenfalls beeinflussen. Um den Effekt von pflanzengenetischer Variation auf die ober- und unterirdischen Interaktionen zu testen, führte ich zum Abschluss ein kontrolliertes Experiment mit einem Regenwurm-Pflanze-Blattläuse-System durch. Die Wirkung von im Boden lebenden Regenwürmern auf die oberirdisch lebende Schwarze Bohnenlaus wurde von der genetischen Variation der Pflanzen beeinflusst.

Insgesamt hat meine Arbeit gezeigt, dass die Interaktionen zwischen den Arten durch verschiedenartige Faktoren beeinflusst werden, wie z.B. Sortenvielfalt, Nutzpflanzenart, Pflanzendichte, die Anwesenheit anderer Arten wie Ameisen und unterirdische Organismen. Ich empfehle deshalb, dass die Landwirte, die Mischkulturen praktizieren, eine Kombination aus Okra und Bohnen in hoher Dichte anbauen, um einen hohen Ertrag bei gleichzeitig geringer Anzahl an Schädlingen zu erzielen. Landwirte, die Okra in Monokulturen anpflanzen, erreichen dadurch eine höhere Dichte an Schwebfliegen-Larven. Das Vorkommen von Ameisen sollte in Okra-Feldern, z.B. durch den Anbau von Sorten mit von Ameisen bevorzugten Perldrüsen, gefördert werden. Weitere Studien sind erforderlich, um Wechselwirkungen zwischen ober- und unterirdischen Organismen in der Landwirtschaft genauer zu untersuchen, da unterirdische Biota den Pflanzenertrag stark beeinflussen können. Das Verständnis von multitrophischen Interaktionen kann dabei helfen, Landwirten bestimmte Schädlingsbekämpfungsmaßnahmen vorzuschlagen, und bietet darüber hinaus weitreichende Möglichkeiten zur Entwicklung robuster ökologischer Agrar-Anbausysteme.

Chapter 1:

General Introduction

Agriculture has significantly modified our environment, total area of cultivated land has increased by 466% from 1700 to 1980 (Meyer and Turner, 1992) and it is estimated that about 38% of the earths' terrestrial surface is used for agricultural activities (Foley *et al.*, 2011). The past few decades have witnessed rapid expansion in food production with an increase of over 100% in some of the staple cereal crop production (Pingali, 2012). Some of this increase has been a result of increased land under cultivation but most of the production gain has resulted from "Green Revolution" (intensive) technologies including fertilizers and pesticides, high-yielding cultivars, mechanization and irrigation (Matson *et al.*, 1997). This increase in food production has come at the cost of environment degradation and current intensive agricultural practices are one of the leading drivers of deforestation, climate change and land and water degradation (Gordon *et al.*, 2008; Power, 2010; Kissinger *et al.*, 2012). Despite the increase in food production, more than one in nine people still lack sufficient nutrients to lead a healthy life (FAO *et al.*, 2015). Over 60% of these undernourished people reside in developing countries where the majority of land is cultivated by small-scale farmers, producing most of the world's food (FAO *et al.*, 2015). With the world's population estimated to increase over 9 billion by 2050 (UN, 2015), there will be immense pressure on the existing land resources (Godfray *et al.*, 2010). In addition, more than half of the population growth between now and 2050 is projected to occur in the developing African countries (UN, 2015), resulting in an increase in demand for food and land in these countries.

Hence, to achieve global food-security there is dire need to enhance and develop sustainable agricultural practices, specifically focussing on small-scale farming.

Sustainable agriculture can be defined as “a process or system where agricultural yields are increased without adverse environment impact and without the conversion of additional non-agricultural land” (Pretty and Bharucha, 2014). Although, sustainable practices such as organic farming often produce lower overall yield than conventional intensive practices (usage of pesticides, fertilizers), the difference between the yields is contextual and depends on the system and site characteristics (Seufert *et al.*, 2012). Sustainable agriculture is known to provide many ecosystem functions such as biodiversity maintenance, regulation of soil quality and carbon sequestration (Bengtsson *et al.*, 2005; Diacono and Montemurro, 2010; Verbruggen *et al.*, 2010). Further, sustainable systems are more resilient as they promote biodiversity and ecosystem services (Ives and Carpenter, 2007; Haddad *et al.*, 2011). The term resilience broadly refers to the limit of a stability domain and can be defined by the magnitude of disturbance that a system can absorb before it changes stable states (Gunderson, 2000). Ecosystem services promoted by sustainable agriculture (e.g. pollination, pest control, nutrient cycling and soil fertility) in turn assist in enhancing sustainability of agroecosystems.

1.1 Biological control

One of the dominant constraints to crop productivity and cause of economic loss are crop pests. Total global potential yield loss due to pests is estimated to be between 30%-50% for economically important crops such as cotton, wheat and soybean (Oerke, 2006). Despite an increase in pesticide usage and production, pest control success ratio using these chemical pesticides has significantly reduced from 1:50,000 in 1995 to 1:140,000 in 2008 (Lenteren, 2011). In fact pests usually develop resistance to pesticides, resulting in even higher economic loss (Palumbi, 2001). Additionally, broad-spectrum pesticides further reduce pest control as their application decreases the diversity of natural enemy populations (Koss *et al.*, 2005; Crowder *et al.*, 2010).

Amongst the many ecosystem services which are necessary for sustainable agroecosystems, natural pest control is considered one of the most important (Rusch *et al.*, 2010). Biological control is one such approach where natural enemy populations are enhanced for pest control and pesticide usage is reduced. “Biological control is the use of living organisms to supress the population of a specific pest organism, making it less abundant or less damaging than it would otherwise be” (Eilenberg *et al.*, 2001). Biological control can further be subdivided into the following:

1.1.1 Classical biological control:

In this strategy there is an intentional introduction of an exotic natural enemy into a new environment so that it becomes established and controls pest population without further intervention. This strategy is usually used to control insect pests and weeds and the primary control agent types used have been insect parasitoids, predators (to control pests) and phytophagous insects (to control weeds) (Hajek, 2004). There have been several successful classical biological control programs such as the introduction of the leaf beetle *Chrysolina quadrigemina* (Suffrian) from Australia to control the St. John’s wort weed *Hypericum perforatum* L. in America; within ten years the introduced beetle successfully reduced the population of *H. perforatum* to less than 1% of its original size (Huffaker and Kennett, 1959).

1.1.2 Augmentative biological control:

In this method, the biological control agent is released but without the goal of permanent establishment. The aim of this strategy is to achieve rapid pest control when natural enemies are absent, when the control due to natural enemies would occur too late to prevent damage, or when natural enemy number is too low for effective pest control (Hajek, 2004). In augmentative control natural enemies are mass reared in bio factories to be released in large numbers and it is estimated that about 170 species of invertebrate natural enemies are produced and sold globally for augmentative control (Lenteren, 2011). Amongst the most successful augmentative control organisms are the hymenopteran egg parasitoids of the genus *Trichogramma*, which are mass produced around the world to control caterpillars in several crops such as cereals, soybean, sugarcane, vegetables, fruits and forest trees (Van Lenteren, 2000).

1.1.3 Conservation biological control:

In this strategy natural enemies are not released; instead, the resident populations of natural enemies are increased and conserved by modification of the environment or the existing practices. Conservation control requires a deep understanding of the biology, ecology and behaviour of pests and its natural enemies (Hajek, 2004). This method is often considered most economically feasible as there is no cost of introduction of a biocontrol agent involved. A popular example of conservation control is the control of the cotton aphid *Aphis gossypii* Glover on cotton by the indigenous entomopathogenic fungus *Neozygites fresenii* in the USA (Steinkraus *et al.*, 1995).

There are several systems where biological control measures have efficiently controlled pest populations; however, the rate of success using these measures is low. It is estimated that the rate of permanent establishment of introduced natural enemies against arthropod pests is about 25%, with complete control of pest population achieved in only 10-15% of the cases (Hill and Greathead, 2000). Unsuccessful biocontrol by natural enemies may occur due to various reasons such as: natural enemies may fail to establish in their introduced range as a result of climate mismatch, predation/parasitizaton by native fauna or lack of alternative food (Stiling, 1993). When natural enemies are reared to be introduced more emphasis is placed on their rapid and cost-effective mass production and less on how these enemies function and affect their associated environment (Lewis *et al.*, 1997). For example, the oligophagous Eurasian weevil *Rhinocyllus conicus* Fröelich was introduced in North America to control the population of exotic thistles as its larvae develops while feeding on thistle seeds. The weevil did effectively reduce exotic thistles but it also reduced dramatically the viable seed set in native thistle species (Louda *et al.*, 2003). There are numerous such examples where the introduced biocontrol agent negatively affected non-target species and even lead to changes in the community food webs (Simberloff, 2012).

Despite decades of research on biological control, it is still unclear which factors are important for maintenance and enhancement of natural pest control (Rusch *et al.*, 2010). Furthermore, biocontrol does not necessarily lead to enhanced plant production. Instead, plant production may be more affected by plant traits (Poveda *et al.*, 2008). Thus, it is evident that for effective and safe natural pest control, which in turn further enhances plant production, it is crucial to gather an overall deeper knowledge of the agricultural system.

1.2 Integrated pest management (IPM)

Integrated pest management is one such method developed by utilising a broader knowledge of the system. “IPM means the careful consideration of all available pest control techniques and subsequent integration of appropriate measures that discourage the development of pest populations and keep pesticides and other interventions to levels that are economically justified and reduce or minimize risks to human health and the environment. IPM emphasizes the growth of a healthy crop with the least possible disruption to agro-ecosystems and encourages natural pest control mechanisms” (ECP, 2010). IPM programs are tailored individually for specific pests and systems utilizing available knowledge of different pest control techniques. Pest control techniques used in IPM include biological control, host plant resistance (using crop varieties which are pest tolerant or resistant) and cultural control (modification of the environment to make it less suitable for pest invasion, for example by crop rotation or tillage practices) (Kos *et al.*, 2009). Within IPM, pesticides are used only when necessary in combination with other approaches and the chemicals are applied in a way that their impact on the environment is minimised (Koul *et al.*, 2004).

Robert van den Bosch was amongst the first entomologist propagating integrated pest management as early as in 1959 (Hajek, 2004). Successful IPM programs have been conducted since their inception. However, there are still relatively few IPM programs which utilize multiple approaches for pest management. More commonly farmer adopted IPM strategies are more simplified and only include one approach for pest management. For example, a threshold is established for pest abundance, when this threshold is reached, pesticides are applied (Brewer and Goodell, 2011). In addition, pest-resistant crop varieties are becoming an integral part of IPM programs and most of the commercially available varieties are bred for direct resistance against pests (Kos *et al.*, 2009). The majority of these varieties express genes which code for proteins that can be harmful against a broad range of non-target herbivores (Aronson and Shai, 2001; Chen *et al.*, 2008). Thus, despite the integrated aim of these programs, a system-wide approach is reduced and instead a more reductionist approach is practiced where only direct interactions are emphasized. It is essential to understand plant associated indirect multitrophic interactions and include this knowledge in the development of IPM programs.

1.3 Potential multitrophic interactions in agroecosystems

Interspecific interactions occur as direct affect between adjacent trophic levels or across multiple trophic levels as indirect effects (Shennan, 2008). Such multitrophic interactions can result in trophic cascades. In addition, these multitrophic interactions may also be affected by secondary crop species. In this review I first focus on multitrophic interactions that can arise from presence of different crop species and then I further elaborate on pest, predator and plant driven interactions.

1.3.1 Crop-diversity driven multitrophic interactions:

Agricultural systems are not just monocultures and small-scale farmers in the tropics often practice more diverse agriculture (e.g. intercropping and agroforestry), growing more than one crop/tree species together in the same piece of land (Vandermeer, 1992; Bhagwat *et al.*, 2008). These diverse farming practices can reduce pests by various mechanisms such as by increasing diversity or abundance of predators (Letourneau *et al.*, 2010; Scherber *et al.*, 2010) or by presence of crops that repel pests (Khan *et al.*, 1997; Cook *et al.*, 2007). On the contrary they may also increase pests by presence of additional crops which give refuge to pests (Room and Smith, 1975; Letourneau *et al.*, 2010) or by hindering predator host search (Ratnadass *et al.*, 2012; Kruidhof *et al.*, 2015).

Besides regulation of pest numbers, diverse farming practices can also increase plant yield by facilitative plant-plant interactions, which are positive non-trophic interactions between physiologically independent plants mediated through the abiotic environment or other organisms (Brooker *et al.*, 2008; Brooker *et al.*, 2015). However, these may also reduce plant yield if the crop species compete with one another for resources (Vandermeer, 1992). Such diverse farming practices can be highly advantageous for small-scale farmers but as mentioned above their effect on crop yield and pest control is varied. A thorough understanding of interspecific interactions, the mechanisms behind such interactions and further understanding their effects on plant yield may play a crucial role in improving these diverse farming practices.

1.3.2Predator driven multitrophic interactions:

Multiple predators occur in a system, interacting with one another and this can have varying effects on the pest and plant species. For example, in an island system Spiller and Schoener (1996) showed that even though lizards were known to reduce arthropod herbivores, their removal did not affect plant fitness. This occurred due to significant increase in the abundance of the web spider in the absence of lizards. Thus, intraguild predation by lizards of spiders was reducing the potential pest control by spiders, indirectly affecting plant fitness. Intraguild predation (IGP) are interactions between multiple predators where a predator consumes another predator with whom it shares a common prey (Polis *et al.*, 1989). In agroecosystems IGP is enhanced by an increased diversity of predators and this in turn can reduce natural enemy impacts on herbivores and dampen the effect of trophic cascades (Finke and Denno, 2004; Mooney *et al.*, 2010). For instance, intraguild predation by the large beetle *Pterostichus melanarius* Illiger of smaller beetles was found to significantly reduce the suppression of their shared prey the anthomyiid flies, negatively affecting biocontrol efficiency (Prasad and Snyder, 2006). However, this effect of intraguild predation on pests also varies with the species involved; Mooney *et al.* (2010) found IGP involving high diversity of vertebrate insectivores to strengthen trophic cascades and even enhances pest suppression (Mooney *et al.*, 2010).

Similar to IGP, intraguild parasitism occurs amongst parasitoids, due to hyperparasitoids. A hyperparasitoid attacks another insect which itself is parasitic on another host insect (Sullivan, 1987); thereby representing a highly evolved fourth trophic level. In a system, several parasitoids can occur which share a common host, leading to multiple trophic levels of hyperparasitism (Brodeur and Rosenheim, 2000). Due to their complicated interactions and biology, establishing effects of hyperparasitoids on biocontrol has been difficult. Nevertheless, recent studies have shown that hyperparasitoids can significantly reduce the numbers of primary parasiotids, which further reduces pest control (Schooler *et al.*, 2011; Gómez-Marco *et al.*, 2015). The intraguild parasitism effects on pests also cannot be generalised and are dependent on several other factors such as foraging efficiency of the primary parasitoid species involved or qualitative differences in the pests diet (Mackauer and Völkl, 1993; Harvey *et al.*, 2003; Vance-Chalcraft *et al.*, 2007). Thus, these predator driven interactions are context dependent and demonstrate that increasing natural enemy abundance and diversity does not automatically reduce pest abundance. Effects of these predator driven multitrophic interactions can further be dependent on the associated pests and crop species.

1.3.3 Pest (prey) driven multitrophic interactions:

In addition to interactions involving predators at multitrophic levels, pests on their own can also mediate these trophic interactions and in turn affect biocontrol efficiency. Factors such as prey diversity, life cycle and distribution play an important role in the degree of pest suppression by predators (Tylianakis and Romo, 2010). Pest driven interspecific interactions occur at both the aboveground and belowground level. Despite of their spatial separation, aboveground herbivorous pests can indirectly affect belowground biota through their effect on plant nutrient content (Barber *et al.*, 2012; Johnson *et al.*, 2012). These effects can be positive or negative for belowground organisms; for example, in some systems foliar herbivory was found to reduce arbuscular mycorrhizal (AM) fungal colonization by reducing plant carbon, whereas, in some systems it was found to increase AM fungal colonisation (Gehring and Bennett, 2009). Similarly, belowground soil decomposers can also have an indirect positive/negative effect on performance of aboveground herbivorous pests by improving nutritional quality of plant tissue (Bonkowski *et al.*, 2001; Newington *et al.*, 2004; Eisenhauer *et al.*, 2010), by reducing aboveground parasitoid recruitment or performance (Bezemer *et al.*, 2005; Rasmann and Turlings, 2007; Soler *et al.*, 2007), reducing shoot/leaf herbivore performance (Bezemer *et al.*, 2005; Johnson *et al.*, 2013) and by increasing/decreasing pollinator visitation (Poveda *et al.*, 2003; Barber *et al.*, 2011). Thus, aboveground-belowground interactions lead to trophic cascades which are crucial in determining pest control efficiency. Despite multiple investigations of such interactions, broad generalities or patterns have been difficult to establish (Johnson *et al.*, 2012; A'Bear *et al.*, 2014) and these are still underutilised in biocontrol programs.

In addition to driving interspecific interactions through their effect on plants, pests themselves can produce compounds to attract opportunistic species such as ants. Mutualistic interactions between ants and honeydew producing hemipterans are well-studied and it is known that ants attend these insects for their honeydew and in return protect them from their predators (Buckley, 1987; Hölldobler and Wilson, 1990). These are widespread interactions occurring in managed agricultural landscapes, grasslands and forests (Buckley, 1987) and many of the honeydew producing hemipterans such as aphids or mealybugs are serious agricultural pests. Honeydew producing hemipterans can support an entire colony of ants, which can help in maintaining large ant densities. (Davidson, 1997; Blüthgen *et al.*, 2000). As ant densities increase, the arthropod community can be significantly affected and shaped by these ant-hemipteran interactions (Styrsky and Eubanks, 2007; Zhang *et al.*, 2012). For example, Kaplan and Eubanks (2005) showed ant-aphid mutualism to significantly reduce herbivore abundance by 27-33% and predator abundance by 40-47%. Similarly, reductions in ant abundance due to aphid removal was found to increase predator and herbivore abundance on plants by ~76% (Wimp and Whitham, 2001). Such interactions have long been utilised for biocontrol of honeydew producing insects in tree crops (Way and Khoo, 1992).

The effect of ant-hemipteran mutualistic interactions on the arthropod community can be beneficial for the plant as ants reduce the more damaging insects (Styrsky and Eubanks, 2007; Layman and Lundgren, 2015). On the contrary, due to ant protection of the hemipterans, their abundance may increase and this can negatively affect plant fitness (Banks and Macaulay, 1967; Renault *et al.*, 2005). However, ant-hemipteran interactions do not always benefit the hemipterans and can vary with several factors such as hemipteran abundance or by the presence of other honeydew producing hemipterans (Rosengren and Sundström, 1991; Stadler and Dixon, 2005). Overall, these pest driven interactions involving ants are highly variable, occur widely in many systems and strongly affect community dynamics. Therefore, these interactions play a crucial role in development of pest control measures which enhance plant fitness.

1.3.4 Plant mediated multitrophic interactions:

Plants are not passive spectators to predator or prey driven interactions and can considerably mediate these interactions. Plants have evolved plethora of defences against herbivorous pests including direct and indirect defence. Plants can directly reduce pest establishment by secreting toxic, antidigestive secondary metabolite compounds (Walling, 2000; Mithöfer and Boland, 2012) or mechanically by the presence of trichomes and increased plant toughness, (Duke *et al.*, 2000; Lucas *et al.*, 2000). Plants also indirectly reduce pests through the production of volatile organic compounds (VOCs) or food rewards, which attract predators and parasitoids (O'Dowd, 1982; Walling, 2000; Heil, 2008; Mithöfer and Boland, 2012). Herbivore induced plant volatiles (HIPVs) are produced after the plant is attacked by a herbivorous pest; HIPVs assist natural enemies in finding their prey and can be detected by a wide range of taxa from insectivorous birds to parasitic nematodes (Dicke and Baldwin, 2010). With their effect on a wide range of taxa HIPVs have also been observed to attract other herbivorous arthropods which reduce plant fitness (Halitschke *et al.*, 2008), interfere with the attraction of beneficial pollinators by attracting bodyguards (Kessler and Halitschke, 2007; Bruinsma *et al.*, 2008) and even reduce natural enemies such as parasitoids by attracting hyperparasitoids (Poelman *et al.*, 2012). Thus, plant produced HIPVs have significant effects on its associated arthropod community.

Food rewards such as extrafloral nectar (EFN) or pearl bodies (food bodies) produced by plants also attract beneficial species such as ants. Ant-plant mutualisms are extensively studied and in many systems ants protect plants from herbivorous pests in return of food rewards (Rosumek *et al.*, 2009; Mayer *et al.*, 2014). These mutualistic interactions have been shown to significantly reduce herbivory and alter arthropod community dynamics (Rosumek *et al.*, 2009), similar to HIPVs. However, as mentioned in the section above honeydew producing hemipteran pests also attract ants and in return ants protect them from their predators. Therefore, ant protection of plants in return of food rewards cannot be generalised and can vary with ant behaviour towards other herbivores. Although, most studies have investigated food reward based ant-plant interactions, it is increasingly being recognised that food rewards are also consumed by many different arthropods such as spiders, parasitoids, predatory wasps and mites (Heil, 2015).

Other than the olfactory and chemical cues, plants can mediate interspecific interactions simply by visual cues. It has been observed that spectra of greenish-yellow light reflected by vegetation or contrast in plant-soil background can strongly influence host finding by pests (Kennedy *et al.*, 1961; Finch and Collier, 2000; Kühnle and Müller, 2011; Döring, 2014). This is more relevant for phytophagous pests, which have not developed detection of these host-specific olfactory cues. Even natural enemies can be affected by visual cues; it has specifically been shown for parasitoids that they learn visual cues which are consistently associated with their host presence (Arthur, 1966; Wackers and Lewis, 1994; Benelli and Canale, 2012). Furthermore, plants can also influence pest or predator abundance by changing their apparency. Plant apparency can broadly be defined as the likelihood of plant being found by herbivores (Feeny, 1976). More apparent plants such as taller plants can be located by certain herbivores more easily than less apparent plants (Lawton, 1983; Castagneyrol *et al.*, 2013), this may further affect predator presence on the plants. Thus, plants mediate multitrophic interactions via numerous extrinsic and intrinsic plant signals. Despite their strong effects on pests and predator abundance, plant signals and their associated multitrophic interactions are largely ignored in pest control programs.

1.3.5 Within-plant variation of multitrophic interactions:

All the interactions discussed until now (predator-pest-plant) can further be mediated by within-plant variation. Intraspecific variation in plant traits has long been utilised in plant breeding to develop high yielding or pest tolerant/resistant varieties. It is well established that genetic variation in plant traits can directly affect herbivore abundance and that herbivore preference and performance varies across difference plant genotypes (Fritz and Simms, 1992; Zytynska and Weisser, 2016). Several mechanisms explain how variations in plant traits (eg. trichomes, resistant genes, leaf water content etc.) across different genotypes alter herbivore abundance. For example, genotypes with stronger resistant genes are more resistant to herbivorous pests (Smith and Clement, 2012); genotypes with high trichome density may protect pests from predators (Johnson, 2008); and those with high leaf water content may increase the nitrogen and sugar processing efficiency of pests (Johnson, 2008). Other than these direct effects, within-plant variation may also indirectly affect herbivores. Variation across plant genotypes in herbivore induced plant volatiles (HIPVs) and plant food rewards has been observed in several agricultural systems (Takabayashi *et al.*, 1991; Hare, 2007; Webber *et al.*, 2007; Schuman *et al.*, 2009). As mentioned in the section above, plant traits such as HIPVs and food rewards strongly affect the associated arthropod community. Thus, when these traits vary across plant varieties, the associated arthropod community may potentially vary across varieties too.

Indeed, there is strong evidence for the effect of within-plant variation on its associated invertebrate community (Whitham *et al.*, 2012). Through its effects on herbivorous pests and their natural enemies, effect of within-plant variation can propagate through the food web as a series of direct effects, influencing the community structure (Bukovinszky *et al.*, 2008). Or, within-plant variation can also affect the community structure by altering indirect interspecific interactions. For example, effect of ant abundance across different plant genotypes on hemipterans such as aphids was also found to vary with the variation in hemipteran honeydew composition across different genotypes (Mooney and Agrawal, 2008; Abdala-Roberts *et al.*, 2012). As plant genotype affects its associated community, it may even regulate aboveground-belowground interactions. For example, the effect of belowground rhizobacteria on aboveground aphids and their parasitoid has been shown to be dependent on plant genotypes (Tétard-Jones *et al.*, 2007; Zytynska *et al.*, 2010). Thus, not only does plant genotype affect the associated community by indirect interspecific interactions, but also the direction (positive or negative) of each of these interactions can vary across plant genotypes. This is crucial to consider for pest control as it indicates that breeding plant varieties which simply reduce pests or enhance their predators may not be effective because direction of pest-predator interactions also varies across varieties.

These overwhelmingly complex multitrophic interactions demonstrate that the output of any pest control strategy is context dependent and varies with species identity and their associated interactions. Most programs still focus on biocontrol and plant breeding as independent pest control strategies (Cortesero *et al.*, 2000). Despite strong effects on their associated community, plant produced compounds such as food rewards and VOCs are so far underutilised in pest control (Stenberg *et al.*, 2015). Further, the effect of plant genetic variation (specifically across plant defence traits) on multitrophic interactions has been largely ignored for agricultural crops. There is an urgent need to bridge these separate measures and to not rely on a single measure for pest control. Considering multiple defences such as: the potential role of vegetational diversity, plant physical and chemical traits, predators, associated arthropods, belowground biota and within-plant variation will help in developing more robust pest control strategies.

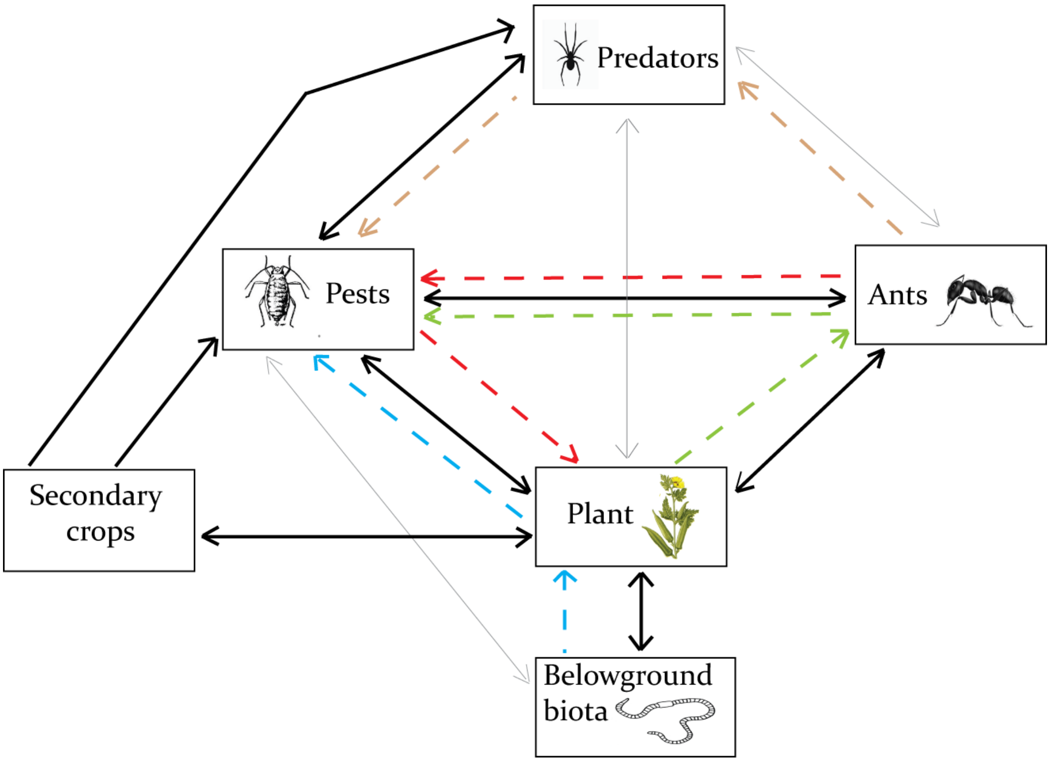
1.4 Research objective

My study focuses on developing sustainable measures to control the cotton aphid (*Aphis gossypii* Glover) pest on okra (*Abelmoschus esculentus* Moench) in Cameroon. Most okra farmers in Cameroon practice small-scale subsistence farming. One of the major concerns regarding agriculture production in Cameroon is that growth in production mainly occurs from an increase in the area of cultivated land rather than from an increase in yield from already cultivated land (Dewbre and Battisti, 2008). With a growing population this is unsustainable as vast tracts of forests are cleared to be converted into agricultural lands. Further, to control pests, okra farmers use large amounts of environmentally damaging pesticides. These pesticides are costly and are not always effective in controlling pest populations.Thus, measures such as pest control under an integrated pest management scheme are essential to obtain increased yield from available agricultural land.

In my thesis I aim to understand species interactions within the okra associated invertebrate community for developing measures for the cotton aphid control. Further, I want to explore how different okra plant varieties and vegetational diversity alter the associated invertebrate community and thus may mediate effects of any pest control measure. The specific objectives are:

1. To understand the effects of intercropping at different planting densities (crop and spatial diversity) on the cotton aphids, their natural enemies, other okra associated herbivores and on okra plant yield.
2. To explore the okra associated multitrophic interactions, their variation across different okra varieties and their effect on okra yield.

3. To investigate the mechanisms behind the okra associated multitrophic interactions and their variation across different okra varieties.
4. To examine if plant variety can mediate aboveground belowground interactions.



Interspecific interactions in agroecosystems. Undashed lines indicate direct and dashed lines indirect interactions. In this thesis I focus on the interactions highlighted in bold lines.

Chapter 2

Methods

2.1 The study system and study species

I conducted controlled and field experiments in Cameroon and in Germany to understand the multitrophic interactions in the aphid-okra system and their effect on okra yield. In Cameroon, the field and controlled experiments were carried out at the research station of International Institute of Tropical Agriculture (IITA) located in Yaounde city (3° 51' 55.43" N, 11° 27' 37.90" E). In Germany the controlled experiments were conducted at the Dürnast experimental station of Technical University of Munich (TUM) (48° 24' 26.24" N, 11° 41' 42.04"E).

2.1.1 Abelmoschus esculentus Moench – Okra:

Okra is an economically important crop grown worldwide and is widely consumed in Cameroon. It is mostly grown in humid climate in sandy and clay loam soils and its optimum growing temperature is estimated to be between 24-30 °C. The plants are annual erect herb (2-4 meters tall) with lobed and hairy leaves (up to 50 cm wide and 35 cm long). It is a self-pollinating crop but insects, especially bees, are attracted to the flowers and hence cross pollination occurs (Tripathi et al., 2011). Regular harvesting of the fruits stimulates continuous fruiting and during rapid growth it might be necessary to harvest okra fruits every day.Okra plants are attacked by many pests at different growing stages such as the polyphagous cotton aphid (*Aphis gossypii* Glover), tobacco whitefly (*Bemisia tabaci* (Gennadius)) and the oligophagous leaf beetle (*Nisotra uniformis* Jacoby) (Benchasri, 2012).

The annual survey conducted by the IITA in Cameroon found that okra farmers grow many different okra varieties. Okra varieties can differ significantly in their mucilage content (Ahiakpa *et al.*, 2014) and in Cameroon varieties with high mucilage content are preferred by the consumers. Approximately 62% of the okra farmers practice intercropping, and maize and bean are the most common crops grown along with okra (IITA annual survey report, 2011). Okra plants also produce pearl bodies (food bodies) on their leaf and stem surfaces (personal observation).

Picture 1: Okra plant with matured fruits and flower

2.1.2. *Aphis gossypii* - *The cotton aphid:*

Aphids are serious pests and responsible for reduction in yield of many agricultural crops worldwide (Van Emden and Harrington, 2007). The cotton aphid colonizes more than 600 host plants across a wide geographic range and vectors more than 50 plant viruses, making it the most diverse aphid species (Blackman *et al.*, 2007). *A. gossypii* is highly colour polymorphic and dependent on temperature its colour can range from pale yellow to dark green to dark brown (Williams and Dixon, 2007). In tropical parts of the world this aphid undergoes mostly parthenogenetic reproduction leading to an exponential growth rate at optimum conditions (Blackman *et al.*, 2007). Despite its polyphagous nature *A. gossypii* has been shown to develop distinct host races with different ability of colonizing different host plants (Guldemond *et al.*, 1994; Carletto *et al.*, 2009). It is also a species highly adaptive to environmental cues as its low genetic polymorphism is associated with a high phenotypic variability (Lombaert *et al.*, 2009). In Cameroon the cotton aphid is one of the dominant pests of okra (Leite *et al.*, 2007; Shannag *et al.*, 2007) and has evolved resistance to pesticides, particularly on cotton plant (Brévault *et al.*, 2008). The annual survey conducted by the IITA of okra farms in Cameroon found that various ant species attend the cotton aphids on okra. Ants of genus *Pheidole* were the dominant ants found attending these aphids on okra in 75% of the surveyed farms (IITA annual survey report, 2011). The cotton aphid is a facultative ant tended species which means that it does not have a close association with ants. Natural enemies of the cotton aphid include number of species in the families Coccinellidae, Chrysopidae, Hemerobiidae, Syrphidae, some small spider species and parasitoids in the order Hymenoptera (Ebert and Cartwright, 1997).

Picture 2: The cotton aphid

2.2 Method summary

All experiments conducted for this thesis were fully factorial with a block design. Treatments were randomly distributed within a block and each block contained all treatments. Linear models (LM) were used to analyse the normally distributed data. For non-normal data I fitted generalized linear models (GLMs). I also calculated correlations between variables and conducted power analysis of the models when necessary. Post-hoc analyses were also conducted to determine significant factors within a treatment. All data was analysed in R version 3.2.2 using RStudio version 0.98.978. For most variables tested, Type I sum of squares was used; I first fitted a full model with all main effects and all interaction effects between the main explanatory variables. Then all the non-significant effects and interactions (starting from the highest interaction order) were removed for simplification of the final model. Mean values and standard deviations were calculated using Microsoft Excel 2010.

Summary of methods are given below.

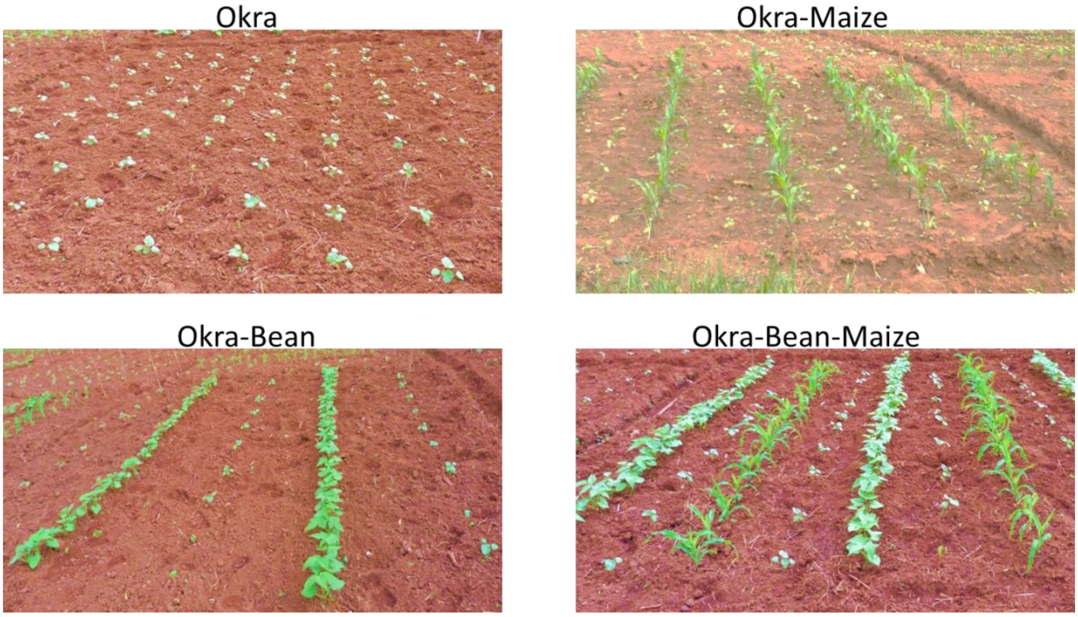
2.2.1 Aim I:

The first experiment was conducted to understand the effects of intercropping (crop diversity) at different plant densities on the okra-associated invertebrates and on okra plant yield. This field experiment was conducted at the IITA research station.

I used three plant species: Okra, Maize (*Zea mays* L.) and common beans (*Phaseolus vulgaris* L.). Four crop species combinations were used: (1) Okra monoculture, (2) Okra-Bean, (3) Okra-Maize and (4) Okra-Bean-Maize. Hence, there were plots with three different diversities i.e. okra monoculture (1-sp), okra with one other crop species (2-sp) and okra with both crop species (3-sp). Each crop combination was grown at two different plant densities within a plot (high and low), resulting in eight treatment combinations, each with nine replicates, distributed within nine spatial blocks. Plant density was manipulated within a plot by having varying distances between planting rows for each plot. In low plant density plots the distance between planting rows was 0.9 m (five rows per plot). In high plant density plots the distance between planting rows was 0.4 m (10 rows per plot). In each of the plots okra plants were selected, marked and data was collected from these selected plants every two weeks.

For the selected plants, on each observation date I counted the number of aphids, leaf beetles, whiteflies, spiders, syrphid larvae, plant height and number of leaves. At the plot level, I recorded weed cover, crop cover and soil CN ratio. I also recorded for each plot total number of okra fruits (marketable and unmarketable), maize fruits, weight of bean fruits and economic profit (total selling price – total cost of inputs).

Data was analysed for the following response variables: aphid abundance, leaf beetle abundance, whitefly abundance, syrphid larvae abundance, plant height, plant biomass, okra fruit number per plant, % marketable okra fruit per plot, land equivalent ratio (LER greater than one indicates that an intercrop gives better yield (overyielding) than a monocrop) and economic profit per plot.



Picture 3: Different crop combinations in the intercropping study

2.2.2 Aim II

I conducted two experiments (one field and one controlled) to explore the okra associated multitrophic interactions, their variation across different okra varieties and their effect on okra yield. These were conducted in the IITA experimental station in Yaoundé in Cameroon.

2.2.2.1 Field experiment: In this first experiment ant and aphid predator access was manipulated. The experiment included 16 treatments: four okra varieties, two ant treatments (Presence/Absence) and two cage treatments [Open/Closed]. Each treatment was replicated ten times (N=160) and distributed in ten blocks, with one potted plant per cage. On day one of the experiment set up, ten aphids were placed on all plants and plant height was measured.

One week after experimental set up, data was recorded weekly over a period of four weeks. Following variables were recorded per plant: plant height, leaf number, aphid number, number of ants attending aphids, ant species, leaf beetle number, foliage remaining, syrphid larvae number, number of parasitoid mummies and spider number. A plant was harvested when the first fruit had matured up to a minimum of 7 cm in length. Both, the fruit and the plant were then dried and their biomass was measured.

Aphid per capita growth rate (aphid GR) was calculated using the formula $(\ln(N_x) – \ln(N_s))/t$. Where, N_x is aphid number in a particular week, N_s is aphid number at the start of the experiment (i.e. ten aphids) and t is the duration of the experiment (days). Plant relative growth rate (plant RGR) was used to correct for plant height variation amongst varieties; it was calculated using the formula: (ln (final plant height) – ln (initial plant height))/total number of days.

Data was analysed for the following main response variables aphid GR, plant RGR, fruit biomass, ant abundance (ant present plants only), syrphid larvae presence/absence, leaf beetle abundance and % foliage remaining (open cages only).

Picture 4: Open cages had opening on all four sides to allow access to flying predators. Closed cages were closed on all four sides. One okra plant was placed inside each cage. A sticky tanglefoot insect barrier was put on stem of the plants with ant absence treatment (Field experiment)

2.2.2.2 Controlled screenhouse experiment: I also conducted a controlled screenhouse study for clearer understanding of the ant-aphid interaction in the okra system. Ant colonies of *Pheidole dea* Santchi species were used as these were found attending aphids on 99% of the plants in the field experiment. This experiment included three okra varieties and two ant treatments (Presence/Absence), resulting in six treatments in total. These were repeated eight times. Each plant was placed inside entirely enclosed plastic-polypropylene insect cages (1,350 μm mesh opening).

On first day I put one plant in each cage, placed ten aphids on these plants and measured plant height. After 48 hours, ant colonies were introduced inside the cages with ant presence treatment. After this, two different forms of observation were conducted of all plants. For the first one, all 48 experimental plants were sampled twice each day (morning and evening) and numbers of ants attending aphids per plant during one minute were recorded. For the second one, six plants were selected every day (two of each variety) out of the 48 plants and these were also observed twice for ten minutes each (morning and evening) to record whether ants were tending the aphids or predating upon them. On the final day (day 15) data was recorded on aphid colony size, plant height and leaf number.

The data were analysed for the response variables: aphid GR, plant RGR and ant abundance.

Picture 5: One okra plant is placed inside each cage. Ant colonies are placed in cages with ant presence treatment (Screenhouse experiment)

2.2.3 Aim III

One field and two controlled studies were conducted to investigate the mechanisms behind ant-aphid interactions on okra and their variation across different okra varieties. The first two experiments (one field and one controlled) were conducted in the IITA experimental station in Yaoundé in Cameroon and the third experiment was conducted in the Dürnast experimental station of Technical University of Munich (TUM) in Freising, Germany.

2.2.3.1 Ant preference field experiment: This experiment included five okra varieties, two aphid treatments (Presence/Absence) and two peal body treatments (PB kept/PB removed), resulting in 20 treatments in total. Each of the treatments were repeated 12 times and distributed within 12 blocks.

Pheidole colonies were marked in the field site of IITA. Each of the ant colonies were the repeat units and consisted of all the treatments. First plant height, number of leaves and pearl body count was recorded. Then pearl bodies were removed from plants with PB removed treatment and 50 aphids were placed on plants with aphid presence treatment. Next day plants were placed outside and grouped around an ant colony with respect to aphid and PB treatment, e.g., all varieties with aphid presence and PB removed were placed closer together as a group. Thus there were four groups around each ant colony (aphid presence+PB kept, aphid absence+PB kept, aphid absence+PB kept and aphid absence+PB removed).

The plants were placed outside early in the morning on the first day between 7:00 AM and 8:30 AM after which observations were conducted every three to four hours, from 8:30 to 18:30 hrs on the first day and from 7:00 to 16:00 hrs the next day. Each group within a block was observed for five minutes during each observation effort. Data was recorded on number of ants on a plant and ant species on a plant.

Data was analysed for the response variables: initial plant height, initial pearl body count, total number of ants on a plant, total number of *Pheidole*/*Camponotus*/'other' ants on a plant and count of presence/absence of *Pheidole*/*Camponotus*/'other ' ants on a plant.

Picture 6: Plants are placed in groups around a single *Pheidole* ant colony. Plants within a group touch one another (Field experiment).

2.2.3.2 Short term pearl body production controlled experiment: This experiment included five okra varieties, two aphid treatments (Presence/Absence) and two peal body treatments (PB kept/PB removed), resulting in 20 treatments in total. Each of the treatments was repeated five times. Ant presence was simulated by PB removal.

First plant height, number of leaves and initial pearl body count of the plants was recorded. Then pearl bodies were removed from plants with PB removed treatment and 50 aphids were placed on the plants with aphid presence treatment. 36 hours after initiating the experiment, final PB count was recorded for all leaves and stem of each experimental plant.

Pearl body replenishment was calculated for PB kept plants as final PB count – initial PB count, and for PB removed plants as final PB count – 0. Data was analysed for the response variables: initial and final PB count, plant height, number of leaves and pearl body replenishment.

2.2.3.3 Long term pearl body production controlled experiment: This experiment included four okra varieties, two aphid treatments (Presence/Absence) and two peal body treatments (PB kept/PB removed), resulting in 16 treatments in total. Each of the treatments was repeated ten times. Ant presence was simulated by PB removal.

First plant height, number of leaves and initial pearl body count of the plants was recorded. Then pearl bodies were removed from plants with PB removed treatment and 30 aphids were placed on the plants with aphid presence treatment. After one week data was recorded on final plant height, final pearl body count and leaf area of each leaf of all plants. Additionally, leaves of similar successional stage were collected for leaf structure analysis.

Data was analysed for the following variables: plant height, number of leaves, initial PB count, total leaf area, plant relative growth rate, total replenishment rate, leaf PB replenishment/cm^2, stem PB replenishment/cm.

Picture 7: Greenhouse experiment conducted in Germany.

2.2.4 Aim IV

This experiment aimed to examine if plant variety can mediate aboveground belowground interactions. An earthworm-plant-aphid model system was used. Species used were bean plants (*Vicia faba* L.), composting earthworms (*Eisenia veneta* (Rosa)) and two aphid species (the legume specialist *Acyrthosiphon pisum* (Harris) and the polyphagous black bean *Aphis fabae* Scop.). This experiment included four varieties of bean plant, two earthworm treatments (Presence/Absence) and four aphid treatments- two single aphid treatments (*A. pisum* or *A. fabae* alone), a paired treatment (*A. pisum* + *A. fabae* together) and a no-aphid control, resulting in 32 treatments in total. Each treatment was repeated 12 times.

First, plant height was measured. Then seven earthworms were added to plants with earthworm presence treatment and six aphids were added in total to aphid presence treatment (six of the same species for the single aphid treatments, or three of each species for the paired aphid treatments). After 14 days data was recorded for the following variables: number of different aphid species on each plant, number of earthworms, plant height, plant biomass and plant CN ratio.

Data was analysed for the following variables: aphid growth rate (for different aphid species), plant biomass and plant CN ratio.

Chapter 3

Manuscript Overview and Author Contribution

This thesis contains four manuscripts. A summary of each manuscript along with author contribution and publication status is given:

3.1 Manuscript I

Minimize space, maximize production: Benefits of intercropping at high densities for okra farmers in Cameroon

Akanksha Singh, Wolfgang W Weisser, Raissa Houmgny, Sharon E Zytynska

Submitted to Agriculture, Ecosystems and Environment journal

Intercropping is a traditional farming practice in the tropics. It can often reduce pests but can also have varying effects on pests, their predators and on crop yield. Furthermore, pest suppression success of intercropping can be crop or pests species-specific. So far, pest control studies have focused on crop diversity and not on planting distances. We suggest that planting distances could also affect pests and yield in intercropped fields. Okra is an economically important vegetable in Cameroon, often grown by small-scale farmers in intercropped fields and is sown with large distance between planting rows (~2 meters). Many pests attack okra, such as aphids, leaf beetles and whiteflies. We intercropped okra with maize and bean in different crop combinations (okra monoculture, okra-bean, okra-maize and okra-bean-maize) and plant densities (high and low) to test how intercropping affected okra pests, their predators and okra yield. We found crop identity, but not diversity to influence okra pests, their predators and yield. Response of pests and predators to crop identity was pest-and crop-specific. Additionally, planting density affected all pests and predators but only leaf beetles affected okra fruit yield. Leaf beetle abundance was reduced at high plant density. Overall, okra grown with bean at high plant density was the most profitable combination. We suggest that legumes such as beans can be included when planting at high densities to provide additional yield and compensate for higher plant nutrient demand. Selecting plants based on

their functional traits may thus help to eliminate yield gaps in sustainable agriculture by maximum utilization of available land.

AS, SZ and WW conceived and designed the experiments. AS and RH conducted the experiment and collected data. AS analysed the data and wrote the first manuscript draft which was commented on by SZ and WW.

3.2 Manuscript II

Ant attendance of the cotton aphid is beneficial for okra plants: Deciphering multitrophic interactions

Akanksha Singh, Sharon E Zytynska, Rachid Hanna and Wolfgang W Weisser

Published in 2016 in Agricultural and Forest Entomology journal

In the previous study additional crop species and planting density influenced okra pests and their predators; this may have confounded the okra-associated multitrophic interactions. Therefore, in this study we focused only on okra plants. Farmers grow different okra varieties in Cameroon which are attacked by the cotton aphid (*Aphis gossypii*). The cotton aphid is a facultative ant-tended species and ants are commonly found attending these aphids on okra. Ant-aphid association can be mutualistic or antagonistic with ants increasing or reducing aphid numbers. Within-species plant variation or other herbivores may further influence these facultative ant-aphid interactions. We conducted field and screenhouse experiments where plant variety, ant presence and predator access were manipulated to investigate the multitrophic interactions on okra and their effects on okra yield. In the field, ants did not protect aphids from their natural predators and syrphid larvae reduced aphids by 42%. Additionally, aphid recruitment of ants reduced chewing herbivore damage by 11% and indirectly increased okra fruit set. We also found aphid numbers, aphid predation by syrphids and chewing herbivory to vary across okra varieties. Finally, in the screenhouse we recorded a 24% reduction in aphid numbers on plants with ant presence. Our study highlights the importance of direct and indirect biotic interactions for pest biocontrol. Tropical agricultural systems are complex and understanding such interactions can help in designing pest control measures in sustainable agriculture.

AS, SZ, RH and WW conceived and designed the experiments. AS conducted the experiment, collected and analysed the data. AS wrote the first manuscript draft which was then commented on by SZ, RH and WW.

3.3 Manuscript III

Effect of ant-plant-aphid interactions on okra pearl body production and its variation across okra varieties

Akanksha Singh, Veronika Mayer, Sharon E Zytynska and Wolfgang W Weisser

In preparation

In our previous study we found that ants reduced aphids in the controlled study but had no effect on aphids in the field study. Ant reduction of aphids was specifically higher on the *Caffeier* variety and ants were even observed to predate on aphids on this variety. Okra plants produce pearl bodies which have been observed to be carried by ants to their nests. We hypothesised that okra pearl bodies may mediate this ant-aphid interaction. Plants produce pearl bodies to attract ants and in return ants are known to protect plants from herbivorous pests. Despite their role in plant protection, pearl bodies are largely ignored in development of pest control measures. Aphid pests also produce honeydew to attract ants and in turn ants may protect them from their predators. Further, these ant-plant/ant-aphid interactions may also vary across plant varieties. It has yet not been studied how tritrophic interactions affect pearl body production and if this varies across plant varieties. We conducted one field and two controlled studies using 4-5 okra varieties and tested: ant preference of okra pearl bodies over aphid honeydew, and the effect of aphid presence and artificial pearl body removal on pearl body production. We found okra pearl body production to vary across okra varieties. Ant preference of pearl bodies was species-specific and ants of genus *Pheidole* favoured pearl bodies over aphid honeydew. These ants also preferred plants of *Caffeier* variety. Additionally, pearl body production was inducible and was higher when they were artificially removed. Aphids induce multiple responses in a plant which can actually benefit the plant. Aphids reduced pearl body count but not when these were artificially removed. Further,

aphids increased plant growth but pearl body production reduced plant growth. With no effect of aphids on pearl bodies in simulated ant presence, their prospective for plant defence by ants is higher. We suggest developing plant varieties favourable to ants to further enhance plant defence by ants.

AS, VM, SZ and WW conceived and designed the experiments. AS conducted the experiment, collected and analysed the data. AS wrote the first manuscript draft which was then commented on by SZ, VM and WW.

3.4 Manuscript IV

Plant genetic variation mediates an indirect ecological effect between belowground earthworms and aboveground aphids

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In this paper we investigated how within-plant variation mediates aboveground-belowground interactions. Interactions between aboveground and belowground terrestrial communities are often mediated by plants, with soil organisms interacting via the roots and aboveground organisms via the shoots and leaves. Many studies now show that plant genetics can drive changes in the structure of both above and belowground communities; however, the role of plant genetic variation in mediating aboveground-belowground interactions is still unclear. We used an earthworm-plant-aphid model system with two aphid species (*Aphis fabae* and *Acyrthosiphon pisum*) to test the effect of host-plant (*Vicia faba)* genetic variation on the indirect interaction between the belowground earthworms (*Eisenia veneta*) on the aboveground aphid populations. Our data shows that host-plant variety mediated an indirect ecological effect of earthworms on generalist black bean aphids (*A. fabae*), with earthworms increasing aphid growth rate in three plant varieties but decreasing it in another variety. We found no effect of earthworms on the second aphid species, the pea aphid (*A. pisum*), and no effect of competition between the aphid species. Plant biomass was increased when earthworms were present, and decreased when *A. pisum* was feeding on the plant (mediated by plant variety). Although *A. fabae* aphids were influenced by the plants and worms, they did not, in turn, alter plant biomass. Previous work has shown inconsistent effects of earthworms on aphids, but we suggest these differences could be explained by plant genetic variation and variation among aphid species. This study demonstrates that the outcome of belowground-aboveground interactions can be mediated by genetic variation in the host-plant, but depends on the identity of the species involved. The effects of plant variety in mediation of aboveground-belowground interactions needs to be included in agricultural studies, specifically as plant varieties are bred to be different.

SZ conceived and designed the experiment, with AS, SZ, JB, ED, SH, AW collecting the data. Analysis and interpretation was done by AS, SZ and WW. AS, JB, ED, SH, AW all contributed to the first draft, completed by AS and commented on by SZ and WW. All authors read and approved the final manuscript.

Chapter 4:

General Discussion

The importance of sustainable agriculture is increasingly being recognised, opening numerous exciting opportunities in agroecological research. Effective research can provide solutions which enhance yield and also reduce environmental impacts of agriculture. Agriculture faces multiple challenges today and with great variability in species interactions, there is no one general solution that can be applied to all crops. Therefore, studies such as my thesis are crucial, which explore the multitrophic interactions in a system and apply that knowledge to suggest pest control measures. Throughout my thesis, I have shown that in the okra system there are numerous multitrophic interactions, which do affect pest and predator abundances and okra yield. Below I discuss my various key findings and their applications for farmers.

4.1 Enhance natural predators

Okra is grown in various systems in Cameroon, in monocultures and also in polycultures. Pest suppression by predators can vary depending on the farming systems and this variation is rarely considered in studies on developing pest control measures. Such information is specifically important for small-scale farmers in the tropics who practice more variable agriculture (Altieri and Koohafkan, 2008), and do not consistently grow crops in monocultures or polycultures. Syrphid larvae predators, which effectively reduced aphids in my okra variety study (Manuscript II), had no effect on aphids in the intercropping study (Manuscript I). Further syrphid abundance was higher than spider abundance in the okra variety study, whereas in the intercropping study spider abundance was higher. Spiders are generalist predators and abundance of generalist predators can increase with increasing plant diversity (more prey) (Langellotto and Denno, 2004; Scherber *et al.*, 2010). On the other hand, syrphid larvae mostly feed on soft-bodies insects but have dominantly been observed to feed on aphids (Almohamad *et al.*, 2007; Smith *et al.*, 2008). Aphid abundance was higher in the okra variety study (mean: 121.9±59.4) than in the intercropping study (mean: 10.4±1.27), and syrphid larvae presence was found to be higher on plants with higher aphid abundance (Manuscript II). Thus, due to higher syrphid abundance, higher aphid reduction was recorded in the okra variety study. It can be suggested that farmers who grow okra in monocultures can enhance syrphid larvae abundance (e.g. by increasing floral resource abundance in the field margins) for aphid control, but this may not be effective in okra intercropping systems.

4.2 Utilize different plant traits

Instead, in intercropping systems plant traits may prove to be more effective for pest suppression. In my intercropping study (Manuscript I) I grew bean and maize with okra in different crop combinations (okra, okra-bean, okra-maize and okra-bean-maize). Plant traits have been utilized for pest control in intercropping systems and push-pull strategies are famous examples of such an approach. Within this strategy, pests are repelled away from the crop (push) by using repellent stimuli (e.g. repellent plants) and are also simultaneously attracted (pull) to an attractive plant (Khan *et al.*, 1997; Cook *et al.*, 2007). These studies usually focus on one particular pest of a crop, whereas crops are often attacked by several serious phytophagous pests, which may respond differently to different stimuli. In the intercropping study it was evident that different okra pests and predators had varied responses to different crop combinations. For example, the abundance of oligophagous leaf beetles which feed dominantly on okra (Pitan and Ekoja, 2011) was highest in okra monocultures, whereas the abundance of polyphagous cotton aphid was not affected by different crop combination.

Overall, lower pest abundance in the intercropping study was recorded in the presence of maize. Thus, growing okra with maize can provide effective pest control. However, this would not be profitable as okra yield in the intercropping study was also recorded to be lowest in maize presence alone. Maize competed with okra for resources as it is a nutrient demanding crop. In contrast, bean plants facilitated okra plants as they fixed nitrogen and increased okra yield. Therefore, plant traits not only affected pest abundances but also okra yield. In fact, it was plant traits and not pests that affected okra fruit production. As pests do not always reduce yield, their lower abundance in a particular intercrop combination (e.g. okra-maize), will not result in an increased yield (Poveda *et al.*, 2008). Hence, in some systems competitive or facilitative interactions amongst crops may have a stronger effect on yield than pests (Poveda *et al.*, 2008). It is crucial that such information is communicated to the farmers. It is often assumed that pests reduce yield and this can lead to more resources being allocated to pest reduction. I do not argue that pests should not be controlled. I only state that with intercropping systems which harbor lower pest abundances, their design should focus on enhancing plant yield. This can specifically be beneficial for improving yield in systems where nutrient limitation impedes productivity.

4.3 Manipulate plant density

Another approach to pest reduction and enhanced yield is by manipulating plant density. In my intercropping study (Manuscript I) plant density was manipulated and each of the different crop combinations were grown at high and low plant densities. Effects of plant density on crop yield are well studied, but the effects on invertebrates are rarely ever considered. Several intercropping studies have found that the inclusion of beans as an additional crop leads to a higher relative yield than from monocultures (Iverson *et al.*, 2014). I found similar positive effects of beans in the intercropping study; in the okra-bean high plant density plots relative yield of okra was higher than in the okra monoculture high plant density plots. Thus, traits of beneficial plants such as bean can be utilized to grow plants at high densities and derive higher yield from available land. In addition to increasing yield, abundances of leaf beetle and whitefly pest species were also reduced at high plant density. This reduction was potentially observed due to change in plant-soil contrast caused due to changing plant density. Changes in plant-soil contrast have been shown to affect plant host search by certain species of aphids and leaf beetles (Kennedy *et al.*, 1961; Döring and Chittka, 2007; Kühnle and Müller, 2011). If density can have such significant effects on invertebrates, it can be used as a powerful tool for pest control. This is specifically beneficial when pests are reduced at high density, as such a system may also provide higher overall yield (e.g. okra-bean high plant density system). Hence, more studies are needed in agricultural monoculture/polyculture systems investigating density effects on invertebrates. I suggest that for okra farmers practicing intercropping, it is beneficial to grow okra with bean at high plant densities.

4.4 Enhance ant abundance

Ants of genus *Pheidole* were consistently found to be the dominant ant species in okra fields in Cameroon (IITA Annual survey report, 2011 and Manuscript II). These were found to attend aphids and also to carry okra pearl bodies (food bodies) back to their nest. *Pheidole* ants did not protect aphids but they were attracted to plants with more aphids (Manuscript II). This aphid attraction of ants was beneficial for okra plants as ants did not protect aphids from their predators, but they did reduce leaf beetle herbivores (Manuscript II). Additionally, aphids did not affect plant yield but leaf beetles did directly (by reducing marketable fruit number) or indirectly (by reducing plant growth rate) reduce okra yield (Manuscript I and II). It has been shown by several systems that recruitment of ants by aphids can reduce other herbivores and enhance plant yield (Styrsky and Eubanks, 2007; Nielsen *et al.*, 2009; Styrsky and Eubanks, 2010). Therefore, the increase in ant numbers in the field can benefit farmers and reduce okra pests, not only in monocultures but potentially also in polycultures. Indeed, ants are abundant in agroecosystems and ant biocontrol has been shown to match synthetic pesticides in many agricultural systems (Offenberg, 2015). Deeper understanding of these ant-aphid-plant interactions may help in maintaining high ant numbers on okra plants.

4.5 Utilize plant defense traits and their variation across varieties

The controlled study facilitated better understanding of the ant-aphid-plant interactions. In this study *Pheidole* ants were found to reduce aphids on all okra varieties. Highest reduction was observed on the *Caffeier* variety and ants even predated upon aphids on this variety (Manuscript II). This interaction was further explored in the third manuscript to understand why ants reduced aphids and why this reduction was mostly on the *Caffeier* variety. Through the ant preference study (Manuscript III), it was evident that ants of genus *Pheidole* favoured okra pearl bodies over aphid honeydew. These ants were also most abundant on the *Caffeier* variety of okra. Thus, okra plants actively manipulated ant-aphid interactions on okra by providing food rewards to ants. Ant protection of plants in return of food rewards is well-established (Rosumek *et al.*, 2009; Mayer *et al.*, 2014) and food rewards are also inducible by ants (Risch and Rickson, 1981). Similarly, pearl body production for okra was found to be inducible by their artificial removal (simulated ant presence) and it was higher when pearl bodies were removed than when they were not removed (Manuscript III).

Production of defensive compounds has been shown to be costly for the plant (Heil *et al.*, 1997; Fischer *et al.*, 2002) and this may explain why okra plants invest in increasing pearl body production only in ant presence. Interestingly, aphids reduced pearl body count only when pear bodies were not removed and had no effect when pearl bodies were removed (Manuscript III). Thus, not only do plants induce pearl bodies in the presence of ants, but they also override the negative effect of aphids with the presence of ants. This emphasises that there are complicated mechanisms inherent in plants, through which they defend themselves. Manipulating inherent plant traits which attract beneficial species, such as ants, provide a vast potential for sustainable pest control. Such measures may not only be cost-effective, but also be harmless to non-target organisms.

In the okra system, ant-plant and ant-aphid interaction is facultative (occasional). This explains the fact that even though ants reduced aphids in controlled conditions, in the field they had no effect on aphids. Instead, in the field ants were attracted to plants with high aphid numbers (Manuscript II). This can occur because even though ants of *Pheidole* genus prefer okra food bodies over aphid honeydew, they do consume aphid honeydew and do not want to predate upon the additional aphid food source. Furthermore, in controlled conditions ants could only interact with aphids and okra plants whereas, in the field ants chose to attend aphids on okra and may have preferred another source of protein (other inverteberates) than aphids. Nevertheless, ant attraction by aphids or by food bodies is beneficial for the plants, as ants reduce herbivory. Thus, growing varieties such as *Caffeier*, which may further enhance ants of genus *Pheidole* on okra plants, can be beneficial for okra farmers in Cameroon.

4.6 Higher emphasis on leaf beetle than aphid reduction

It was consistently recorded that aphids had no effect on okra yield (Manuscript I and II). Instead, it was leaf beetles that reduced okra yield. In natural field conditions, aphid numbers were reduced due to aphid predators (Manuscript I), or were found to be low overall (Manuscript II). Such low numbers may explain why aphids had no effect on okra plant biomass or yield. As mentioned above, ant attraction by aphids actually benefited okra plants. Furthermore, aphids were also found to increase plant growth and leaf area (Manuscript III). This suggests that okra plants show certain levels of overcompensation against aphids. When plants overcompensate, their fitness is actually increased following moderate herbivore damage (Agrawal, 2000). In the controlled experiment, only 30 aphids were added to plants and allowed to reproduce for only one week, hence, aphid numbers potentially did not reach a damaging level. I do hypothesize that high aphid density will be harmful for okra plants thus, measures should be taken to maintain low aphid densities. However, under natural field conditions aphids do not reach high densities and in such a scenario a moderate aphid presence may actually benefit okra plants.

4.7 Future research

All my work related to okra focused on aboveground interactions, but belowground interactions too strongly mediate these aboveground interactions. Therefore, I used a model earthworm-bean-aphid system to explore if belowground-aboveground interactions would be mediated by plant variety (Manuscript IV). Plant varieties did mediate the effect of earthworms on aphids, with earthworms increasing aphid growth rate on three varieties but decreasing it on one variety. In agricultural systems, practices such as fertilizers application, weeding and tilling may significantly influence belowground biota (Edwards and Lofty, 1982; Haines and Uren, 1990; Chan, 2001). This change in belowground biota may in turn affect the interactions occurring aboveground. For example, if earthworm abundance is increased by fertilizer application (Edwards and Lofty, 1982), then it is important to select plant varieties whose biomass increases with earthworm abundance. Moreover, as belowground organisms can alter plant nutrient content, variation in this content may make a plant less or more attracted to pests or ants. The effects of plant variety in mediation of aboveground-belowground interactions needs to be included in agricultural studies, specifically as plant varieties are bred to be different.

A deeper understanding of pearl bodies' role in plant defence can be beneficial for okra plants. Future studies can be conducted to test if there is another (other than ants) beneficial species that consumes pearl bodies and how this affects ant-plant interaction and okra yield. It would also be interesting to explore the chemical variation in okra pearl bodies or aphid honeydew across different varieties, and test how this variation affects ant attendance of aphids, or their interaction with plants. For example, if a particular compound is favoured by ants in okra pearl bodies, varieties can be bred with enhanced secretion of this compound in pearl bodies. Breeding crops with enhanced capacity for ant attraction can significantly contribute to sustainable agriculture (Stenberg *et al.*, 2015).

4.8 Conclusion

Overall, my thesis shows that species interactions are context dependent and vary with several factors such as plant variety, presence of additional plant species, plant density, presence of other species such as ants and even with belowground organisms. Agriculture systems in the tropics, particularly small-scale farming systems, are complex and harbour a diversity of species and improving such systems requires a more holistic approach. In my thesis I did use a more holistic approach by focusing on okra-associated multitrophic interactions. Understanding these interactions helped in developing multiple solutions to reduce pests on okra and enhance okra yield. Furthermore, exploration of the ant-aphid-plant interaction in my thesis proved that when species interact, the direction of their effect varies with many factors and is not always net-positive (mutualism) or net-negative (antagonism). Knowledge of such interaction variability is crucial as it suggests that introduction of biocontrol agents or a pest resistant plant variety does not automatically lead to pest reduction. In my thesis ants were beneficial for okra plants. Moreover, plants were found to have evolved complex mechanisms to attract ants and defend themselves. I urge future pest control studies to investigate the role of ants in their system and also test if plant traits can be manipulated to attract beneficial species. Application of such multiple solutions can help in development of resilient agroecosystems.

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Appendix A

Manuscripts Included in the Thesis

Manuscript I

Minimize space, maximize production: Benefits of intercropping at high densities for okra farmers in Cameroon

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Abstract

Intercropping is a traditional farming practice in the tropics that can help reduce pest attacks but can also have varying effects on crop yield. Furthermore, pest suppression success of intercropping can be pest-or crop-specific. Okra is an economically important vegetable in Cameroon, often grown by small-scale farmers in intercropped fields and is sown with large distances between planting rows (~2 meters). Many pests attack okra, such as aphids, leaf beetles and whiteflies. We conducted a field experiment where we tested for the effects of diversity, crop identity and planting distances, as these may also affect pests and yield in intercropped fields. We intercropped okra with maize and bean in different crop combinations (okra monoculture, okra-bean, okra-maize and okra-bean-maize) and plant densities (high and low) to test how intercropping affected okra pests, their predators and okra yield. We found crop identity, but not diversity to influence okra pests, their predators and yield. Response of pests and predators to crop identity was pest-and crop-specific. Additionally, planting density affected all pests and predators but only leaf beetles affected okra fruit yield. Overall, okra grown with bean at high plant density was the most profitable combination. We suggest that legumes such as beans can be included when planting at high densities to provide additional yield and compensate for higher plant nutrient demand. Selecting plants based on their functional traits may thus help to eliminate yield gaps in sustainable agriculture by maximum utilization of available land.

Keywords: Intercropping, species-specific, high plant density, pest, predators, yield

1. Introduction

Agriculture is the primary driver of deforestation today, responsible for ~80% deforestation worldwide (Kissinger *et al.*, 2012). With the world human population estimated to increase by 2.3 billion by 2050, there is a growing demand for food, putting high pressure on remaining forest resources (FAO, 2009). It is well known that current intensive agricultural practices are not sustainable in the long run as they contribute to depleting ecosystem services and increased greenhouse emissions (Foley *et al.*, 2011). Hence, efforts are needed to link traditional non-intensive practices with modern ecological knowledge, to understand the ecology of these systems and develop effective agricultural designs. Intercropping is a practice that has been carried out traditionally in many parts of the world, especially in the tropics and subtropics (Vandermeer, 1992). In fact, in Africa, the majority of farmers are small-land holders with farm sizes of less than 2 ha, producing the majority of the continent's food with little fertilizer or improved seeds via traditional practices such as intercropping (Altieri and Koohafkan, 2008). Intercropping has recently received a theoretical boost from functional biodiversity research that has shown that plant communities with more species show higher ecosystem functioning than plant communities with few species diversity (Naeem *et al.*, 2012). A number of these functions are relevant for agriculture including a higher number of naturally occurring predators (Scherber *et al.*, 2010) and also plant productivity (Weigelt *et al.*, 2009). Hence, intercropping systems provide vast possibilities to experiment and produce effective farming designs which are sustainable and profitable.

Intercropped fields have higher vegetation diversity than monocultures and this has been shown to reduce pest insects by increasing the diversity and abundance of predators and parasitoids (Root, 1973; Letourneau *et al.*, 2010; Scherber *et al.*, 2010), by growing barrier crops which obstruct the movement of pests (Perrin and Phillips, 1978), or by growing crops that repel pests (Khan *et al.*, 1997; Cook *et al.*, 2007) or act as trap crops (Moore and Watson, 1991; Badenes-Perez *et al.*, 2004). Furthermore, as stated by the *"resource concentration"* hypothesis (Root, 1973) pest numbers can simply be reduced due to their reduced colonization of diverse fields. Root (1973) suggested that herbivores with a narrow host range are more likely to find, remain and increase in densities in pure crop strands. Although, most studies have recorded pest densities to be higher in monocultures, such hypothesis cannot be generalized and do not consistently predict the influence of crop diversity on arthropods (Risch *et al.*, 1983; Smith and McSorley, 2000; Altieri and Nicholls, 2004; Letourneau *et al.*, 2010).

Increasing vegetation diversity can also reduce pest suppression by hindering predator host-searching due to increasing vegetation complexity or, due to different herbivore species that distract predators away from target pests (Perfecto and Vet, 2003; Gols *et al.*, 2005; Ratnadass *et al.*, 2012; Kruidhof *et al.*, 2015). Pest numbers can also increase in intercropped fields with the presence of additional crops or weeds which give refuge to pests (Room and Smith, 1975; Letourneau *et al.*, 2010). Additionally, the response of pests to intercropping can be species-specific and vary with their host-finding mechanisms, such as differences in their olfactory or visual cues (Poveda *et al.*, 2008). Most pest colonization hypotheses apply to specialist pests. Crops are often attacked by several polyphagous pests, eg. certain polyphagous whitefly or aphid species, which have not developed host-specific olfactory cues and usually respond to the spectra of greenish-yellow light reflected by vegetation or to the contrast in the plant-soil landscape (Kennedy *et al.*, 1961; Smith, 1976; Finch and Collier, 2000; Döring, 2014). The relevance of visual cues in host-searching abilities has also been shown for oligophagous leaf beetles (Stenberg and Ericson, 2007; Kühnle and Müller, 2011). Therefore, it is evident that in intercropping systems the identity of the additional crop species, and of the pest, can be crucial in determining the pest suppression success of the intercropped field design. Within this study, we use the description by Cates (1980) to define herbivores with different host plant feeding ranges. Polyphagous herbivores are defined as those that feed on multiple plant families, oligophagous herbivores are those that feed on multiple genera within the same plant family and monophagous herbivores are those that feed on one or more plant species within the same genus.

In addition to crop diversity, the density of crop plants within a plot can also affect pest numbers. Monoculture studies on plant density have shown that pests can locate their host plants more effectively in high plant density plots (Douwes, 1968; Ralph, 1977; Obermaier *et al.*, 2008). If the lack of plant-soil contrast and homogenous visual cues can distract herbivorous pests, then higher plant densities could further hinder pest host-searching. In intercropped fields, the effect of density on crop yields has been studied (Agegnehu *et al.*, 2006; Gooding *et al.*, 2007), but its effect on pests is often overlooked. Some studies have used additive designs to test for the effect of diversity on pests, where primary crop density does not change but secondary species are added to the plot, increasing total plant density in an intercropped plot (Vandermeer, 1992; Bukovinszky *et al.*, 2004; Gianoli *et al.*, 2006; Ponti *et al.*, 2007). The interpretation of any density effect from such a design is confounding as total plant density is not constant between treatments and the results observed may only be driven by diversity. Only one study so far to our knowledge has tested for the effect of diversity and density on pest assemblages (not on plant yield) where abundance of the cucumber beetle was found to be higher in monocultures irrespective of plant density (Bach, 1980). Hence, as pest/predator responses to diversity are species-specific and affected by density, understanding the impact of diversity and density together can be important for pest suppression in an intercropping system.

Besides regulation of pest numbers, intercropping can also increase crop yield by facilitative plant-plant interactions, which are positive non-trophic interactions between physiologically independent plants mediated through the abiotic environment or other organisms (Brooker *et al.*, 2008; Brooker *et al.*, 2015). Such interactions occur in most legume-cereal intercropping systems and are prevalent worldwide, as nitrogen-fixing legumes improve soil fertility and transfer N to co-occurring plants; e.g. 98% of cowpea in Africa and 90% of soybean in Colombia are intercropped, often with staple cereal crops (Vandermeer, 1992). Although there is ample literature on how intercropping can improve yield and reduce pest damage, most intercropping studies have measured biocontrol or crop yield parameters separately and only 26 studies so far have measure these two together (Iverson *et al.*, 2014). Hence, there is little data to measure the effect of biocontrol services on yield and specifically how these are altered by plant density. With the current scenario of increasing food demand and limited available land, understanding these effects is crucial for development of optimized agroecosystems.

Okra (*Abelmoschus esculentus* Moench) is an economically important vegetable crop grown worldwide, and is popularly consumed in Cameroon. It is attacked by several pests such as the cotton aphid (*Aphis gossypii* Glover) and a leaf beetle (*Nisotra uniformis* Jacoby) (Benchasri, 2012). A survey conducted by the International Institute of Tropical Agriculture (IITA) in Cameroon (2011) found that ~62% of okra farmers practice intercropping and maize and bean were the most common crops grown along with okra. Okra was always found to be planted in rows and often the fields were sparsely planted with a distance between two consecutive okra rows recorded to be as large as 2 meters. It is unknown if intercropping at higher density has a negative effect on okra fruit yield or not. Thus, we conducted a field study where we intercropped maize and bean with okra (2 and 3-species combinations) at different plant densities (high and low). The aim of our study was to answer the following questions: (a) Does crop diversity, crop identity and plant density affect okra pests, their natural enemies and the okra fruit yield? (b) Does plant density mediate (alter) any effects of crop diversity and crop identity?

2. Methods

2.1. Study site and study system

The field experiment was conducted at the International Institute of Tropical Agriculture (IITA) research station in Yaoundé, located in the central region of Cameroon (West Africa). The experiment was initiated on the 15th of April, 2014 and terminated once all plants had yielded fruits and completed their life cycle, Average temperature: 23.6±0.07°C (range: 18.1-33.2 °C), average humidity: 90.7±0.24% (range: 51.9-100%), average rainfall: 7.6±1.49mm (range: 0-90.7mm) and natural light at approximately 12:12(light:dark) .

We used three plant species: Okra (*Abelmoschus esculentus* Moench), Maize (*Zea mays* L.) and common beans (*Phaseolus vulgaris* L.). The varieties used were Clemson (okra), CMS8704 (maize) and ECAPAN-21 (beans), which are popular commercially sold varieties in Cameroon.

Okra is mostly grown in well-drained sandy and clay loam soils in a humid climate, and the optimum temperature for its growth is 24-30 °C. The plants are annual erect herb (2-4 m tall) with lobed and hairy leaves (up to 50 cm wide and 35 cm long). Okra plants are attacked by many pests at different growing stages such as the polyphagous cotton aphid (*Aphis gossypii* Glover), tobacco whitefly (*Bemisia tabaci* (Gennadius)) and an oligophagous leaf beetle (*Nisostra uniformis* Jacoby) (Benchasri, 2012).

Maize is an annual grass, usually with one stem (1-4 m tall) and the optimum temperature for its growth is estimated to be between 20-30 °C (Hardacre and Turnbull, 1986). It is a nutrient demanding crop and requires large quantities of nitrogen during its growth (Roy *et al.*, 2006).

In sub-Saharan Africa dominant maize pests are stem and cob borer species belonging to the families Noctuidae, Pyralidae and Crambidae (Chabi-Olaye *et al.*, 2005). The main okra pests (tobacco whitefly and leaf beetles), do not attack maize, but the cotton aphid is known to feed on, but is not a dominant pest of, maize.

Common bean is a polymorphic, herbaceous annual plant which can grow at temperatures between 10-27 °C (FAO, 2015). We used the erect bush bean type ECAPAN-21 for our experiment which grows up to 20-60 cm high and has a thin multi-branched stem (Heuze *et al.*, 2015). Amongst many bean pests in Africa, the bean stem maggot (*Ophiomyia* spp.) and bruchids (Family: Chrysomelidae, sub-family: Bruchinae) are considered dominant pests (Abate and Ampofo, 1996). The cotton aphid, tobacco whitefly and leaf beetle, which are pests of okra also attack bean but these are not dominant pests.

2.2. Experimental design

We used four crop species combinations: (1) Okra monoculture, (2) Okra-Bean, (3) Okra-Maize and (4) Okra-Bean-Maize. Hence, we had plots with three different diversities i.e. okra monoculture (1-sp), okra with one other crop species (2-sp) and okra with both crop species (3-sp). Each crop combination was grown at two different plant densities within a plot (high and low), resulting in eight treatment combinations, each with nine replicates, in total 4 x 2 x 9 = 72 plots. A block design was used with nine spatial blocks each containing one replicate of each treatment. Within a block different plot treatments were randomly distributed. Blocks 1- 5 and 6 - 9 were set up on two consecutive days. Due to lack of space we could not include bean and maize monoculture plots in our study, and so in our study we focus on the effect of intercropping on okra. The experimental field site (~1800 m^2) was situated on a hillside and was surrounded by cassava and plantain fields and set aside land.

The plots measured 12.96 m^2 (3.6×3.6 m) in size and were at distance of 1.4 m from adjacent plots, on all sides. We manipulated plant density within a plot by varying the distances between planting rows. In low plant density plots the distance between planting rows was 0.9 m, i.e. five rows per plot. In high plant density plots the distance between planting rows was 0.4 m, i.e. 10 rows per plot. These distances were chosen because the recommended distance between rows for growing okra ranges from 0.3 m to 1.0 m (Splittstoesser, 1990; Ijoyah *et al.*, 2010). Within a row, the planting distance used was 40 cm for okra (10 plants/row), 20 cm for maize (19 plants/row) and 10 cm for bean plants (36 plants/row). Hence, for a particular crop combination, density was only changed due to the distance between rows, not within. Within an intercropped plot, we planted rows of okra alternating with rows of the other crops. Whenever the number of rows was unequal among species, okra was planted in the higher number rows, such that one of the outermost edges of plots always had a row of okra. For example, in low plant density plots (five rows total) for each 2-species plot we had three rows of okra interspersed with two rows of maize (okra-maize combination) or two rows of bean (okra-bean combination). For the 3-species plots, three rows of okra were interspersed with one row of bean and one of maize. For more information on the planting design see supplementary material (Table S1, Figure S1).

2.3. Experimental set-up

The field site was weeded and cleared, and then small gullies were dug around each plot to direct rain water. The experiment was conducted during the wet season (optimal time for okra growth) and heavy rain water can cause soil erosion if allowed to run directly across the growing seedlings. Seeds were placed directly into the ground at a depth of 2.5 cm. We planted three seeds per hole, and removed any additional germinated seedling two weeks later to ensure we had one established plant per planting site. All invertebrates were allowed to colonize naturally.

2.4. Weeding and fertilizing

Weeding was done by hand, starting from day 18 and repeated every three weeks (four times in total during the experiment). Okra plants were fertilized twice, in week four and six. Maize was fertilized once, in week three. We used 9.5 g of 20:10:10 (N:P:K) solid fertilizer per plant (Yara company, Cameroon), for both maize and okra and this was placed into the soil next to each plant.

2.5. Data collection

Data was collected every two weeks, on weeks 3, 5, 7 and 9, each time over two consecutive days, i.e. in blocks 1-5 on one day and in blocks 6-10 the next day. At the plot level, we recorded weed cover (% weed ground cover) and crop cover (% of the plot surface covered by overhead canopy of the experimental plants).

We collected more specific data on the abundance of pest species and their natural enemies using a subset of plants in each plot. We selected three okra plants in low plant density and five okra plants in high plant density plots to account for differences in plant number. We used the same plants on each observation date. In total, data were collected from 288 okra plants. For these selected plants, on each observation date we counted the number of aphids (using a hand tally counter), leaf beetles, whiteflies, spiders and syrphid larvae. We also measured plant height and number of leaves. Carbon and nitrogen content of okra leaves was also measured from plants from which data had been collected during the experiment, see below.

Once okra fruits started to develop (week 8) we conducted observations of all plants every two days and harvested the fruits when they were at least 7 cm in length. A newly developed okra fruit pod can take 4-5 days to mature and one plant can produce multiple fruits (at different time periods) for up to one month, after its first fruit production. Thus, within a plot, not all fruits were harvested at the same time and fruits from a single plant were also harvested at multiple times. We aggregated the data to obtain total fruit number and total fruit fresh weight per plot for all plants within a plot. Further, fruits were separated into two categories per plot, marketable and unmarketable. We classified fruits as unmarketable when more than 50% of their surface was black (by bacterial, leaf beetle or *Dysdercus* sp. damage), when there was fruit borer damage and when the fruits were rotting. The remaining fruits were classified as marketable fruits. For maize, fruits were harvested once the ears (top part) were filled out and were round in shape (weeks 12 and 13) (harvest method used by maize farmers in Cameroon (R. Houmgny, author comm.)). We recorded the number and weight of maize fruits per plot. Bean fruits were harvested once the bean pods had turned fully yellow (week 11) and we recorded the total weight of bean pods per plot.

Further, we collected soil samples from the center of each plot on week 8. The samples were air-dried for a week at room temperature and then analyzed for carbon and nitrogen content in the soil analysis laboratory of IITA, Cameroon. Once all okra plants had stopped producing fruits (week 15), the experiment was terminated. The okra plants were harvested, dried in paper bags at 60°C for three days and their biomass measured.

2.6. Yield calculations

Okra is sold by numbers and not by weight in Cameroon, hence the response variables were calculated using fruit number. High plant density (HD) plots had almost double the number of okra plants than low plant density (LD) plots and, okra monoculture plots had greater numbers of planted okra than intercropped plots. Hence, for unbiased investigation of the effect of our treatments on okra yield, we calculated the percentage marketable fruit M_P:

$$M_P = (M/N_F) \times 100.$$

Here, M is the total number of marketable fruits per plot and N_F is the total number of fruits per plot.

We also calculated okra yield per plant per plot Y:

$$Y = N_F / N$$

Here N_F is the total number of fruits per plot and N is the total number of okra plants per plot.

We also calculated relative land equivalent ratio ($RLER$) per plot. Land equivalent ratio (LER) is used to judge the effectiveness of an intercrop and is defined as relative area required for sole crops to produce the same yield as intercropping (Mead and Willey, 1980). LER greater than one indicates that an intercrop gives better yield (overyielding) than a monocrop. Calculating LER requires monocultures yield for all crop species. As we only had okra monoculture, we have modified the formula to allow for calculation focused on okra

$$RLER = Y_{inter}/Y_{mono}.$$

Here Y_{inter} is the okra yield per m^2 in an intercropped plot and Y_{mono} is the okra yield per m^2 in a monoculture plot. Okra plants were grown in 50% of the area in high plant density and in 60% of the area in low plant density intercropped plots. Our total plot area was $3.6 \times 3.6 = 12.96$ m^2. Hence, to derive Y_{inter} in a high plant density intercropped plot, okra yield per plot was divided by 50% of the plot area, i.e. $1.8 \times 3.6 = 6.48 m^2$ and for a low plant density plot it was divided by 60% of the plot area, i.e. $2.16 \times 3.6 = 7.78 m^2$ .To derive Y_{mono} in a monoculture plot, okra yield per plot was divided by the total plot area (12.96 m^2). $RLER$ was calculated for each 2- and 3-species plot separately.

Further, we determined the market selling price of our crops. For this we first interviewed ten vegetable selling vendors and asked them the price at which they sell okra, maize and bean during the crops respective growing seasons (okra and maize are sold by numbers and bean is sold by weight in Cameroon). As the price is always as a range rather than a fixed number, we noted the highest price of the range. For example, when we were told that 10-15 okra fruits were sold for 100 CFA then we would note the price of 10 fruits to be 100 CFA. We averaged the selling price for each crop species across vendors, and used it to calculate the selling price of the produce from our plots (average selling price for each crop species was multiplied with total fruit number (okra and maize) or fruit weight (bean) per plot for the respective crop species). We also calculated the total inputs (seeds and fertilizer) cost per plot by adding price of total fertilizer and seed weight (for each crop species) used per plot (Table S2). Finally we calculated the economic profit per plot by subtracting the total selling price by total input cost. The economic profit is presented in US dollars (USD) for ease of comparison, using the exchange rate of 1 USD to 584.58 CFA.

2.7 Data analysis

The abundance values used for invertebrates (aphids, whiteflies, leaf beetles, spiders and syrphid larvae) and plant height variables in all our analyses are cumulative averages. We first calculated averages per plot from each reading (3 plants for the LD or 5 for HD plots) for each of these variables. Then the average values of all readings were added, separately for each of these variables, to yield the cumulative average abundance per plant. Plant biomass values are also average values from each plot calculated from sample plants.

Linear models were used to analyse the data. First, two separate models for each of the response variables were run to test for the effects of crop diversity (i.e. 1-3) and crop combination (okra, okra-bean, okra-maize, okra-bean-maize) separately. Diversity significantly affected only two response variables but crop combination affected seven response variables. Further, average adjusted R squared values derived from different models of all response variables using crop diversity was 0.17 and from models using crop combination was 0.41. Thus, crop combination explained more variation than crop diversity for all response variables, in all our further analyses we focused on crop combination.

For syrphid larvae abundance as a response variable we ran a generalized linear model (GLM) with quasipoisson distribution and for *RLER* we ran a GLM with poisson distribution. For all other response variables we ran linear models (LM) with normal distributions, since these gave the optimal model fits. To analyse the effect of plot plant density and crop combination on all response variables (okra pests and their predators, okra plant height and biomass, okra yield (yield per plant and % marketable fruit per plot), soil CN ratio, *RLER* and economic profit), our models included block as a fixed effect, plot plant density (high/low) and crop combination as main explanatory variables and the interaction between plot plant density and crop combination. The significance of our blocking factor in the analyses showed there was significant spatial variation with higher invertebrate abundances in block 9 (next to a Cassava field) and lower values in block 1 (next to a fallow field). By including block in all models, along with the randomized block design, we have minimized any bias the spatial effect may have on our results. For our response variables soil CN ratio, *RLER* and economic profit we did not include any covariates in the model. For other response variable we included suitable covariates in each of the models to account for additional variation across our blocks.

For the okra pests/predator response variables (aphids, whiteflies, leaf beetles, spiders and syrphids) we included- soil CN ratio, plant CN ratio, % crop cover, % weed cover, plant height. The abundance of aphids, leaf beetles, whiteflies, spiders and syrphid larvae were included as covariates when these were not the respective response variables.

For the okra plant biomass and plant height response variables, we included- soil CN ratio, % crop cover, % weed cover and abundance of whiteflies, leaf beetles, aphids, spiders and syrphid larvae.

For the okra yield per plant *(Y)* and percentage marketable fruit per plot *(M_P)* we included- soil CN ratio, plant CN ratio, % crop cover, % weed cover, plant height, biomass and abundance of whiteflies, leaf beetles, aphids, spiders and syrphid larvae. M_P data was arcsine transformed before analysis.

As density had a significant effect on all our response variables, for each response variable tested we also further analysed the data within each density level to investigate the effect of crop combination within the different densities.

All data were analysed in R version 3.2.2 using RStudio version 0.98.978. We first fitted full models with all main effects and the interaction between the main explanatory variables, plus covariates. Then, all non-significant covariates were removed followed by the non-significant interaction, using model fit comparisons.

3. Results

Okra pests and their predators started colonizing plants from the first observation week and were recorded in all plots throughout the experiment. Bean plants flowered the earliest (week 5), followed by okra (week 6) and maize plants (week 8). Okra plants started to produce fruits by week 8 and peak fruit production weeks were 9, 10 and 11, after which most plants had completed their life cycle and fruit production decreased (total okra fruits harvested: week 9=3096, week 10=2351, week 11=1818, week 12= 702). Our field site was situated on a hill and different parts of the site were surrounded by cassava or plantain or set aside land. Hence, due to these spatial variations we recorded a strong effect of block on okra invertebrates and plants traits (Table 1 and 2).

Table 1: Effect of main experimental variables and covariates on okra invertebrates

	Response variables				
	Aphid abundance	**Leaf beetle abundance**	**Whitefly abundance**	**Spider abundance**	**Syrphid larvae abundance**
Block	$F_{8,58}=3.02$, $P=0.006$	$F_{8,55}=2.63$, $P=0.016$	$F_{8,58}=4.74$, $P=0.001$	$F_{8,58}=1.18$, $P=0.327$	$F_{8,54}=20.14$, $P<0.001$
Soil carbon-nitrogen ratio	-	-	-	-	↑$F_{1,54}=6.39$, $P=0.014$
% crop cover	-	-	↓$F_{1,58}=8.79$, $P=0.008$	-	-
Plant height	-	↑$F_{1,55}=9.29$, $P=0.004$	-	-	↑$F_{1,54}=5.63$, $P=0.021$
Spider abundance	↓$F_{1,58}=3.24$, $P=0.077$	-	-		-
Leaf beetle abundance	-		-	↑$F_{1,58}=6.23$, $P=0.015$	-
Plot plant density	↑$F_{1,58}=7.16$, $P=0.009$	↓$F_{1,55}=55.32$, $P<0.001$	↓$F_{1,58}=3.12$, $P=0.049$	↓$F_{1,58}=14.17$, $P=0.003$	↓$F_{1,54}=4.15$, $P=0.046$
Crop combination	$F_{3,58}=0.072$, $P=0.975$	$F_{3,55}=32.24$, $P<0.001$	$F_{3,58}=4.32$, $P=0.008$	$F_{3,58}=3.57$, $P=0.019$	$F_{3,54}=3.11$, $P=0.033$
Density×Combination	-	$F_{3,55}=7.87$, $P=0.001$	-	-	$F_{3,54}=3.90$, $P=0.044$

Linear models were used. '-' indicates when the value was not significant in a model and was removed from the final minimal model. Only those values are shown which were kept in the final minimal model. Covariates which were not significant for any of the response variables are not mentioned in the table. Arrow symbols indicate the direction of effect; ↑indicates a positive effect and ↓ indicates a negative effect.

3.1. Effect of main experimental variables and covariates on okra pests and their predators

Plant density of plots affected all invertebrates, specifically a strong effect was observed for leaf beetles, aphids and spiders (Table 1). Overall, the abundance of leaf beetles, whiteflies, spiders and syrphid larvae per plant was reduced in high plant density (HD) plots, whereas, aphid abundance increased in these plots (Table 3, Figure 1). We found that the plot crop diversity explained a significant proportion of variation only in the abundance of leaf beetles

($F_{1,69}$=22.84, P<0.001), with more leaf beetles in okra monocultures and fewer in higher crop diversity plots (Figure 1). There was no influence of crop diversity on any other invertebrate group (Table S3). However, crop combination (i.e. including the identity of the crop species) explained a significant amount of variation for all invertebrates, except aphids (Table 1). This suggests that crop identity is crucial in determining pest and predator abundances. Overall, abundance of pests was highest in okra monoculture (83.8±13.02), followed by okra-bean (82.6±14.90), okra-bean-maize (66.1±11.84) and least in okra-maize plots (64.5±12.43). Spider abundance was highest in okra monoculture and in 3-species okra-bean-maize plots, whereas, whitefly abundance was highest in okra monoculture plots and in the presence of bean (Figure 1). We found that the effect of plant density on the abundance of leaf beetles and syrphid larvae varies across crop combinations (significant 2-way interaction; Table 1). In comparison to LD plots, syrphid larvae abundance was higher only in HD okra-bean plots but lower in other crop combinations, suggesting an attraction of syrphids to bean plants (Figure 1). In comparison to LD plots, leaf beetle abundance was similar in okra monoculture HD plots, but was lower in other intercropped HD plots (Figure 1).

There was no direct effect of syrphids on any of the okra pests, but we did observe spiders to have a marginally negative effect on aphid abundance (Table 1). Spider abundance was also higher in plots with higher leaf beetle abundance (Table 1). Taller plants had a higher abundance of both leaf beetles and syrphid larvae (Table 1) and whitefly abundance reduced with an increase in crop cover (Table 1).

Furthermore, we recorded an increase in syrphid larvae numbers with an increase in soil CN ratio (Table 1), likely driven by the positive effect of soil CN on plant height (Table 2) and the positive association between plant height and syrphid larvae abundance. Soil CN ratio was also higher in LD plots ($F_{1,59}$=4.87, *P*=0.031; Table 3) but was not affected by crop combination ($F_{3,59}$=1.82, *P*=0.153).

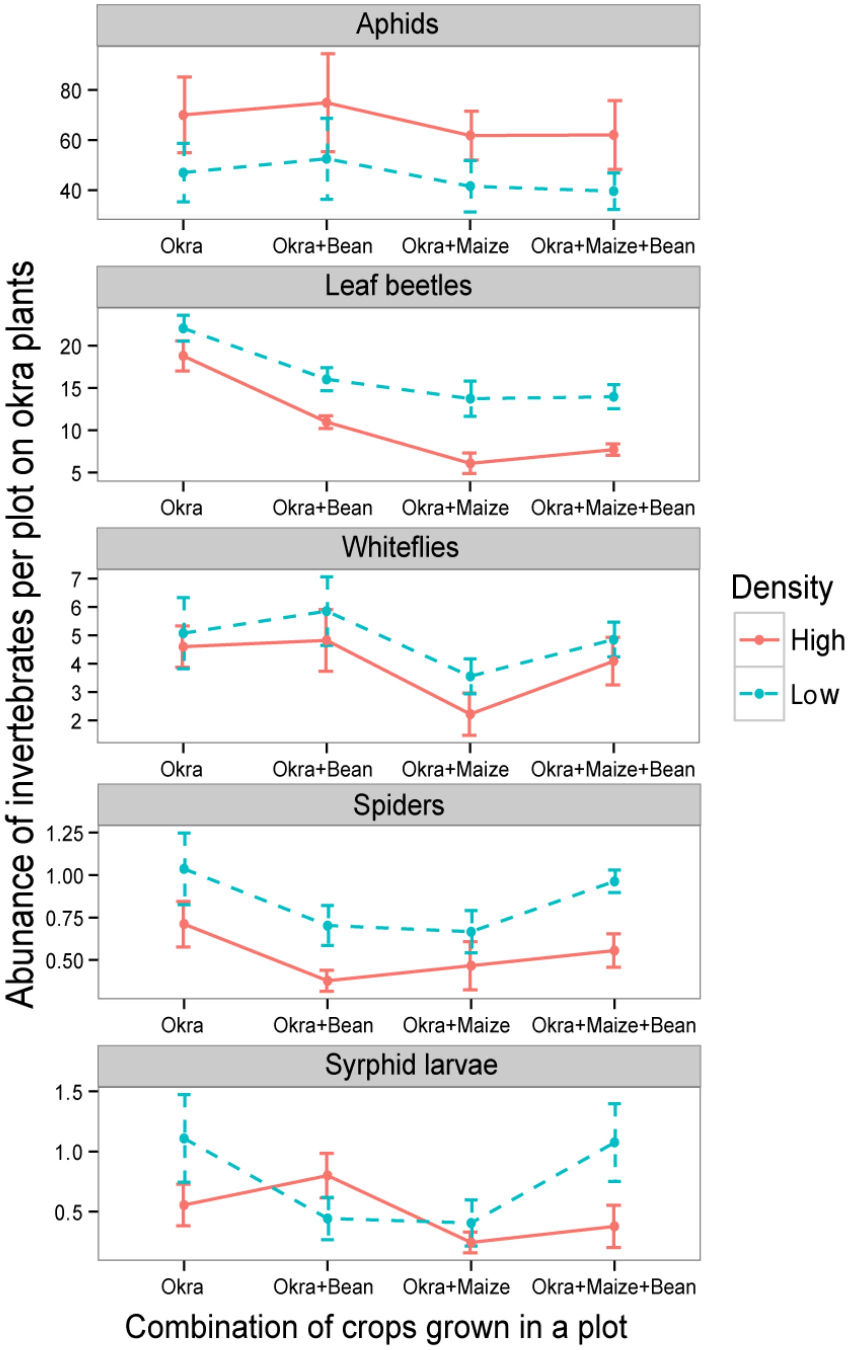

Figure 1: (a) Cumulative average abundance of okra pests (aphids, whiteflies and leaf beetles) and predator (spider and syrphid larvae) in different combination of crop plants grown in a plot, at different plant density. Error bars represent ± 1 SE.

3.2. Effect of main experimental variables and covariates on okra plant traits

Plant density of plots significantly affected okra plants and their yield (Table 2) and okra plant biomass, yield per plant (Figure 2) and % marketable fruits were all greater in LD than HD plots (Table 3), indicating some negative effects of high density. Higher yield per plant was also recorded in plots with a lower crop cover (Table 2); crop cover was lower in LD plots (Table 3).

Table 2: Effect of main experimental variables and covariates on okra plant traits

	Response variables			
	Plant biomass	**Plant height**	**Yield per plant**	**% marketable fruits**
Block	$F_{8,58}=5.39$, $P=0.001$	$F_{8,53}=2.23$, $P=0.039$	$F_{8,56}=12.89$, $P<0.001$	$F_{8,55}=0.99$, $P=0.452$
% crop cover	-	↑$F_{1,53}=9.61$, $P=0.003$	↓$F_{1,56}=11.66$, $P=0.001$	-
Soil carbon-nitrogen ratio	-	↑$F_{1,53}=4.09$, $P=0.048$	-	-
Plant height			↑$F_{1,56}=11.24$, $P=0.001$	
Plant biomass			↑$F_{1,56}=23.24$, $P<0.001$	-
Leaf beetle abundance	↑$F_{1,58}=25.79$, $P<0.001$	↑$F_{1,53}=8.73$, $P=0.005$	-	↓$F_{1,55}=11.35$, $P=0.001$
Plot plant density	↓$F_{1,58}=33.41$, $P<0.001$	↑$F_{1,53}=3.04$, $P=0.037$	↓$F_{1,56}=12.89$, $P=0.006$	↓$F_{1,55}=9.49$, $P=0.003$
Crop combination	$F_{3,58}=28.15$, $P<0.001$	$F_{3,53}=2.59$, $P=0.062$	$F_{3,56}=17.44$, $P<0.001$	$F_{3,55}=1.33$, $P=0.273$
Density×Combination	-	$F_{3,53}=3.89$, $P=0.014$	-	$F_{3,55}=2.07$, $P=0.115$

Linear models were used with normal distribution. '-' indicates when the value was not significant in a model and was removed from the final minimal model. Only those values are shown which were kept in the final minimal model. Covariates which were not significant for any of the response variables are not mentioned in the table. Arrow symbols indicate the direction of effect; ↑indicates a positive effect and ↓ indicates a negative effect.

Crop combination also affected plant biomass (Table 2) and individual okra plant biomass was highest in okra monocultures (11.2±0.94 g), followed by okra-bean (8.5±0.93 g), okra-bean-maize (7.1±0.91 g) and lowest in okra-maize (4.8±1.25 g) plots. Plant biomass in return had a strong effect on okra yield per plant (Table 2) and plants with higher biomass had a higher yield per plant. Yield per plant was therefore also highest in okra monoculture along with okra-bean plots and lowest in okra-maize plots (Table 2, Figure 2). Thus, there seems to be some negative effect of intercropping with maize on okra plants. However, this negative effect of maize on okra was negated to some extent by the presence of bean. This was evident in our 3-species plots where okra yield per plant was higher than in the 2-species okra-maize plots (Figure 2). Further, the increase in okra yield per plant from okra-maize to okra-bean-maize plots (despite no significant interaction), was higher in LD than in HD plots (Figure 2).

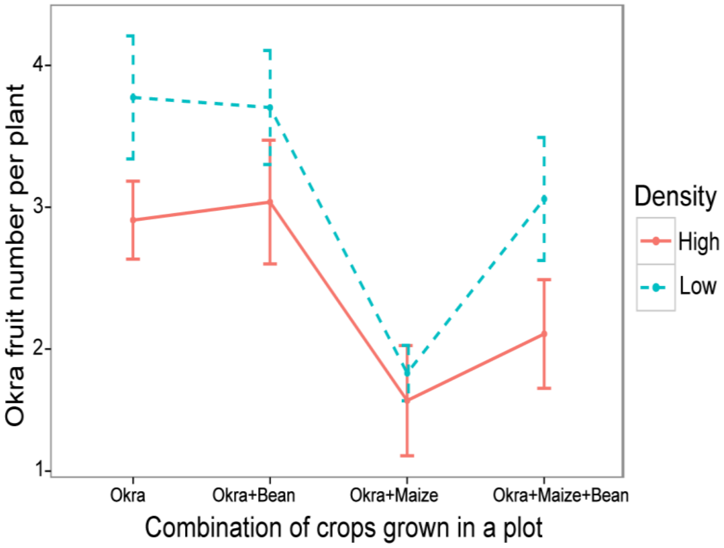

Figure 2: Okra fruit number per plant in different combination of crop plants grown in a plot, at different plant density. Error bars represent ± 1 SE.

Unlike plant biomass and yield, plant height was greater in HD plots (Table 3) and we recorded a significant 2-way interaction between density and combination on plant height (Table 2). Here, plant height was similar in okra monocultures in both low and high plant density plots; however, it was higher in intercropped HD plots (Figure S2). We also recorded plant height to increase with an increase in crop cover (Table 2).

Crop combination affected the % of marketable fruits but only in LD plots ($F_{3,23}$=3.04, P=0.049) and within the LD plots highest % of marketable fruits was recorded in okra-maize-bean (80.4±3.98%), followed by okra-maize (72.8±6.51%), okra monocultures

(67.2±6.26%) and least in okra-bean plots (58.4±6.11%). In HD plots all plants had lower % of marketable fruits irrespective of crop combination.

Table 3: Measure of different variables per plot at low and high plant density

Variable	Low Plant Density	High Plant Density
Plot variables		
% crop cover	↓ 53.1±1.42%	↑ 84.9±1.81%
% weed cover	↑ 46.7±2.75%	↓ 37.8±2.90%
Soil carbon-nitrogen ratio	↑ 12.2±0.14	↓ 11.8±0.18
Total okra fruit number	↓ 74.1±6.33	↑ 105.4±11.62
Invertebrates		
Aphid abundance	↓ 45.2±5.66	↑ 67.2±7.13
Leaf beetle abundance	↑ 16.5±0.90	↓ 10.9±0.92
Whitefly abundance	↑ 4.8±0.53	↓ 3.9±0.41
Spider abundance	↑ 1.2±0.07	↓ 0.5±0.06
Syrphid larvae abundance	↑ 0.8±0.14	↓ 0.5±0.08
Plant traits		
Okra plant height	↓ 47.2±1.02 cm	↑ 52.8±1.37 cm
Okra plant biomass	↑ 9.6±0.87 g	↓ 6.3±0.72 g
% marketable fruits	↑ 69.7±3.23%	↓ 55.9±4.40%

The values are given as mean ± 1 SE

Amongst the invertebrates only leaf beetles affected okra plant biomass and % marketable fruits (Table 2) and we recorded no effect of other invertebrates on okra plant biomass, yield per plant or on % marketable fruits. Percentage marketable fruits reduced with an increase in leaf beetle abundance (Table 2). However, plant biomass was positively associated with leaf beetle abundance (Table 2) and it was higher in plots with higher abundance of leaf beetle. This could have occurred as leaf beetle abundance was positively correlated with leaf numbers (r= +0.77, df= 70, P<0.001) which in turn was positively correlated with plant biomass (r= +0.88, df= 70, P<0.001), suggesting that leaf beetles colonized plants with more leaves, rather than leaf beetles positively affecting plant biomass.

Plant carbon-nitrogen ratio had no effect on any invertebrate abundance or on okra plants.

3.4. Relative land equivalent ratio (*RLER*), overall yield and resultant profit

RLER >1 indicates overyielding and *RLER*<1 indicates underyielding by an intercropped plot. Okra *RLER* significantly varied across crop combinations (X^2=142.85, df=2, *P*<0.001), and was affected by plot plant density (X^2=22.09, df=1, *P*<0.001). Okra *RLER* was higher in HD than in LD plots and it was greater than one in the presence of bean alone but in the presence of maize alone it was lower than one at both densities (Figure 3). We also recorded a 2-way interaction between crop combination and density on *RLER* (X^2=13.30, df=2, *P*=0.002); in the 3-crop combination bean negated the negative effect of maize on okra to some extent, but only in LD plots (Figure 3).

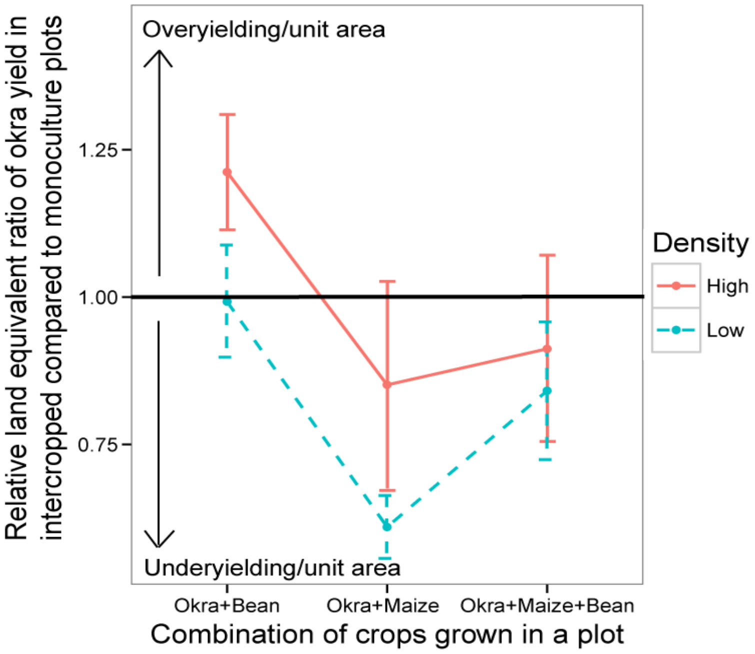

Figure 3: Relative land equivalent ratio of intercropped plots at low and high plant density. This was calculated by dividing okra yield per m^2 in an intercropped plot with okra yield per m^2 in a monoculture plot. Error bars represent ± 1 SE.

The resultant profit (total selling price – total input cost) to be gained varied significantly across crop combinations ($F_{3,59}$=8.32, *P*<0.001) and marginally by plot density ($F_{1,59}$=2.90, *P*=0.093), it was highest in okra-bean plots and lowest in okra-maize plots (Figure 4). For the okra-bean combination, the profit was higher for the HD than for the LD plots (t_{12}=1.89, *P*=0.041, Figure 4), even though there was no overall significant interaction between combination and plant density ($F_{3,64}$=0.51, *P*=0.674). Further, fertilizer usage (added per plant) was lowest in okra-bean (no fertilizer added to beans) and highest in HD okra monoculture plots (highest number of okra plants) (Table S2). Weed cover was also lower in HD than in LD plots and it was highest in okra monoculture plots (Figure S3). The most profitable design in our study was okra-bean grown at high plant density, whereas, okra-maize grown at low plant density was the least profitable (Figure 4).

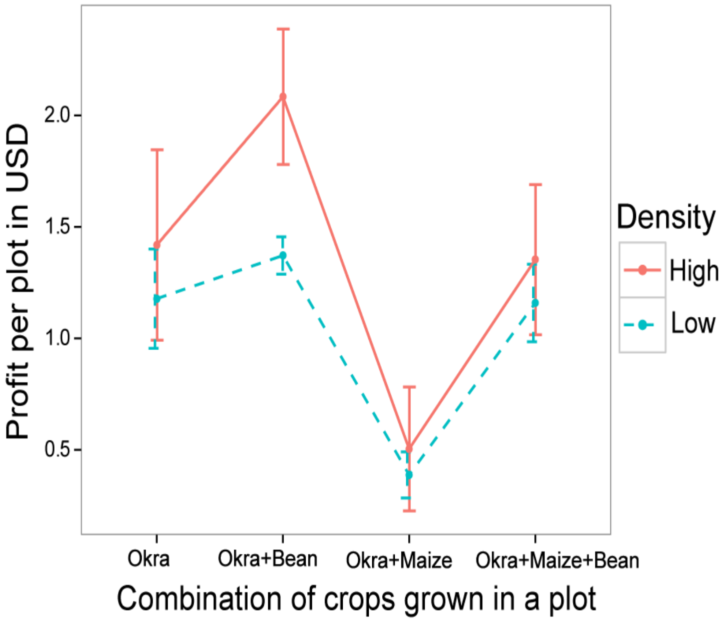

Figure 4: Economic profit per plot in USD in different combination of crop plants grown in a plot, at different plant density. Profit was calculated by subtracting the total selling price obtained from all crops per plot with cost of total inputs (seeds and fertilizers) used per plot. Error bars represent ± 1 SE.

4. Discussion

Overall, we found that crop identity was important in our system with bean plants benefiting the production of okra, and maize having a strong negative effect. Crop identity also had varying effects on different okra-associated invertebrate species with low abundance of okra pests and their predators in the presence of maize alone. Additionally, plant density within a plot, which is often ignored in intercropping studies, significantly affected not only plant traits but also invertebrate species as leaf beetles and whitefly pest species were reduced in plots with high plant density (HD). The optimal strategy in our study for highest resultant profit, lowest fertilizer input and overyielding (relative land equivalent ratio >1) was to grow okra at high plant density in combination with bean plants. From additive studies it is well-established for intercropping that per unit yield of primary crops is increased most when legumes are the secondary crop. In such systems, the polyculture yield often exceeds the monoculture yield (Iverson *et al.*, 2014). Unlike crop identity and density, we did not observe an effect of crop diversity on any invertebrate species except leaf beetles. There are a myriad of factors which can affect invertebrate abundance such as vegetation structure (Langellotto and Denno, 2004; Castagneyrol *et al.*, 2013), visual cues of plant species (Smith, 1976; Benelli and Canale, 2012; Döring, 2014), feeding range and host-specificity of pest and predator species (Root, 1973; Sheehan, 1986; Dassou and Tixier, 2016), presence of floral structures (Lavandero *et al.*, 2006; Ramsden *et al.*, 2015) and even plant nutrients (Joern *et al.*, 2012). Further, even studies showing positive effects of diversity on predator abundances have shown that this effect can vary at different trophic levels or due to factors such as intraguild predation and these can also be species-specific (Bianchi *et al.*, 2006; Straub *et al.*, 2008; Scherber *et al.*, 2010). Hence, simply increasing diversity in agroecosystems does not automatically reduce pests and enhance their predators (Poveda *et al.*, 2008). Functional traits and their dissimilarity across species play a crucial role in determining crop yield and pest and predator abundances (Heemsbergen *et al.*, 2004), and may even override the effects of diversity.

Variation in pest abundances across our different crop combinations was species-specific and could potentially be explained by their plant host-specificity and feeding range. Whitefly abundance was highest in okra monoculture plots and in the presence of bean, possibly as both bean and okra are their host plants. Whereas, leaf beetle (*N. uniformis*) abundance was highest in okra monoculture plots (1-species), and reduced with an increase in diversity in intercropped plots. Leaf beetles are oligophagous pests with a small host range, feeding dominantly on okra (Pitan and Ekoja, 2011). In accordance to the '*resource concentration*' hypothesis, a smaller host range could have led to observing highest leaf beetle abundance in okra monocultures (Root, 1973). On the contrary, the abundance of the highly polyphagous cotton aphids was not influenced by crop combinations as they feed on maize, bean and okra (Van Emden and Harrington, 2007). Further, reduction of leaf beetles and whiteflies in plots with maize could also be attributed to it being an effective barrier plant. Maize is taller and has a larger canopy than both okra and bean, possibly hindering pest host search and ultimately their abundance (Perrin, 1976).

We allowed natural colonisation of natural enemies in this experiment, and the main species were syrphid larvae and spiders (i.e. predators). Syrphid larvae abundance across crop combinations was mediated by density and it was higher in HD okra and bean plots. As okra and bean plants flowered earlier than maize, higher attraction of syrphid flies to HD okra-bean plots may have occurred due to high abundance of floral resources in these plots (Ramsden *et al.*, 2015). Pest abundance and habitat complexity affected the abundance of spiders. Spider abundances significantly increased with an increase in leaf beetles abundances, indicating that they perhaps share a similar habitat preference. Similar to leaf beetles, we recorded highest spider abundance in okra monocultures and in low density plots. Spider abundance was also higher in 3-crop plots probably due to increased habitat complexity in these plots, which in turn has been known to attract more invertebrates (more spider prey) (Langellotto and Denno, 2004). Thus, we can state that response of invertebrates in intercropping can be both, invertebrate and plant species-specific.

There was no strong effect of predators on any of the pest species, which can be attributed to low predator numbers (Table 3). However, spiders did have a marginal negative effect on aphids. Previous studies also found spiders to reduce aphids by only a small percentage (Birkhofer *et al.*, 2008; Diehl *et al.*, 2013), this may occur due to the wide-host range of generalists spiders as they get distracted by other pests. Studies so far have not tested which predators are specifically effective against *N. uniformis* leaf beetle species, but the control of whiteflies and other beetle species using predators has been mostly unsuccessful (Hare, 1990; Oliveira *et al.*, 2001).

Plant density (leading to variation in plant-soil contrast) and habitat structure had a strong effect on the invertebrates. Both, leaf beetles and syrphid flies (flying species), were attracted to taller okra plants, which would have been more visible and accessible than shorter plants (Lawton, 1983; Castagneyrol *et al.*, 2013). Higher abundance of aphids in HD plots occurred as it was easier for them to search for their host plants and they remained in plots with higher resources (Ralph, 1977; Bach, 1980; Obermaier *et al.*, 2008). However, whitefly abundances reduced in HD plots (more resources) and with an increase in crop cover. In HD plots there was higher crop cover, which could have hindered host-searching of whiteflies as these are flying species, particularly if they rely on the plant-soil contrast as a cue. The plant-soil contrast may also explain low abundances of leaf beetles in HD plots. Certain species are more attracted to green than brown backgrounds (Smith, 1976; Döring, 2014). Finch and Collier (2000) illustrated this further in a study where they intercropped cabbage with green and brown clover. They found that the eggs laid by cabbage pest species reduced only when the surrounding clover was green and not when it was brown. In LD intercropped plots okra rows were replaced by additional crops, whereas, in HD intercropped plots the bare soil was replaced with secondary crops (additional green surfaces). Hence, in HD intercropped plots leaf beetles possibly landed more on the additional green surfaces, resulting in their reduced numbers on okra. In HD okra monocultures, the additional green surfaces were okra plants and thus leaf beetle numbers only slightly reduced in these plots in comparison to other HD plots. Importance of visual contrasts on beetles has also been shown by previous studies (Stenberg and Ericson, 2007; Kühnle and Müller, 2011).

Leaf beetles were the only pests that affected okra yield through reducing the number of marketable fruits but this did not lead to leaf beetles reducing okra yield per plant. Instead yield per plant was significantly influenced by crop combination. It has been suggested that other than pests, competitive or facilitative interactions amongst crops strongly affect yield (Poveda *et al.*, 2008). For example, in HD plots with beans there was likely a higher availability of plant resources as beans can fix soil N (Brooker *et al.*, 2015), facilitating greater okra yield even in the absence of additional fertilizer to the bean plants. On the contrary, maize requires large amount of nutrients (Roy *et al.*, 2006) and is a tall plant with a wide canopy, leading to reduced sunlight for okra plants (crop cover was ~60% in LD and ~95% in HD okra-maize plots). Beans did not cover okra plants. Therefore, when okra was grown alone with maize, competition for nutrient and light resources was higher, resulting in reduced okra yield. However, the competitive effect of maize on okra was reduced in the presence of bean as okra yield increased when bean was grown along with okra and maize (3-species plots). Nevertheless, this positive effect of bean was stronger in LD plots, possibly due to even greater competition for resources (more abiotic stress) with higher maize plant numbers in 3-species HD plots (Callaway, 2007; García-Cervigón *et al.*, 2013).

Reduced sunlight due to maize could also explain why we recorded okra plant height to increase in maize presence in HD plots and with an increase in crop cover. Increase in stem elongation in dense vegetation is generally believed to be induced by canopy shading as plants grow tall to obtain sufficient light (Anten *et al.*, 2005) and allocate more resources to stem than to other parts (Poorter *et al.*, 2012). We did not record significant variation in soil CN across crop combinations likely because samples were taken only once at the end of the season potentially after the main facilitation time point. However, N-fixing by beans, high nutrient requirements and large canopy cover of maize are all well-established mechanisms, so their potential effect on the okra plants cannot be ruled out.

We obtained highest total yield and economic profit from HD plots, and even overyielding ($RLER$>1) from HD okra-bean plots. Weed cover was also lower in HD and intercropped plots, which would further reduce labour cost and also competition for the harvested crops. Hence, we suggest that okra-bean grown at high plant density is the most efficient design in our system.

Overall, our study focuses on the importance of suitable functional plant diversity. Functional traits of secondary crop species should be screened to determine key elements which affect pest and predator abundances and ultimately result in improved yield (Landis *et al.*, 2000). Furthermore, we also show how plant density affects pests and even mediates the effect of crop identity. With an ever growing human population and continually exploiting natural resources, it is crucial to design sustainable cropping systems with an aim to achieve the maximum yield from available land. Legumes such as beans can be included in designs when nutrient limitation is the major obstacle and barrier crops such as maize can be included in designs when pests are the major obstacle in crop production. Our study adds to the much needed knowledge in agroecological research to eliminate yield gaps in sustainable agriculture (Ponisio *et al.*, 2015). A system such as ours built on traditional practices would be more acceptable by subsistence farmers and meet their socio-economic needs.

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Supplementary material

Minimize space, maximize production: Benefits of intercropping at high densities for okra farmers in Cameroon

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Table S1: Total number of okra, bean and maize plants and total number of okra, bean and maize planting rows, within a plot for each treatment combination.

Crop combination in a plot	Density	Crop	Number of rows	Number of plants
Okra monoculture	Low	Okra	5	50
Okra monoculture	High	Okra	10	100
Okra+Maize	Low	Okra	3	30
Okra+Maize	Low	Maize	2	38
Okra+Maize	High	Okra	5	50
Okra+Maize	High	Maize	5	95
Okra+Bean	Low	Okra	3	30
Okra+Bean	Low	Bean	2	72
Okra+Bean	High	Okra	5	50
Okra+Bean	High	Bean	5	180
Okra+Bean+Maize	Low	Okra	3	30
Okra+Bean+Maize	Low	Maize	1	19
Okra+Bean+Maize	Low	Bean	1	36
Okra+Bean+Maize	High	Okra	5	50
Okra+Bean+Maize	High	Maize	2-3	38-57
Okra+Bean+Maize	High	Bean	2-3	72-108

Table S2: Total weight of fertilizer used and seeds used in each plot, for each treatment combination

Crop combination in a plot	Density	Total fertilizer used (gms)	Total weight of okra seeds sown (gms)	Total weight of bean seeds sown (gms)	Total weight of maize seeds sown (gms)
Okra	Low	950.0	9.0	-	-
Okra	High	1900.0	18.0	-	-
Okra+Maize	Low	931.0	5.4	-	36.5
Okra+Maize	High	1852.5	9.0	-	91.2
Okra+Bean	Low	570.0	5.4	73.4	-
Okra+Bean	High	950.0	9.0	183.6	-
Okra+Bean+Maize	Low	750.5	5.4	36.72	18.24
Okra+Bean+Maize	High	1311.0/1491.5	9.0	73.4/110.2	36.5/54.7

Table S3: Effect of crop diversity on okra invertebrates, okra plant height, biomass and yield

	Crop diversity index of a plot		
Response variables	**Df**	**F**	**P**
Aphid abundance	1,69	0.12	0.736
Leaf beetle abundance	**1,69**	**22.84**	**<0.001**
Whitefly abundance	1,69	0.15	0.697
Spider abundance	1,69	0.76	0.386
Syrphid abundance	1,69	0.21	0.645
Okra plant height	1,69	1.39	0.241
Okra plant biomass	**1,69**	**7.41**	**0.008**
Okra yield per plant (Y_p)	1,69	1.39	0.241
% marketable fruits (M_p)	1,69	1.82	0.182

Linear models were used to analyse the data. All significant values are highlighted in bold.

O	Okra high density
M	Maize high density
B	Bean high density
O	Okra low density
M	Maize low density
B	Bean low density

Legend

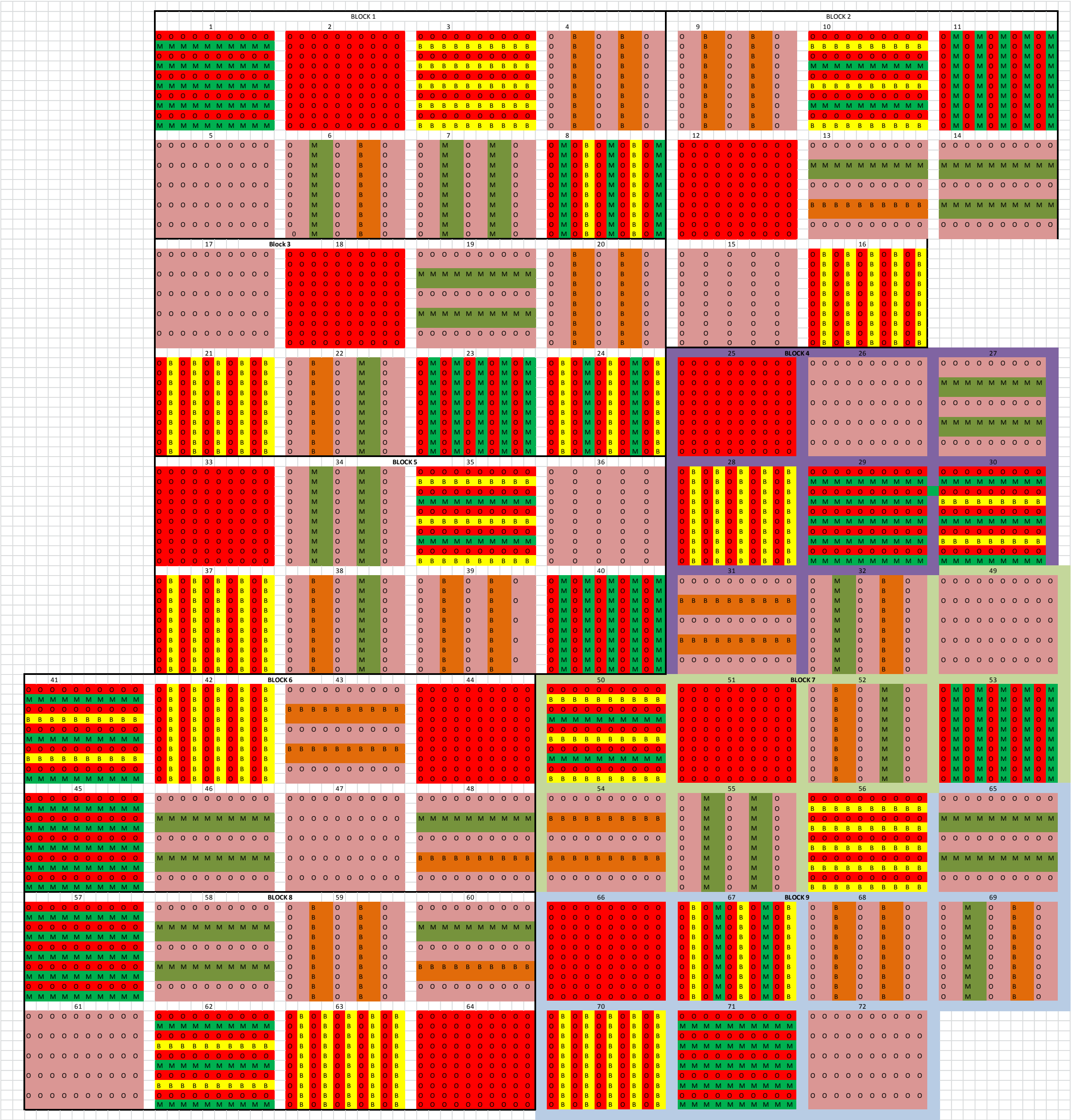

Figure S1: Layout of blocks and treatments in the field.

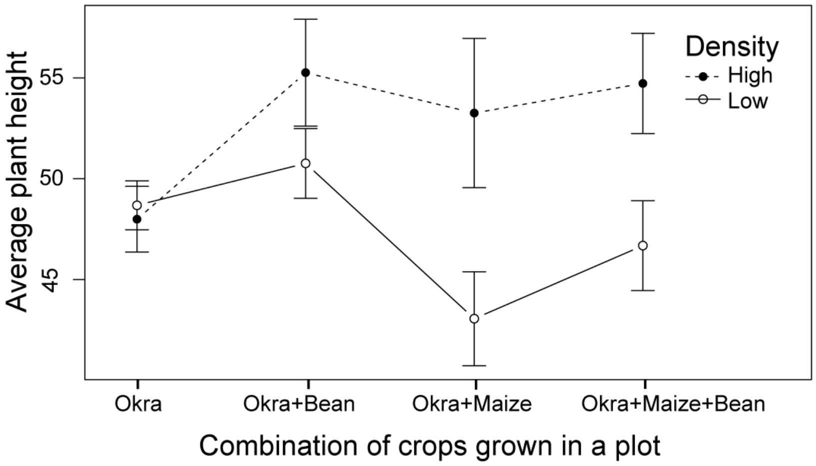

Figure S2: Cumulative average plant height per plot in different crop combinations at different plant density. Error bars represent ± 1 SE.

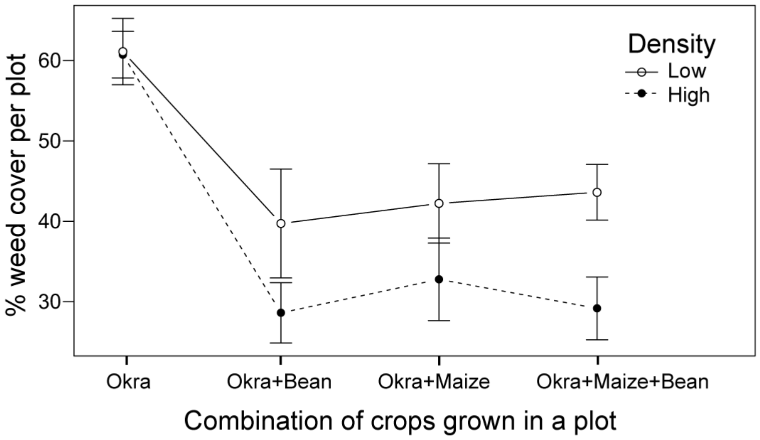

Figure S3: Percentage average weed cover per plot in different crop combinations at different plant density. Error bars represent ± 1 SE.

Manuscript II

Ant attendance of the cotton aphid is beneficial for okra plants: Deciphering multitrophic interactions

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Ant attendance of the cotton aphid is beneficial for okra plants: deciphering multitrophic interactions

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Abstract

1 Aphids are pest species of many crops and biocontrol methods are often ineffective. Ant–aphid associations can be mutualistic or antagonistic, with ants increasing or reducing aphid numbers. Within-species plant variation or other herbivores may further influence these ant–aphid interactions.

2 Okra is an economically important crop in Cameroon. Several okra varieties are grown here and attacked by the facultatively ant-tended cotton aphid *Aphis gossypii*. We conducted field and screenhouse experiments where plant variety, ant presence and predator access were manipulated to investigate the multitrophic interactions on okra and their effects on okra yield.

3 In the field, ants did not protect aphids from their natural enemies and syrphid larvae reduced aphids by 42%. Additionally, aphid recruitment of ants reduced chewing herbivore damage by 11% and indirectly increased okra fruit set. We also found aphid numbers, aphid predation by syrphids and chewing herbivory to vary across okra varieties. Finally, in the screenhouse, we recorded a 24% reduction in aphid numbers on plants with ant presence.

4 The present study highlights the importance of direct and indirect biotic interactions for pest biocontrol. Tropical agricultural systems are complex and understanding such interactions can help in designing pest control measures in sustainable agriculture.

Keywords Ant–aphid, biocontrol, interactions, multitrophic, plant varieties.

Introduction

Aphids are economically important pests that are responsible for a reduction in yield on many agricultural crops worldwide (van Emden & Harrington, 2007). Pest-resistant plant varieties can help to reduce the impact of aphid outbreaks on crops, although these can be expensive and time-consuming to develop (McCouch *et al.*, 2013). Alternatively, biological control measures can be used to control aphid populations, which usually focus on enhancing aphid natural enemy abundance (Powell & Pell, 2007). The introduction of a novel biocontrol agent is not always successful in the long-term regulation of pest populations, mainly as a result of a mismatch in climate between the native and introduced range of the agent, the lack of an alternate food source and/or predation/parasitism by native fauna of the agent (Stiling, 1993). Native fauna such as ants are known mutualists with aphids and often protect aphids against their natural enemies in return for the aphid honeydew (Way, 1963; Buckley, 1987; Völkl *et al.*, 1990; Kaplan & Eubanks, 2005). Such interactions can hinder biocontrol efficiency and the factors that can maintain or enhance natural enemy populations for pest regulation are still relatively unclear (Rusch *et al.*, 2010).

Other than protecting the aphids from natural enemies, ants can further benefit aphids by removing sticky honeydew and fungal-infected aphid cadavers, which would otherwise support fungal growth, leading to reduced aphid survival (Nixon, 1951; Nielsen *et al.*, 2009). Ants can also benefit aphids by increasing their body size, longevity and reproduction rate (Stadler & Dixon, 1999; Flatt & Weisser, 2000). However, ant–aphid mutualisms do vary from obligate (close) to facultative (occasional) and it is well reported that obligate ant-tended aphids are better protected by ants than facultative ant-tended aphids

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(Stadler & Dixon, 2005). Furthermore, ants do not always benefit aphids and their association with aphids can be antagonistic; for example, when ants negatively affect aphid growth and development (Stadler & Dixon, 1998; Yao *et al.*, 2000; Stadler *et al.*, 2002) or even prey on aphids (Rosengren & Sundström, 1991; Sakata, 1995; Stadler & Dixon, 2005).

More recently, plant genotype has been shown to influence whether ant–aphid associations are mutualistic or antagonistic (Mooney & Agrawal, 2008; Abdala-Roberts *et al.*, 2012). Ant attendance has been shown to increase on higher quality host plants, probably as a result of higher quality honeydew (Stadler *et al.*, 2002). Aphid performance and preference also varies across different plant genotypes or varieties (Zytynska & Weisser, 2016). This could further influence the effect of ants on the aphids, particularly if the interaction is density-dependent, with ant predation being more likely with an increase in aphid numbers (Rosengren & Sundström, 1991; Sakata, 1995). If ants and aphids are influenced by host-plant quality, then other factors that alter host-plant quality could also indirectly mediate ant–aphid interactions. For example, leaf chewers both reduce the biomass of a plant and can induce anti-herbivore plant chemical defences (Walling, 2000). There is strong evidence for the effect of within-species plant variation on its associated invertebrate community (Whitham *et al.*, 2012) and this could further mediate the ant–aphid interaction (e.g. flea beetle abundance has been shown to vary across soybean plant genotypes) (Underwood & Rausher, 2000). Although studies have investigated the effect of plant traits on biocontrol efficiency (Cortesero *et al.*, 2000; Inbar & Gerling, 2008), the effect of plant within-species variation on multitrophic interactions is still understudied in agricultural systems. Furthermore, understanding ant–aphid associations is crucial because these can be keystone interactions influencing the arthropod communities on plants and, in return, influence plant fitness (Styrsky & Eubanks, 2007; Zhang *et al.*, 2012).

Okra (*Abelmoschus esculentus* Moench) is an economically important vegetable crop grown worldwide and is widely consumed in West Africa. In Cameroon, the cotton aphid (*Aphis gossypii* Glover) is one of the dominant pests of okra (Leite *et al.*, 2007; Shannag *et al.*, 2007) and has evolved resistance to pesticides, particularly on cotton plant (Brévault *et al.*, 2008). An annual survey conducted by the International Institute of Tropical Agriculture (IITA) in Cameroon (2011) found that okra farmers grow many different okra varieties, various ant species attend aphids on okra, and ants of genus *Pheidole* were the dominant ant species found attending aphids on okra plants in 75% of the surveyed okra farms (IITA annual survey report, 2011). The cotton aphid is a facultative ant-tended species and therefore its interaction with ants may vary. When suggesting aphid control measures, it is crucial not only to understand the ant–aphid interaction, but also to find varieties with lower pest abundances and a higher yield. Thus, we conducted a field and a screenhouse experiment to test the hypotheses: (i) predators reduce aphid numbers in the field; (ii) ants protect aphids from their predators; (iii) okra variety influences the ant–aphid association; (iv) the ant–aphid association will influence plant growth and okra yield; and (v) the ant–aphid association can affect okra-associated invertebrates (aphid predators and additional okra herbivores), or vice versa, and this in turn can affect okra plants.

Materials and methods

Study site and study species

The experiments were conducted at the IITA research station in Yaoundé, located in the central region of Cameroon (West Africa). We conducted a field experiment and a controlled screenhouse experiment within the research site. Screenhouses are made of a greenhouse frame but are covered with a double layer of fine net to avoid any insects from entering, at the same time as allowing air to circulate from the outside. Our study consisted of cotton aphids (*A. gossypii* Glover) and ants (*Pheidole dea* Santschi) on okra *A. esculentus* Moench.

Okra is mostly grown in humid climate in sandy and clay loam soils and its optimum growing temperature is estimated to be between 24 and 30 °C. The plants are an annual erect herb (height 2–4 m) with lobed and hairy leaves. It is a self-pollinating crop, although insects, especially bees, are attracted to the flowers and hence cross-pollination occurs (Tripathi *et al.*, 2011). Okra plants are attacked by many pests at different growing stages, such as the cotton aphid and beetles (Benchasri, 2012). In our experiment, four varieties of okra were used: *Clemson* (Les Doigts Verts, France), *Bangourain*, *Caffeier* and *Kirikou* (obtained locally from Dschang, Northwest Cameroon). These differ in their growth pattern (*Clemson* and *Kirikou* grow faster than *Caffeier* and *Bangourain*), leaf size (*Caffeier* and *Bangourain* have larger leaf size area than *Clemson* and *Kirikou*) and fruit shape (*Clemson* has longer, slender fruits and the others have broader, shorter fruits) (Akanksha Singh, personal observation). These also vary in their mucilage content (a trait associated with consumer preference), with high mucilage content in *Caffeier* and low content in *Clemson* (Albert Abang, personal communication) and such variation in mucilage content has been studied amongst okra accessions (Ahiakpa *et al.*, 2014).

Aphis gossypii colonizes more than 600 host plants across a wide geographical range and vectors more than 50 plant viruses (van Emden & Harrington, 2007). In tropical climates, this facultative ant-tended aphid undergoes mostly parthenogenetic (i.e. asexual) reproduction, leading to an exponential growth rate at optimal conditions. The aphids were reared on *Clemson* okra in an insectary in IITA Cameroon under a 14 : 10 h light/dark photocycle at 24.1 °C and 71.2% relative humidity prior to use in the experiments.

Pheidole dea are ground-dwelling ants that form large colonies. This species has been recorded in afro-tropical countries such as Cameroon and Uganda and details of their diet are relatively unknown (Fischer *et al.*, 2012). The ants used in the screenhouse experiment were collected from the experimental field site and maintained in field soil, inside plastic containers (depth 8 cm, diameter 14 cm) as a queen and approximately 100 workers. We first applied tape at the rim of the container and then Tanglefoot (a sticky insect barrier; Contech Inc., Spartanburg, South Carolina) was spread on the tape to prevent ants from escaping. Ants were given sugar solution and insect protein (crickets) to maintain the colony.

Seed germination

The seeds were soaked in water in darkness for 24 h. Then one seed per pot (depth 8 cm, diameter 14 cm) was sown in sterilized soil (25% sand, 25% fowl manure and 50% soil) and left to germinate for 10 days in the open. From the date of sowing, we used 5-week-old plants for the field and 3-week-old plants for the screenhouse experiment. We used older plants for the field experiment because they are more resilient against weather conditions and damage from other invertebrates experienced in the field.

Field experiment

We first conducted a field study to test: whether predators would reduce aphid numbers in the field; whether ants would protect aphids from their predators; whether the ant–aphid association would vary across okra varieties; and the effect of ant–aphid association on okra plants and other okra-associated invertebrates (aphid predators and additional herbivores).

Experimental design. We used a fully factorial randomized block design with 16 treatments including four okra varieties (*Clemson*, *Kirikou*, *Caffeier* and *Bangourain*), two ant treatments (presence and absence) and two cage treatments [open (predator/chewing herbivore presence) and closed (predator/chewing herbivore absence)]. The insect cages were 0.4 × 0.4 × 0.7 m (length × breadth × height), constructed as a frame of polyvinyl chloride piping, covered with a white fine-mesh cotton cloth. Our 'closed' cages were completely covered with mesh, whereas 'open' cages had an opening on all four sides measuring 0.3 × 0.5 m to allow colonization of the plant by the natural invertebrate community. We used 'open' cages for predator/herbivore presence rather than no cage to ensure that the results were not biased as a result of a cage effect. For ant presence, small V-shaped wooden bridges were constructed connecting the ground with the soil in the pot and, for ant absence, we applied Tanglefoot at the base of the stem of the plants. Each treatment was replicated 10 times ($n = 160$), with one potted plant per cage. We placed these in 10 blocks to control for spatial variation across the field, with one replicate per treatment in each block and treatments randomized within block (4 × 4 cages). Within a block, each cage was 0.6 m from the adjacent cages, with a distance of 1.6 m between blocks. The field experimental site measured 27 × 18 m and was surrounded by two plantain fields, an old okra field and fallow land.

Experimental set-up. The experiment started on 1 April 2013. Blocks 1–5 and 6–10 were set up on two consecutive days. Pots were placed on the ground within the cages. After measuring initial plant height and leaf number, 10 aphids (four or five adults and the remainder of earlier ages) were introduced to each plant. Any vegetation around the pots that was touching the experimental pots was removed. Ants and other invertebrates colonized the plants naturally.

Data collection (8 April to 7 May 2013). One week after the experiment was set up, we began to take readings. Data were collected once per plant per week over two consecutive days (one day from blocks 1 to 5 and the consecutive day from blocks 6 to 10) over a period of 4 weeks. The variables recorded per plant were: leaf number, aphid number (total per plant, using a hand tally counter), ant attendance (total number of ants per plant attending aphids during 1 min), ant species, leaf beetle number, foliage remaining (percentage residual leaf tissue after damage from chewing herbivores of all leaves combined, per plant), syrphid larvae number, number of parasitoid mummies and spider number. In the final observation, we also recorded the plant height. A plant was harvested when the first fruit had matured up to a minimum of 7 cm in length. We also recorded the day (number of day after sowing the seeds) on which fruit was collected from each plant. We starting collecting okra fruits on day 78 and fruit collection continued until day 105. Fruits were bagged in paper bags and dried in an oven for 3 days at 60 °C to measure the dry biomass.

We also measured temperature and humidity in open and closed cages using Hobo data loggers (Onset, Cape Cod, Massachusetts). In the open cages, the mean temperature was 24.3 ± 0.1 °C (range 21.0–32.9 °C) and mean humidity was 87.4 ± 0.5% (range 51.6–100%). In closed cages, we recorded a mean temperature of 24.9 ± 0.3 °C (range 20.9–34.7 °C) and a mean humidity of 85.1 ± 0.9% (range 42.9–100%). Mean rainfall during the course of the experiment was 10.3 ± 2.8 mm (range 0–55.9 mm) with a 12 : 12 h light/dark photocycle.

Screenhouse experiment

In the field experiment, aphid predators and herbivory by a leaf beetle could have influenced the ant–aphid interactions on okra. Hence, we also conducted a controlled screenhouse study for a clearer understanding of whether ants benefit aphids in our system or not.

Experimental design. We used a fully-factorial randomized block design with three okra varieties (*Clemson*, *Kirikou* and *Caffeier*) and two ant treatments (presence and absence). In total, there were six treatments with eight repeats per treatment combination (48 plants). We used eight blocks within the screenhouse with one repeat of each treatment in each block. The cages were placed on two separate tables (four blocks per table). Each plant was placed inside entirely enclosed plastic-polypropylene insect cages (1350 μm mesh opening) measuring 30 × 30 × 30 cm (length × breadth × height) (Megaview Science, Taiwan).

Experimental set-up. This experiment was set up on 29 May 2013 and terminated on 13 June 2013. On day 1, one plant was placed inside each insect cage and 10 adult aphids were introduced onto each plant using a fine paintbrush. Ant colonies were introduced 48 h after (on day 3) the introduction of aphids using a small V-shaped wooden bridge connecting the ant colony with plant. In addition, throughout the duration of the experiment, ant colonies were provided with protein (crickets collected from experimental field site) to avoid forced predation on aphids by the ants. Mean temperature in the screenhouse was 25.2 ± 0.2 °C (range 21.3–31.4 °C), mean humidity was 79.4 ± 0.5% (range 37.4–97.8%) and during the 12 : 12 h light/dark natural photocycle, additional lighting was used.

Data collection (29 May to 13 June 2013). On first day, we measured the height and leaf number of the plant. On day 5, 48 h after introduction of ant colonies (day 3), we started our observations. We conducted two different forms of observations of the experimental plants for 9 days. For the first one, all 48 experimental plants were sampled twice each day (morning and evening) and the numbers of ants attending aphids per plant during 1 min were recorded. For the second one, we selected six plants every day (two of each variety) out of the 48 plants and these were also observed twice for 10 min each (morning and evening) to record whether ants were tending the aphids or predating upon them. For these second observations, the same plants were observed morning/evening on the same day but different plants chosen across the 9 days, resulting in two or three observations per plant. On the final day (day 15), we recorded data on aphid colony size, plant height and leaf number.

Statistical analysis

Field experiment. Ants attended aphids on all plants with ant presence treatment except on four plants where ants were never observed throughout the experiment. Similarly, on four plants with ant absence treatment, ants of *Camponotus* and *Pheidole* genus were observed attending aphids as a result of the ants building a soil-bridge to navigate across the tanglefoot barrier. Cages effectively excluded predators from all but two cages in which syrphid larvae were observed; these two plants were removed from our analysis. In addition, we removed the following plants from our analysis: two plants in open cages that died as a result of excessive herbivory during week 1 by individuals of an unidentified grasshopper; a further 41 plants in which aphid extinctions occurred in weeks 1 (88% of the extinctions), 2 and 3; and three plants in which only one aphid was present throughout the experiment. Hence, in total, 48 plants were removed from our analysis, giving us a final sample size of 112 plants, with six to eight repeats per treatment.

Aphid per capita growth rate (aphid GR) was calculated using the formula: $[\ln(N_x) - \ln(N_s)]/t$, where, N_x is aphid number in a particular week, N_s is aphid number at the start of the experiment (i.e. 10 aphids) and t is the duration of the experiment (days). Plant relative growth rate (plant RGR) was used to correct for plant height variation amongst varieties; this was calculated using the formula: [ln(final plant height) − ln (initial plant height)]/total number of days. Linear models were used to analyze the data.

We first tested for the effect of our main explanatory variables cage treatment (predator present and absent), ant treatment (ant present and absent) and plant variety. For the aphid extinctions in week 1 (1, 0; extinction, no extinction) we fitted a generalized linear model (GLM) with quasibinomial distribution. For aphid GR (week 3), plant RGR and fruit biomass, we fitted normal linear models; for day of fruit collection, we fitted GLM with quasipoisson distribution with additional covariates plant RGR and aphid GR, which were included when these were not the respective response variables. Additionally, day of fruit collection was included as a covariate in our analysis for fruit biomass. The main explanatory variables, and their interactions, were included in all five models described above.

Because ant abundance varied across ant-present plants, we also analyzed the effect of ant abundance on aphid GR, plant RGR and fruit biomass. Only data originating from ant-present plants were used. Here, our model included plant variety and cage treatment as the main explanatory variables and ant abundance (mean number of ants recorded per observation) as a covariate. Plant RGR and aphid GR were included as covariates when these were not the respective response variables.

Furthermore, leaf beetle abundance, percentage foliage remaining and frequency of presence of syrphid larvae also varied across plants. Hence, we analyzed the effects of the degree of herbivory and predation by syrphid larvae on aphid GR, plant RGR and fruit biomass. For this, only data originating from open cages were used. Our linear model included plant variety and ant treatment as the main explanatory variables and foliage remaining (percentage residual leaf tissue), leaf beetle abundance (mean number of leaf beetles recorded per observation) and syrphid larvae (presence/absence) as covariates. Plant RGR and aphid GR were included as covariates when these were not the respective response variables.

Finally, we analyzed the effect of our treatments on the secondary response variables ant abundance (week 3) (data used: ant-present plants), syrphid larvae presence/absence, leaf beetle abundance (week 3) and foliage remaining (week 3) (data used: open cages plants). Here, for ant abundance, our model included cage treatment (predator present and absent) and plant variety as main explanatory variables and plant RGR and aphid GR as covariates.

For syrphid larvae (presence, absence), we applied a GLM model with quasibinomial distribution. Foliage remaining data (%) were arcsine transformed before the analysis. Standard linear models were used for foliage remaining and leaf beetle abundance analysis. Ant treatment and plant variety were included as main explanatory variables and aphid GR, plant RGR and ant abundance as covariates. Foliage remaining and leaf beetle abundance were included as covariates when these were not the respective response variables.

Screenhouse experiment. One ant-present plant of *Clemson* variety was removed from the analysis because no ant attendance was observed on it during the experiment. The data were analyzed for two main response variables: aphid GR and plant RGR. For these, we fitted linear models and our main explanatory variables were ant treatment and plant variety. To analyze the effect of ant abundance on our main response variables, data originating from ant-present plants were used. Here, plant variety was our only explanatory variable. Aphid GR and plant RGR were included as covariates for both the total and split data when these were not the respective response variables.

We also analyzed data for ant abundance on a plant, as a response variable and, for this, only ant-present plants were used. The observation time for ant attendance was 2 min per plant per day for focal plants (0.1% of the day) and ants were not always present on the plant during the observation. Thus, we chose the maximum ant number per plant over the full observation period to be included in our analysis as a variable for the effective representation of ant abundance. Here, we applied a GLM model with quasipoisson distribution with plant variety as our main explanatory variable and plant RGR and aphid GR as covariates.

Table 1 The number of extinctions and aphid numbers throughout the field study

	Open (predator present)		Closed (predator absent)	
Data collection week	Number of extinctions	Aphid number	Number of extinctions	Aphid number
Week 1	19	54.4 ± 16.0	17	72.31 ± 9.6
Week 2	2	129.0 ± 23.4	0	336.0 ± 45.2
Week 3	3	77.9 ± 12.9	0	687.5 ± 76.2
Week 4	5	121.9 ± 59.4	0	753.5 ± 101.2

Aphid number is given as the mean ± SE.

All data were analyzed in R, version 3.2.2 (The R Project for Statistical Computing, Austria) using RStudio version 0.98.978 (Rstudio, Boston, Massachusetts). For all the variables that we tested, we used Type I sum of squares; we first fitted a full model with all main effects and all interactions between the main effects. Then, all the nonsignificant effects and interactions (starting from the highest interaction order) were removed for simplification of the final model. Descriptive statistics are reported as the mean ± SE.

Results

Field experiment

Aphid, ant, predator and herbivore observations. Thirty-six plants had no aphids in the first week; out of these, 19 plants (9/19 in ant presence) were in open cages and 17 in closed cages (7/17 in ant presence) (Table 1). The number of extinction events was significantly higher in ant absence than in ant presence ($F_{1,142} = 4.20$, $P = 0.042$) and there was an interaction between plant variety and cage treatment ($F_{3,142} = 2.95$, $P = 0.034$). On *Caffeier* and *Clemson*, aphid extinction events were lower in open cages; on *Bangourain*, these were similar in open and closed cages and, on *Kirikou*, these were higher on plants in open cages (predator present). In the subsequent weeks, there were much fewer extinction events and all occurred in open cages (Table 1). Aphid numbers increased in closed cages from week 1 to week 4 but, in open cages, aphid numbers fluctuated across the weeks (Table 1).

In open cages, we observed predators in 57% (31/54) of the cages. Amongst the predators, we recorded syrphid larvae on 54% (29/54) and spiders on 12% (7/54) of the plants. Aphid parasitoid mummies were found on 5% (3/54) of the plants. *Pheidole dea* was the dominant ant species recorded on ant-present plants and was found attending aphids on 99% (66/67) of the ant-present plants. Other ant species observed were: *Tapinoma carininotum* (Weber) on 22% (15/67) and *Camponotus flavomarginatus* (Mayr) on 6% (4/67) of the plants. We observed 14.1 ± 1.8 ants per sampling effort (i.e. per plant). Ant presence on plants increased from 47% (32/67) on the first week up to 79% (53/67) in the subsequent weeks.

We also observed leaf beetles (*Nisotra uniformis* Jacoby) on 83% (45/54) of the plants in open (chewing herbivore present) cages. Mean leaf beetle numbers per plant increased from 0.4 ± 0.2 at week 1 to 2.1 ± 0.5 and 2.2 ± 0.4 at weeks 3 and 4, respectively. No leaf tissue loss or leaf beetles were recorded in closed (chewing herbivore absent) cages plants. Foliage remaining (percentage residual leaf tissue) also reduced over the weeks from a mean of 89.8 ± 2.1% at week 1 to 78.9 ± 2.8% and 70.8 ± 2.7% at weeks 3 and 4, respectively.

Few predators and ants were observed on the plants in week 1 but, by week 2, most okra plants were colonized by invertebrates and okra fruits had also started to appear by observation week 4. Hence, we report the results from week 3 to explain the effect of our treatments on our response variables. Descriptive statistics are given as the mean ± SE.

Effect of main experimental variables. Aphid GR was lower in open (predator present) cages than in closed (predator absent) cages and differed across okra varieties (Table 2). Overall, aphid GR was higher on larger plants (Table 2). In open cages, the highest aphid GR was observed on *Caffeier* and least on *Bangourain*, whereas, in closed cages, *Caffeier* and *Bangourain* both had the highest aphid GR (Fig. 1). In addition, we found no overall main effect of ant presence/absence on aphid GR (Table 2).

Plant RGR varied amongst the different varieties (Table 2). *Caffeier* and *Clemson* grew the least and *Kirikou* showed the highest growth. Cage treatment affected plant RGR (Table 2) and plants grew more in the closed cages (12.2 ± 0.5 cm) than in open cages (9.3 ± 0.6 cm). This is probably the result of a lack of chewing herbivory.

Table 2 Effect of main experimental variables on aphid growth rate, plant relative growth rate and the resulting biomass of harvested fruit from the okra plants

	Aphid growth rate			Plant relative growth rate			Fruit biomass		
Explanatory variables	d.f.	*F*	*P*	d.f.	*F*	*P*	d.f.	*F*	*P*
Day of fruit collection							–	–	–
Aphid growth rate				**1,103**	**11.14**	**↑0.001**	–	–	–
Plant relative growth rate	**1,103**	**10.92**	**↑0.001**				**1,100**	**3.98**	**↑0.042**
Ants (P/A)	1,103	1.77	0.186	1,103	0.58	0.447	1,100	0.72	0.399
Cage (open/close)	**1,103**	**76.26**	**<0.001**	**1,103**	**3.54**	**0.050**	1,100	0.03	0.617
Plant variety	**3,103**	**2.94**	**0.036**	**3,103**	**12.51**	**<0.001**	3,100	2.05	0.479
Cage × Ants	–	–	–	–	–	–	**1,100**	**6.48**	**0.013**

–, Term removed from the minimal adequate model because it was not significant. Linear models were used with normal error distribution; all higher-order interaction terms were included in the maximal model. P/A, predator absent. Significant values are given in bold.

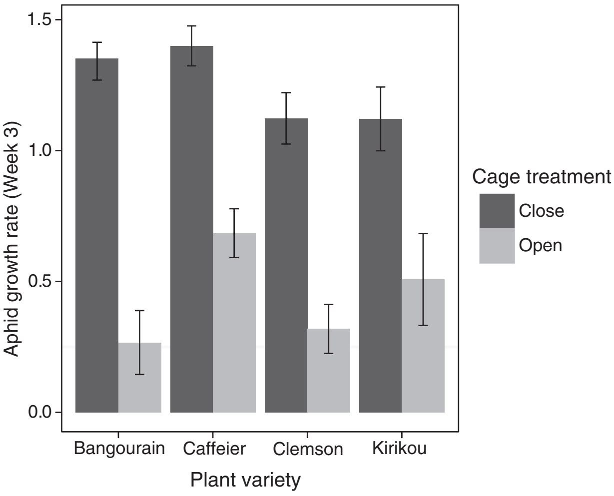

Figure 1 Aphid growth rate on different okra varieties in open and closed cages. Error bars indicate the SE.

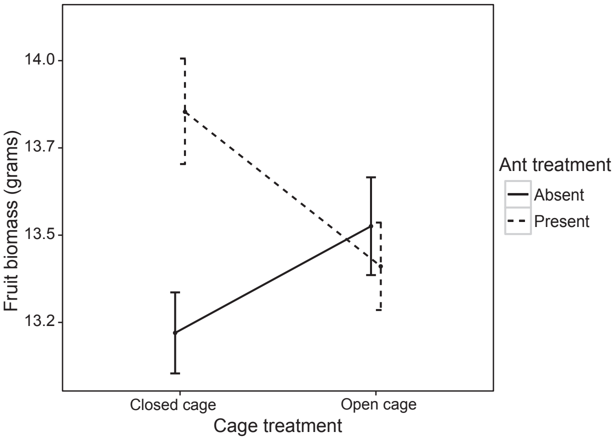

Figure 2 Okra fruit biomass in open and closed cages in the presence and absence of ants. Error bars indicate the SE.

There was no effect of okra variety on fruit biomass (Table 2); however, there was a significant interaction between cage and ant treatment (Table 2), with an increase in fruit biomass in ant presence in closed cages and no such effect in the open cages (Fig. 2). Fruit biomass was recorded to be higher with an increase in plant RGR (Table 2), although there was no effect of aphid GR ($F_{1,98} = 1.25$, $P = 0.266$) on fruit biomass. Plant variety did effect the day of fruit collection ($F_{3,100} = 29.56$, $P < 0.001$) and plants of *Clemson* and *Kirikou* fruited earlier than plants of *Caffeier* and *Bangourain* (see Supporting information, Fig. S1). Ant presence/absence ($F_{1,100} = 0.81$, $P = 0.371$) or cage treatment ($F_{1,100} = 0.49$, $P = 0.485$) did not affect the day of fruit collection.

Effect of ant abundance. Although there was no effect of ants on aphids, we did find a positive association between ant abundance and aphid GR ($F_{1,59} = 19.63$, $P < 0.001$) (see Supporting information, Table S1). Because there was no effect of ant presence/absence on aphid GR (Table 2), we assume that the causal relationship is the result of more aphids attracting more ants. Ant abundance had no effect on plant RGR or fruit biomass (see Supporting information, Table S1).

Effect of degree of herbivory and predation. Aphid GR at week 3 was higher in syrphid larvae presence ($F_{1,45} = 16.20$, $P = 0.002$), suggesting an attraction of syrphids to plants with higher aphid numbers. This effect was mediated by okra variety (aphid GR × okra variety: $F_{3,45} = 3.17$, $P = 0.033$). On three of the four okra varieties, aphid GR was higher with syrphid larvae presence, whereas, on *Caffeier*, it was the opposite (Fig. 3a). To analyze the effect of predation by syrphid larvae on the aphids, we calculated the change in aphid GR from week 3 to week 4 and found that syrphid larvae presence significantly reduced aphids by 42% ($F_{1,48} = 5.15$, $P = 0.027$). Although there was no significant interaction between plant variety and syrphid larvae presence ($F_{3,45} = 0.55$, $P = 0.653$), we did observe that syrphids reduced aphid GR on three of the four okra varieties but not on *Caffeier* (Fig. 3b). The abundance of leaf beetles ($F_{1,43} = 2.39$, $P = 0.129$) or foliage remaining ($F_{1,44} = 0.97$, $P = 0.329$) had no effect on aphid GR.

Plant RGR increased with an increase in foliage remaining (i.e. decrease in herbivory) ($F_{1,48} = 7.75$, $P = 0.007$) but was not affected by leaf beetle abundance ($F_{1,46} = 1.88$, $P = 0.177$). There was also no effect of leaf beetle abundance ($F_{1,44} = 0.17$, $P = 0.683$) or syrphid larvae presence ($F_{1,46} = 1.08$, $P = 0.305$) on fruit biomass.

Effect on ant abundance, syrphid larvae, leaf beetle abundance and chewing herbivory. We found no effect of cage ($F_{1,57} = 0.78$, $P = 0.382$) or plant variety ($F_{3,57} = 0.06$, $P = 0.981$) on ant abundance. As noted above, ant abundance on a plant increased with aphid abundance ($F_{1,57} = 5.46$, $P = 0.023$).

The presence of syrphid larvae was not affected by the presence of ants ($F_{1,48} = 0.57$, $P = 0.452$) or by plant variety ($F_{3,48} = 1.55$, $P = 0.214$). Aphid GR did affect syrphid larvae presence on a plant ($F_{1,48} = 9.98$, $P = 0.002$), probably because more syrphid larvae were attracted to plants with a higher aphid GR.

We found an effect of okra variety on foliage remaining ($F_{3,49} = 3.01$, $P = 0.038$) and leaf beetle abundance ($F_{3,47} = 3.25$, $P = 0.030$). Highest foliage remaining was recorded for *Bangourain* and *Caffeier* and lowest for *Clemson* (see Supporting information, Fig. S2). Leaf beetle abundance was lowest on *Bangourain* and highest on *Clemson* and *Caffeier* (see Supporting information, Fig. S3). There was a moderate negative correlation between leaf beetle abundance and foliage remaining ($r = -0.46$, d.f. = 52, $P < 0.001$) and thus leaf loss was explained only partly by leaf beetle herbivory. Ant presence had a positive effect on foliage remaining ($F_{1,49} = 5.86$, $P = 0.010$), with a higher amount of foliage remaining (i.e. less herbivory) in ant presence (83.2 ± 3.8%) than in ant absence (72.3 ± 3.9%). Ant presence/absence had no effect on leaf beetle abundance, although leaf beetle abundance decreased with an increase in ant abundance ($F_{1,47} = 5.38$, $P = 0.024$).

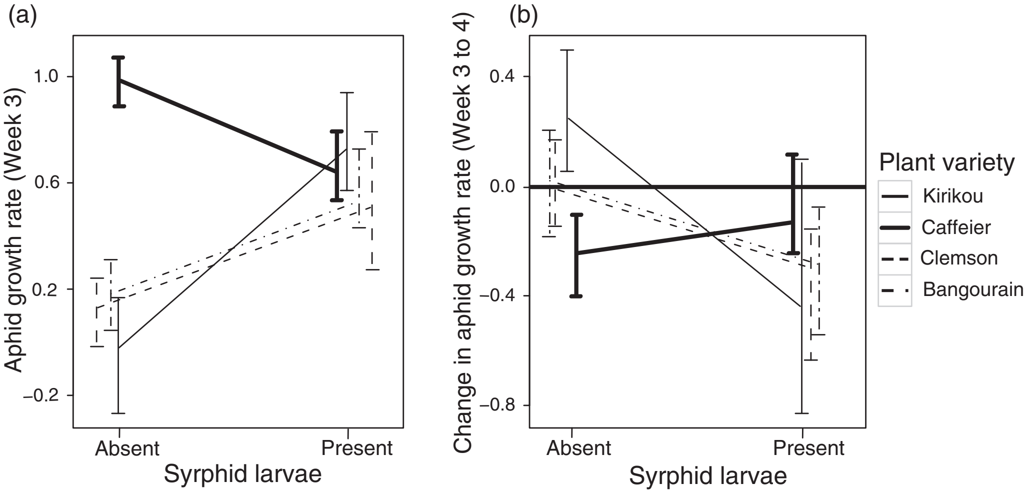

Figure 3 (a) Aphid growth rate at week 3 on different okra varieties in the presence and absence of syrphid larvae in open cages. (b) Change in aphid growth rate from week 3 to 4 on different okra varieties in the presence and absence of syrphid larvae in open cages. The horizontal line in (b) shows no change in aphid growth rate. Error bars indicate the SE.

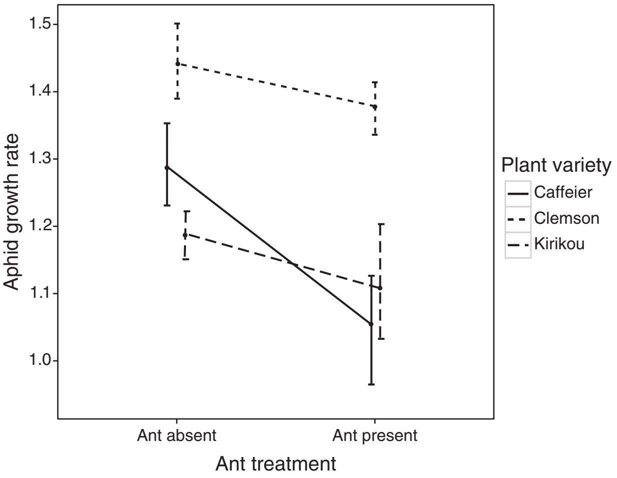

Figure 4 Aphid growth rate in the presence and absence of ants on different okra varieties in the screenhouse. Ten adult aphids were added to the plants and allowed to reproduce. Error bars indicate the SE.

Screenhouse experiment

Plant variety influenced aphid GR ($F_{2,43} = 8.66$, $P = 0.006$) and highest aphid numbers were recorded on *Clemson*, followed by *Caffeier* and *Kirikou*. Ants reduced aphid GR on all okra varieties ($F_{1,43} = 5.19$, $P = 0.023$) (Fig. 4) by 24% and, although there was no significant interaction between ant and plant variety ($F_{2,40} = 1.05$, $P = 0.359$), aphid reduction in ant presence was strongest on *Caffeier* (aphid number: ant presence 216.0 ± 42.9; ant absence 362.3 ± 38.9) (Fig. 4). During one sampling, we observed an ant predating on the aphids on *Caffeier*. Plant RGR did not vary across plant varieties ($F_{2,43} = 1.06$, $P = 0.354$) and was not affected by aphids ($F_{1,18} = 2.19$, $P = 0.156$). However, it was influenced by ants ($F_{1,43} = 4.16$, $P = 0.048$) (see Supporting information, Table S2) and plants grew less in the presence of ants (ant present 14.2 ± 1.0 cm; ant absent 16.4 ± 1.1 cm).

We observed a mean of 4.4 ± 0.7 ants per observation. In accordance with the field experiment results, there was no effect of plant variety on ant abundance ($F_{2,20} = 0.14$, $P = 0.869$). By contrast to the field results, we found no association between aphid GR and ant abundance on the plants ($F_{1,19} = 0.002$, $P = 0.965$).

Discussion

Overall, our results show that ants had neither a positive, nor negative effect on the aphids in the field experiment. However, in the screenhouse, ants had a negative effect on the aphids and ant predation on aphids was observed. Thus, ant–aphid interactions on okra are more complex than a standard model of mutualistic or antagonistic relationships. Similar to our study, previous studies have also found facultative ant–aphid associations to vary, where ants that tend aphids also predate upon them dependent on external food source (Offenberg, 2001), plant genotype (Mooney & Agrawal, 2008) or increasing aphid density (Sakata, 1995). Hence, we argue that, because *A. gossypii* is a facultative ant attended species, the nature of its association with ants can vary and be mediated by a diversity of factors.

In the present study, predators (specifically syrphid larvae) significantly reduced aphids on the plants in the field and were more often present on plants with higher aphid numbers. This suggests that the female syrphid chose to oviposit on plants with more food resource for her offspring (Gripenberg *et al.*, 2010). Aphid extinctions were high in the first week of the experiment, influenced by a cage-by-plant variety interaction, irrespective of ant presence. Because we found little effect of plant variety on predator abundance, this effect may be driven by reduced settling behaviour and acceptance of the plant by aphids (Sauge *et al.*, 1998). From the screenhouse experiment, we know that each plant variety is a suitable host, although potential variation in acceptance could lead to aphids leaving a plant in the field experiment and not returning, thus impacting the future growth and chance of extinction.

Although ants did not protect aphids from predation, their abundance on plants did increase with an increase in aphid abundance suggesting a more opportunistic ant–aphid interaction in our system. Recruitment of ants was beneficial for okra plants because plant herbivory and leaf beetle abundance reduced with an increase in ant abundance. In turn, this indirectly benefited the plant because more ants meant less herbivory, which was associated with a higher plant RGR; fruit biomass was positively correlated with a higher plant RGR. Indeed, an increasing density of flea beetles has previously been shown to reduce okra yield (Pitan & Ekoja, 2011). It is known in many systems that the recruitment of ants by aphids can reduce leaf-herbivory by beetles and caterpillars of the plant (Floate & Whitham, 1994; Styrsky & Eubanks, 2007). Furthermore, in a study on *A. gossypii*, Styrsky and Eubanks (2010) also found that ant attendance of the aphids increased cotton-plant reproduction as a result of a reduction in leaf-chewing herbivores.

We also found that ants benefited fruit biomass; however, this was only apparent in the closed cages and is thus independent of herbivory effects. One possible mechanism might be through efficient removal of honeydew from the aphids by the ants because aphid numbers were higher in the closed cages (possibly leading to higher honeydew production) and this would have attracted more ants. Efficient removal of honeydew by ants will benefit the plant because honeydew left on the plant can encourage the growth of harmful mould (Way, 1963). Okra varieties did not differ in their fruit biomass, although they did differ in the time of reaching fruit maturity (day of fruit collection), which was expected because it known that okra varieties differ in the time that they take to produce mature fruits (Saifullah & Rabbani, 2009).

By contrast to the field study, in the screenhouse, there was no association between ant abundance and aphid numbers. Indeed, ants were observed to prey upon aphids and aphid numbers were reduced on all plants with ant presence. This is in accordance with ant colonies mostly foraging for insect prey (protein source) during their larval growing season (Edwards, 1951). However, we provided an external protein source to our ant colonies and so protein limitation is not considered to explain our results. Possibly, ants preyed upon aphids because of high aphid numbers on the plant resulting in honeydew production that was in excess of the demands of the ant colony (density-dependent predation) (Rosengren & Sundström, 1991; Sakata, 1995). Our experiment did not specifically test for density-dependent predation, although we know from our field experiment that ants colonized plants with a higher aphid growth rate (i.e. *Caffeier* variety).

In the field, plant variety influenced aphid GR and highest aphid GR was recorded on *Caffeier*. Aphids are known to vary in their performance and preference, in various systems, across different plant genotypes or varieties, and this cascades to affect natural enemy abundances (Zytynska & Weisser, 2016). Because more syrphid larvae were observed on plants with a higher aphid GR overall, we might assume that a plant with high aphid performance (i.e. *Caffeier* variety) will also host more syrphid larvae. At week 3, on all varieties except *Caffeier*, we recorded high aphid GR in syrphid larvae presence, suggesting that there was such a high aphid GR on *Caffeier* that syrphid females did not need to particularly seek out plants with high aphid loads. Furthermore, there was little effect of syrphids on the change in aphid GR from week 3 to week 4 on *Caffeier*, whereas there was a negative effect on the other three varieties. This could be explained by such a high aphid performance on *Caffeier* that it negated any effect of predation by the syrphid larvae.

Similar to the field study, plant variety affected aphid GR in the screenhouse; however, here, the highest aphid GR was observed on *Clemson* and lowest on *Caffeier*. Aphid numbers were reduced in the screenhouse in ant presence. In particular, ant presence strongly reduced aphid numbers on *Caffeier* variety. In both studies, ants were observed collecting plant-produced pearl bodies, which have been shown to be produced by plants to attract ants in exchange for protection from phytophagous insects (Dutra *et al.*, 2006; Mayer *et al.*, 2014). The presence of pearl bodies has been little studied in okra, although it may mediate the aphid–ant interactions in our system. For example, the relative suitability of the aphid honeydew versus the plant pearl bodies for resources, and the variable ant preferences for this, could explain why ants might switch between tending the aphids and predating the aphids on okra (Mooney & Agrawal, 2008). Indeed, this might also explain why we recorded a reduced plant growth rate in ant presence in the screenhouse because it is well known that plant fitness can be reduced by investing in defensive compounds (Frederickson *et al.*, 2012; Mayer *et al.*, 2014). However, in the field with multiple food sources and okra-associated invertebrates, we did not record this negative effect of ants on plants or aphids. Controlled studies can only test a limited range of possible outcomes amongst species (Stadler & Dixon, 2005). Hence, in the screenhouse, where ant colonies were restricted to a plant for their nutritional requirements, there was a stronger effect of their presence on aphids and the plant than in the field study.

We also found that herbivory differed across plant varieties, with much less herbivory on *Bangourain* and *Caffeier* than on *Clemson.* A previous study by Underwood and Rausher (2000) found that flea beetles showed a preference for different soybean genotypes. Although we did not specifically test for preference effects, there were fewer beetles on *Bangourain* (with higher foliage remaining) and more on *Clemson* (with lower foliage remaining), suggesting some preference for *Clemson* against *Bangourain*. However, on *Caffeier*, the low rate of herbivory (high foliage remaining) was not explained by low leaf beetle abundance and, indeed, the leaf beetles were most abundant on this variety (see Supporting information, Figs S2 and S3). This suggests that, even though they may be attracted to *Caffeier*, they do not consume as much leaf material, which is potentially explained by a higher nutritional value for the beetle (i.e. low C : N value) (Mattson, 1980), although this remains to be studied. Alternatively, the high aphid GR on *Caffeier* in the open cages would have attracted more ants, which in turn was found to reduce herbivory on the plant (Styrsky & Eubanks, 2007, 2010; Zhang *et al.*, 2012).

Overall, we show that predators significantly reduced aphid numbers on okra and ants did not protect aphids. Furthermore, aphids did not influence okra fruit yield, although there was substantial herbivory on the plants by leaf beetles, which indirectly impacted yield. Aphid recruitment of ants was beneficial for okra plants because they reduced the number of leaf beetles and had an indirect positive effect on fruit yield. We also found aphids and leaf beetle numbers to vary across okra varieties, although the ant–aphid interaction on okra was not mediated by okra varieties. Despite minimal effects of aphids on plant yield, it is still important to maintain low aphid and leaf beetle population sizes because aphids can potentially transmit plant viruses (Katis *et al.*, 2007) and the spread of okra mosaic virus by beetles is well documented (Pitan & Ekoja, 2011; Benchasri, 2012). Enhancing ant abundance in okra farms can be useful for leaf beetle control (Styrsky & Eubanks, 2007). With respect to biocontrol measures, because ants do not protect aphids, the efficiency of an introduced predator for *A. gossypii* can be higher and syrphid larvae species can be tested to determine suitable species for biocontrol. Finally, understanding the mechanisms behind the negative effect of ants on aphids can further help in the development of efficient biocontrol measures.

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AS, SZ, RH and WW designed the experiment, with AS collecting the data. Analysis and interpretation was carried out by AS and SZ. AS, SZ, RH and WW all contributed to the first draft, which was completed by AS and commented on by SZ, RH and WW. All authors read and approved the final manuscript submitted for publication.

Supporting information

Additional Supporting information may be found in the online version of this article under the DOI reference:
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Table S1. Ant-present plants. Effect of experimental variables on aphid growth rate, plant relative growth rate and resulting biomass of harvested fruit from the okra plants with ant presence (Field experiment).
Table S2. Effect of experimental variables on aphid growth rate and plant relative growth rate (screenhouse experiment).
Fig. S1. Variation in day of fruit collection across okra varieties. Error bars indicate the SE.
Fig. S2. Mean leaf remaining per plant on different okra varieties (open cages). Error bars indicate the SE.
Fig. S3. Mean leaf beetle number per plant on different okra varieties (open cages). Error bars indicate the SE.

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Supplementary material

Ant attendance of the cotton aphid is beneficial for okra plants: Deciphering multitrophic interactions

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Table S1: Ant present plants. Effect of experimental variables on aphid growth rate, plant relative growth rate and resulting biomass of harvested fruit from the okra plants, in ant presence (Field experiment)

	Aphid growth rate (GR)			**Plant relative growth rate (RGR)**			**Fruit biomass**		
	Df	**F**	**P**	**Df**	**F**	**P**	**Df**	**F**	**P**
Aphid GR				1,59	2.47	0.121	1,55	0.51	0.477
Plant RGR	**1,59**	**4.07**	**0.048**				1,56	1.08	0.303
Ant abundance	**1,59**	**19.63**	**<0.001**	1,60	1.58	0.213	1,57	0.42	0.518
Cage (Open/Close)	**1,59**	**43.13**	**<0.001**	1,61	1.71	0.195	3,58	0.29	0.590
Plant variety	**3,59**	**2.70**	**0.053**	**3,61**	**6.65**	**0.005**	1,58	0.82	0.493

Linear models were used with normal error distribution; all higher-order interaction terms were included in the maximal model but not retained as they were not significant

Table S2: Effect of experimental variables on aphid growth rate and plant relative growth rate (Screenhouse experiment)

	Aphid growth rate (GR)			**Plant relative growth rate (RGR)**		
	Df	**F**	**P**	**Df**	**F**	**P**
Aphid GR				1,42	0.49	0.485
Plant RGR	1,42	0.66	0.421			
Plant variety	**2,43**	**8.66**	**0.006**	2,43	1.06	0.354
Ant (P/A)	**1,43**	**5.19**	**0.023**	**1,43**	**4.16**	**0.048**

Linear models were used with normal error distribution; all higher-order interaction terms were included in the maximal model but not retained as they were not significant

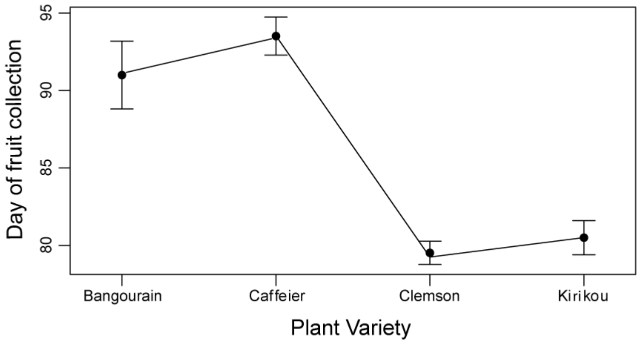

Figure S1: Variation in day of fruit collection across okra varieties. Error bars represent ± 1 SE

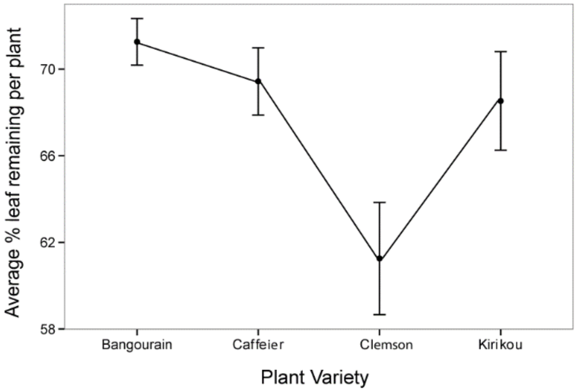

Figure S2: Average leaf remaining per plant on different okra varieties (open cages). Error bars represent ± 1 SE.

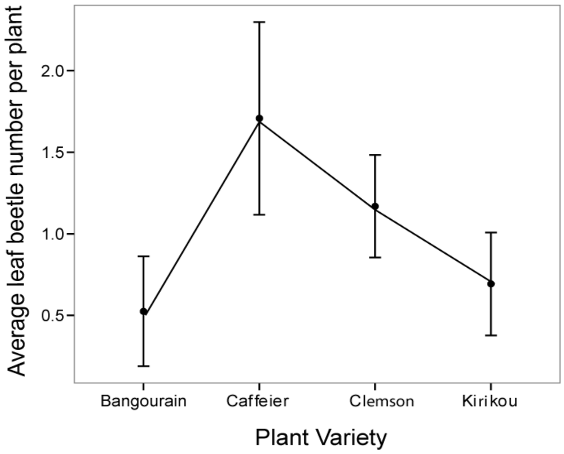

Figure S3: Average leaf beetle number per plant on different okra varieties (open cages). Error bars represent ± 1 SE.

Manuscript III

Effect of ant-plant-aphid interactions on okra pearl body production and its variation across okra varieties

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Abstract

Plants produce pearl bodies to attract ants and in return ants are known to protect plants from herbivorous pests. Despite their role in plant protection, pearl bodies are largely ignored in development of pest control measures. Aphid pests also produce honeydew to attract ants and in turn ants may protect them from their predators. Further, these ant-plant/ant-aphid interactions may also vary across plant varieties. It has yet not been studied how tritrophic interactions affect pearl body production and if this varies across plant varieties. Okra is an economically important crop in Cameroon. Several okra varieties are grown here and attacked by the ant-tended cotton aphid *Aphis gossypii.* Okra plants produce pearl bodies which have been observed to be carried by ants to their nests. We conducted one field and two controlled studies using 4-5 okra varieties and tested: ant preference of okra pearl bodies over aphid honeydew, and the effect of aphid presence and artificial pearl body removal on pearl body production. We found okra pearl body production to vary across okra varieties. Ant preference of pearl bodies was species specific and ants of genus *Pheidole* favoured pearl bodies over aphid honeydew. These ants also preferred plants of *Caffeier* variety. Additionally, pearl body production was inducible and was higher when they were artificially removed. Aphids induce multiple responses in a plant which can actually benefit the plant. Aphids reduced pearl body count but not when these were artificially removed. Further, aphids increased plant growth but pearl body production reduced plant growth. With no effect of aphids on pearl bodies in simulated ant presence, their prospective for plant defence by ants is higher. We suggest developing plant varieties favourable to ants to further enhance plant defence by ants.

Keywords: pearl bodies, ant-plant, ant-aphid, tritrophic, preference, inducibility, plant varieties

Introduction

Plants have evolved numerous defence traits against herbivorous insects such as trichomes, release of volatile organic compounds (VOCs), excretion of extraforal nectar (EFN) or production of food bodies (or pearl bodies) (Heil, 2015; Mithöfer & Boland, 2012; O'Dowd, 1982; Walling, 2000). Despite their potential in pest reduction, these defence traits have been largely underused in integrated pest control programs (Stenberg et al., 2015). Many plant defence traits are indirect where they increase the number of members of another trophic level (carnivore) and thereby reduce the number of herbivores on a plant (Heil, 2008a). Amongst multitudes of such indirect defences, ant-plant mutualisms have been extensively studied and it is known from many systems that ants defend plants and, in return, plants provide food rewards or shelter to ants (Mayer et al., 2014; Rosumek et al., 2009). Ants are also known to have mutualistic association with herbivores such as aphids, where they tend aphids for their honeydew and in return protect them from their natural enemies (Buckley, 1987; Kaplan & Eubanks, 2005; Völkl et al., 1990). Hence, ant defence of plants in return of food rewards cannot be generalised and can potentially vary due to their association with herbivorous pests such as aphids. Exploiting plant defence traits and understanding these tritrophic interactions (ant-plant-herbivore) can be crucial in development of integrated pest control measures.

Pearl bodies are produced as food rewards for ants, these are food bodies found on plant surfaces with lustrous pearl like appearance and are easily detached from the plants (O'Dowd, 1982). The role of pearl bodies as plant food is well established and pearl bodies of some plant species are known to be a high-energy food source for ants due to their large content of lipids, amino acids and carbohydrates (Fischer et al., 2002; Heil et al., 1998; O'Dowd, 1982; Webber et al., 2007). In several specialised ant-plant systems pearl bodies production is known to be induced by ants (Risch & Rickson, 1981) and even by their artificial removal

(Folgarait et al., 1994). The fact that pearl body production is inducible suggests that production of pearl bodies is possibly costly for plants (Mayer et al., 2014). In fact, plants have been found to allocate around 10% of their aboveground biomass to food body production (Fischer et al., 2002; Heil et al., 1997). An increase in plant fitness via ant protection possibly balances out the negative cost born by the plant (Heil, 2008b; Rosumek et al., 2009).

The majority of studies on pearl bodies so far have been conducted on specialised obligate (close) ant-plant associations such as those found in *Cercopia*, *Macaranga* or *Piper* plants (Fiala & Maschwitz, 1992; Fischer et al., 2002; Folgarait et al., 1994; Risch & Rickson, 1981). In such associations the direction of the ant-plant interaction is positive and ants defend plants efficiently against herbivores. The role of pearl bodies in facultative (occasional) ant-plant associations has been often overlooked (but see Buono et al., 2008; Dutra et al., 2006; Paiva et al., 2009). When ant-plant associations are facultative (occasional), it can lead to conditional mutualism where these associations may vary in space and time (Becerra & Venable, 1989; Rico-Gray et al., 2008). Furthermore, the majority of ant associations with herbivores such as aphids are also facultative and vary with a diversity of factors (Stadler & Dixon, 2005). Hence, when a facultative ant-associated plant harbours facultative ant-associated aphids, competition may rise between plants and aphids for ant protection. All studies with pearl bodies until now have only investigated the role of ant-plant interactions in pearl body production and have ignored the role of herbivores such as ant-tended aphids in these interactions.

Herbivores have been shown to have varying effects on the qualitative and quantitative production of plant compounds. Smith et al. (1990) found an increase in amino acid concentration following herbivory but found no increase in nectar volume. On the other hand, Koptur (1989) found nectar volume to increase by 2.5 fold in response to herbivory. Aphids were also shown to effect EFN production by Mondor and Addicott (2003) and here *Vicia faba* beans increased the production of nectaries following aphid attack. When herbivores affect plant compounds, they indirectly influence the presence of ants on the plant. This was demonstrated in a study on *Catalpa bignonioides* plant in which total EFN volume increased 2-3 fold after herbivore attack, which in turn significantly increased ant presence on the plant and protection against herbivores (Ness, 2003). Similar to EFN, pearl body production might also be altered by the presence of herbivores and this may influence ant presence on a plant.

Other than the possible alteration of pearl body composition by herbivores, it may differ across different plant varieties. Indeed, abundance and composition of EFN has been shown to be genetically determined (Webber et al., 2007). Thus, plant food rewards for ants can differ in quantity and quality across plant varieties and this may cascade down to affect ant community on a plant and their interaction with herbivores such as aphids. Such effects were shown by Rudgers (2004), where heritable variation in wild cotton traits influenced the abundance of nectar-feeding ants and ant predation of cotton herbivores. Despite strong evidence of pearl bodies as ant food, such tritrophic interactions and differences across plant varieties has never been studied. We suggest that enhancing our understanding of pearl bodies in agriculture and applying it for enhancing suitable crop varieties can be beneficial for integrated pest control measures.

Okra (*Abelmoschus esculentus* Moench) is an economically important vegetable crop grown worldwide, and is widely consumed in West Africa. In Cameroon the facultative ant-tended cotton aphid (*Aphis gossypii* Glover) is a dominant pest of okra (Leite et al., 2007; Shannag et al., 2007). An annual survey conducted by the International Institute of Tropical Agriculture (IITA) in Cameroon (2011), found ants of genus *Pheidole* to be present on okra plants in 75%

of the surveyed okra farms (IITA annual survey report, 2011). In our previous study we found that ants did not protect aphids from their natural enemies, but they were observed carrying pearl bodies from the plant to their nest and in controlled condition ants even reduced aphid numbers. In an observational study, *Pheidole dea* Santschi ants were also found to predate upon aphids, in particular on the *Caffeier* variety (Singh et al., 2016). Hence, we conducted several field and controlled studies to understand this ant-aphid-pearl body interactions on okra by answering the following questions: (a) Does okra pearl body production vary across okra varieties? (b) Do ants prefer okra pearl bodies over aphid honeydew and does this vary across okra varieties? (c) How does aphid infestation effect okra pearl body production across plant varieties? (d) How does artificial pearl body removal effect okra pearl body production across plant varieties? Additionally we also describe the structure and development of okra pearl bodies as these have not been described before for okra.

Materials and methods

Study system

Okra (*Abelmoschus esculentus* Moench) is mostly grown in a humid climate in sandy and clay loam soils and its optimum growing temperature is estimated to be between 24-30 °C. The plants are annual erect herb (2-4 meters tall) with lobed and hairy leaves. It is a self-pollinating crop but insects especially bees are attracted to the flowers and hence cross pollination occurs (Tripathi et al., 2011). Okra plants are attacked by many pests at different growing stages such as the cotton aphid (*Aphis gossypii* Glover) and whiteflies (*Bemisia tabaci* (Gannadius)) (Benchasri, 2012). Okra plants produce pearl bodies (PBs) on their leaf and stem surfaces (personal observation).

The cotton aphid colonizes more than 600 host plants across a wide geographic range and vectors more than 50 plant viruses (Van Emden & Harrington, 2007). In tropical climates this facultative ant-tended aphid undergoes mostly parthenogenetic (i.e. asexual) reproduction, leading to an exponential growth rate at optimal conditions.

Pheidole genus is one of the two most diverse ant genera along with *Camponotus* genus and, ~900 *Pheidole* species are known worldwide; *Pheidole* species are particularly dominant in the tropics (Economo et al., 2015; Wilson, 2003). Most *Pheidole* species colony can consist of multiple queens and *Pheidole* species are often dimorphic, this means that the workers are subdivided into relatively slender minors and stronger, conspicuously large-headed majors (Wilson, 2003).

Study sites and study species:

Field and screenhouse experiments conducted in Cameroon (experiment 1 and 2): These were conducted within the research site of the International Institute of Tropical Agriculture (IITA) in Yaoundé, in Cameroon (West Africa). Screenhouses are made of a greenhouse frame but are covered with double layer of fine net to avoid any insects from entering, yet allowing air to circulate from the outside. In the first field experiment our study consisted of cotton aphids on okra and ant species of genus *Pheidole*. In the second screenhouse experiment our study contained cotton aphids on okra without ants. In both experiments five okra varieties were used: *Clemson, Hire, Paysan* (Les Doigts Verts, France), *Kirikou* (obtained locally from Dschang town (Northwest Cameroon) and *Caffeier* (G.M.R. Sarl, Cameroon). The aphids used in these experiments were obtained from the screenhouse in IITA where they were reared on *Clemson* variety of okra, inside entirely enclosed plastic-polypropylene insect cages (1,350 µm mesh opening) of 30×30×30 cm dimensions (Megaview Science, Taiwan).

Greenhouse experiment in Dürnast, Germany (experiment 3): This was conducted inside a greenhouse located at the Dürnast experimental station of Technical University of Munich (TUM) in Freising, Germany. Here our study consisted of cotton aphids on okra. Four okra varieties were used: *Kirikou, Paysan, Hire* and *Clemson*. The cotton aphids used for the experiment belong to the 'Darmstadt' strain (undergoes parthenogenetic reproduction) and were obtained from Dürnast greenhouse where they are maintained at temperatures of 20°C/18°C and 16:8 (light:dark) hours light cycle, in plastic-polypropylene fine mesh insect cages of 30×30×30 cm dimensions.

Seed germination

Field and screenhouse experiments conducted in Cameroon (experiment 1 and 2): The seeds were soaked in water under complete darkness for 24 hours. Then one seed per pot (14 cm deep, 16 cm diameter) was sown in sterilized soil (25% sand, 25% fowl manure and 50% soil) and left to germinate inside entirely enclosed cages for five weeks. The cages were of 0.4x0.4x0.7 m (length x breadth x height) dimensions, constructed as a frame of PVC piping and were covered with a white fine-mesh cotton cloth. Plants used for both experiments were five weeks old from the data of sowing.

Greenhouse experiment in Dürnast, Germany (experiment 3): The seeds were soaked in water under complete darkness for 24 hours. Then one seed per pot (9 cm deep , 10 cm diameter) was sown in potting soil (Einheitserde profi substrat, Germany) and left to germinate inside the greenhouse. Plants used for the experiment were five weeks old from the date of sowing.

1. Ant preference experiment (field experiment in Cameroon), 22nd April – 8th May, 2014

Experimental design and set-up: We used a fully-factorial block design with 20 treatments including five okra varieties (*Clemson, Caffeier, Kirikou, Paysan* and *Hire*), two aphid treatments (presence and absence) and two pearl body (PB) treatments (PB kept and PB removed), i.e. 5 varieties×2×2= 20 treatments. This was replicated 12 times using 12 blocks, with one treatment replicate in every block. Each block was positioned around a *Pheidole* ant colony, allowing natural ant colonisation and plants of the same PB/aphid treatment were grouped resulting in four groups (aphid presence+PB kept, aphid absence+PB kept, aphid absence+PB kept and aphid absence+PB removed) containing plants of different varieties. In this way, ants were first able to choose the PB/aphid treatment and subsequently the plant variety within this. Due to insufficient plants (-)Aphid(-)PB treatments of *Caffeier* and *Clemson* plants were repeated 11 times and distributed within 11 blocks; hence, we had 238 plants in total. We conducted observations on six of the blocks in the first week, on three blocks the following week and on further three in the third week, to allow sufficient time for observations of the plants.

Two days before the experimental plants were placed outside for observation, *Pheidole* ant colonies were marked in the field site of IITA. Out of the ant colonies selected for observation, four colonies were of *Pheidole dea* Santschi, three of *Pheidole nigeriensis* Santschi, three of an unidentified *Pheidole* species (*Pheidole 1*) and two of a second, and different, unidentified *Pheidole* species (*Pheidole 2*). Ant species were identified by Dr. Apollin Fotso at IITA. Above each ant colony we built a rain shelter using wooden sticks and a plastic cover of ~120×100 cm (length × breadth) dimensions (Figure SI 1), to reduce the impact of heavy rain on the plants. All the vegetation around the ant colony was removed.

A day before the experimental plants were placed outside, we first measured their height and leaf number. Pearl body numbers were also recorded but only on the plants (leaves and stem) with PB kept treatment; PBs were removed from the plants with PB removed treatment using a paint brush. Then 50 aphids of mixed instar were placed on the corresponding plants using a fine paint brush. This was done to allow aphids' sufficient time to settle on the plants so that they would be less affected by wind and rain in the field.

The next day we dug holes around the ant colony and inserted the experimental pots in them to allow ants convenient access to the plants (level of top of the pot and the ground was the same). Each of the treatment groups (as described above) was placed at equal distance from the ant colonies and within a group all plants touched one another (Figure SI 1).

Data collection: The plants were placed outside early in the morning on the first day between 7:00 AM and 8:30 AM after which we conducted our observation in daylight (not during the night) every three to four hours, from 8:30 to 18:30 hrs on the first day and from 7:00 to 16:00 hrs the next day. Each group within a block was observed for five minutes during each observation effort. Hence, it took 20 minutes to observe one block during each observation, during which we recorded the number of ants on a plant and ant species on a plant. Pearl bodies were removed after each observation for plants with PB removed treatment, i.e. every three to four hours.

The average temperature during the course of the experiment was 24.1±0.17°C (range: 18.6-32.6 °C) and average humidity was 89.4%±0.61% (range: 53.1% - 100%). Average rainfall was 9.9mm±3.80mm (range: 1.5mm – 68.6 mm) with natural 12:12 (light:dark) cycle.

2. Short term pearl body production experiment (screenhouse experiment in Cameroon), 10th – 11th June, 2014

We did not use ants in this experiment but simulated their presence by artificial pearl body removal.

Experimental design and set-up and data collection: We used a fully factorial randomized design with 20 treatment including five okra varieties (*Clemson, Caffeier, Kirikou, Paysan* and *Hire*), two aphid treatments (presence and absence) and two pearl body treatments (PB kept and PB removed). Each of our treatments was repeated five times giving us 100 plants in total.

We first measured the plant height and leaf number of all plants. Then pearl body numbers were recorded only on plants (leaves and stem) with PB kept treatment and pearl bodies were removed from plants with PB removed treatment. Then 50 aphids of mixed instar were placed on the plants with aphid presence treatment. Thirty-six hours after initiating the experiment, final PB count was recorded for all leaves and stem of each experimental plant.

During the experiment, the average temperature of the screenhouse was 25.2±0.16°C (21.3-31.4 °C), average humidity was 83.4%±0.50% (55.4%-97.8%) and additional lighting of 12:12 (light:dark) was used.

3. Long term pearl body production experiment (greenhouse experiment in Germany), 26th February – 6th March, 2015

We again did not use ants in this experiment but simulated their presence by artificial pearl body removal. This experiment ran longer than the first two experiments and we also measured leaf area and recorded PB count of each individual leaf of all plants. This experiment was repeated the following year from 2nd June – 11th June, 2016. The main results were similar and as such the 2016 results are only presented in the secondary supplementary material. The dissimilar results are mentioned and discussed in this manuscript.

Experimental design and set-up: We used a fully factorial randomised block design with 16 treatments including four okra varieties (*Clemson, Paysan, Kirikou* and *Hire*), two aphid treatments (presence and absence) and two pearl body treatments (PB kept and PB removed) i.e 4 varieties×2×2= 16. Each of our treatments was repeated ten times and distributed within ten blocks with each of the blocks containing one repeat of all treatments, giving us 160 plants in total. We conducted observations on the first five blocks on the first day and the next five blocks on the consecutive day.

The day before the initiation of the experiment we numbered the plants, measured their height, and numbered all the leaves of each plant. Each plant was placed on top of a plastic pot on a water-filled tray (to prevent escape of unwinged aphids). We first counted pearl bodies (PBs) on all plants and recorded them separately for each leaf and stem of a plant. After this, PBs were removed using a paint brush from plants with PB removed treatment. Then, 30 aphids of mixed instar were placed on each plant with the aphid present treatment. After this plants were left for one week and watered daily.

Data collection: One week after setting up the blocks, data were recorded on final plant height and final PB count on all plants separately for each leaf and stem. After collecting PBs, we measured leaf area separately of each leaf of a plant using a LI-3100 C area meter (LI-COR, USA). Additionally, leaves were collected for leaf structure analysis.

During the experiment temperature of the greenhouse was 24/20 °C (day/night) and additional lighting of 16:8 hours (light:dark) was used.

Okra pearl body structure: Young and mature leaves of all varieties (*Clemson*, *Hire*, *Kirikou* and *Paysan*) were fixated in FAA (formaldehyde – acetic acid – 70% ethanol) and after fixation stored in 70% ethanol until further processing. For morphological pearl body investigation from each okra variety two young and two mature leaves were randomly chosen and virtually divided into 4 sectors: basal part left and right from the main rib, apical part left and right from the main rib. From each sector two pieces of 5mm^2 were cut out, critical point dried and the upper and lower surface investigated with a Jeol JSM IT300 scanning electron microscope (SEM) at 10 kV. For anatomical investigation, two parts per leaf sector were taken from *Clemson* and *Paysan*, soaked and embedded in a resin based on hydroxyethylmethacrylate (Kulzer Histo-Technique), cut in 2.5–3 µm cross-sections with a microtome, stained with Ruthenium Red/Astra Blue (both Sigma Aldrich) and investigated with an Olympus BX50 microscope.

Data analysis

1. Ant preference experiment (field experiment in Cameroon): Five plants were removed from the analysis, three of them [*Hire* (+Aphid-PB), *Clemson* (-Aphid+PB) and *Paysan* (+Aphid-PB)] were damaged due to strong wind and two of them [*Hire* (+Aphid+PB) and *Clemson* (+Aphid-PB)] were partially eaten by an unidentified spiny caterpillar within six hours of putting the plants in the field. Hence, we had a final sample size of 233 plants and 11-12 repeats for all our treatments. For initial PB count, plant height and leaf number as response variables we ran linear models (lm) with normal distribution including only plant variety as the main explanatory variable as other treatments had not been applied to the plants. The ant species observed were grouped into three main types: *Pheidole*, *Camponotus* and rest as 'other' species. To test the effect of our treatments on the response variables total ant, *Pheidole*, *Camponotus* and 'other' ant number, we ran Generalised Linear Models (glm) with quasipoisson distribution including plant variety, aphid treatment (presence and absence) and PB treatment (PB kept and PB removed) as main explanatory variables, and block was included as a fixed effect.

To analyse the effect of initial PB count on numbers of total and different ant groups only data of PB kept plants was used. We ran glms with quasipoisson distribution including plant variety and aphid treatment as our main explanatory variables, block as a fixed effect and initial PB count as a covariate.

Observed numbers of solitary forager species like of genus *Camponotus* were much lower on the plants as compared to of group foragers like ants of genus *Pheidole.* Therefore, to determine the preference of different ant groups (between aphids and pearl bodies), we also ran a glm with binary distribution including presence/absence of ant groups as the main response variable and type of ant groups, plant variety, aphid treatment and PB treatment as main explanatory variables.

Interactions between the main explanatory variables were tested in all the models mentioned above.

2. Short term pearl body production experiment: We only counted initial pearl bodies for PB kept plants and pearl body replenishment for these plants was calculated as final PB count – initial PB count. Final PB count for PB removed plants was equivalent to their PB replenishment (Final PB count – 0), as all PBs were removed at the beginning from these plants. For our response variables initial PB count (PB kept plants only), plant height and leaf number we ran linear models including only plant variety as the main explanatory variable. For final PB count (PB kept plants only) we ran a glm with quasipoisson distribution including plant variety and aphid treatment (presence and absence) as the main explanatory variables. For PB replenishment we ran a linear model including plant variety, aphid treatment and PB treatment (PB kept and removed) as main explanatory variables. As pearl body treatment had a strong effect on PB replenishment, we also split the data between PB kept and PB removed plants. Here linear models were run including PB replenishment as the main response variable and plant variety and aphid treatment as main explanatory variables. In all the above models interactions between the main explanatory variables were tested.

3. Long term pearl body production experiment: Two experimental plants were removed from our analysis as these died during the course of the experiment. One was of *Hire* and one of *Kirikou* variety and both were with (+)Aphid(-)PB treatment. Hence, our final sample size was 158 plants. Plant relative growth rate (Plant RGR) was used to correct for initial plant height variation amongst varieties; it was calculated using the formula: (ln (Final plant height) – ln (Initial plant height)). We also calculated the replenishment rate of pearl bodies each for the total number, and then separately for the leaf and stem: replenishment = Final PB count – Initial PB count, using initial PB count of zero for PB removed plants. We also calculated replenishment per cm^2 leaf from the leaf area and per cm from final plant height variables.

For our response variables initial PB count, plant height and leaf number we ran linear models including only plant variety as the main explanatory variable. For all other response variables mentioned below we included aphid treatment (presence/absence) and plant variety as main explanatory variables. For total final PB count (PB kept plants only) and total PB replenishment we applied glms with quasipoisson distribution. For total final PB count our model included final plant height as a covariate. For total PB replenishment our model also included PB treatment (keep and removed) as a main explanatory variable and plant RGR as a covariate. To analyse the effects of our treatments on final leaf PB replenishment/cm^2 ,final stem PB replenishment/cm, total final leaf area and plant RGR we appled linear models and also included PB treatment as a main explanatory variable. Further, for plant RGR we included total replenished PB count as a covariate and for total leaf area we included final plant height as a covariate.

We further split data between PB kept and PB removed plants and analysed the effect of aphids and plant variety on total PB replenishment, final leaf PB replenishment/cm^2 and final stem PB replenishment/cm .

The data for all three experiments was analysed in R version 3.2.2 using RStudio version 0.98.978. For all the variables that we tested, we used Type I sum of squares; we first fitted a full model with all main effects and all interaction effects between the main explanatory variables. Then all the non-significant effects and interactions (starting from the highest interaction order) were removed for simplification of our final model.

Results

Plant morphology and pearl body count variation across okra varieties

Okra varieties differed in their height and leaf number (Table SI 1), plants of *Paysan* variety were found to be the tallest and of *Kirikou* the shortest (Table SI 2). There was little variation (<1 leaf) recorded between plant varieties in their leaf numbers except for *Clemson* (expt. 1 and 2) and *Hire* (expt. 3) which had the lowest leaf numbers (Table SI 2).

In expt. 1 and 2 initial pearl body count was recorded only for PB kept plants but in the expt. 3 it was recorded on all plants. Initial pearl body (PB) counts differed across okra varieties (Table SI 1). Highest initial PB count was recorded for plants of *Caffeier* (expt. 1 and 2) and *Clemson* varieties (expt. 3) (Table SI 3). Despite being the tallest, *Paysan* plants had the lowest PB count and the lowest PB count per cm^2 (Table SI 3 and SI 4). Pearl body count increased on all PB kept plants during the course of the experiments; in our long term study (expt. 3) it increased by 37.8% and in the short term study it increased by 3.3% of the initial count (expt. 2). The trend across varieties for final PB count was always similar as that for initial count (Table SI 3). In the long term study, plants with pearl bodies removed replenished 67.2% of their initial PB count.

We only recorded leaf area in our long term study. Leaf area differed across varieties (Table SI 1) and followed an opposite trend than plant height; *Hire* variety with shortest plants had the largest and *Clemson* variety with taller plants had the smallest total leaf area (Table SI 4). Area of an individual leaf was also highest for *Hire* variety (Table SI 4). Plants with largest leaf area did not have the highest PB count and *Clemson* plants with smallest total leaf area had the highest PB count and also the highest PB count per cm^2 (Table SI 3 and SI 4).

Okra pearl body structure: Pearl bodies occur on okra stems and both leaf surfaces, whereas, pearl body density is found to be much higher on the abaxial surface (Figure 1 a). On the upper surface pearl bodies are rare and only in the vicinity of the veins (Figure 1 b). Okra pearl bodies are multicellular and of epidermal origin, cells of the subepidermis are not involved (Figure 1 e, f). Their elaboration is through periclinal cell divisions with subsequent anticlinal division of the two apical cells (Figure 1 c, e, f). Two basal cells remain as “stalk”. They are usually composed of 8 cells surrounded by a very robust cuticle (Figure 1 d). During their enlargement the characteristic spherical shape is reached.

Young leaves have plenty of initial stages of pearl bodies on their abaxial surface (Figure 1 a) but on old leaves much lower numbers occur. This strongly suggests that pearl body replenishment rate is correlated with leaf age.

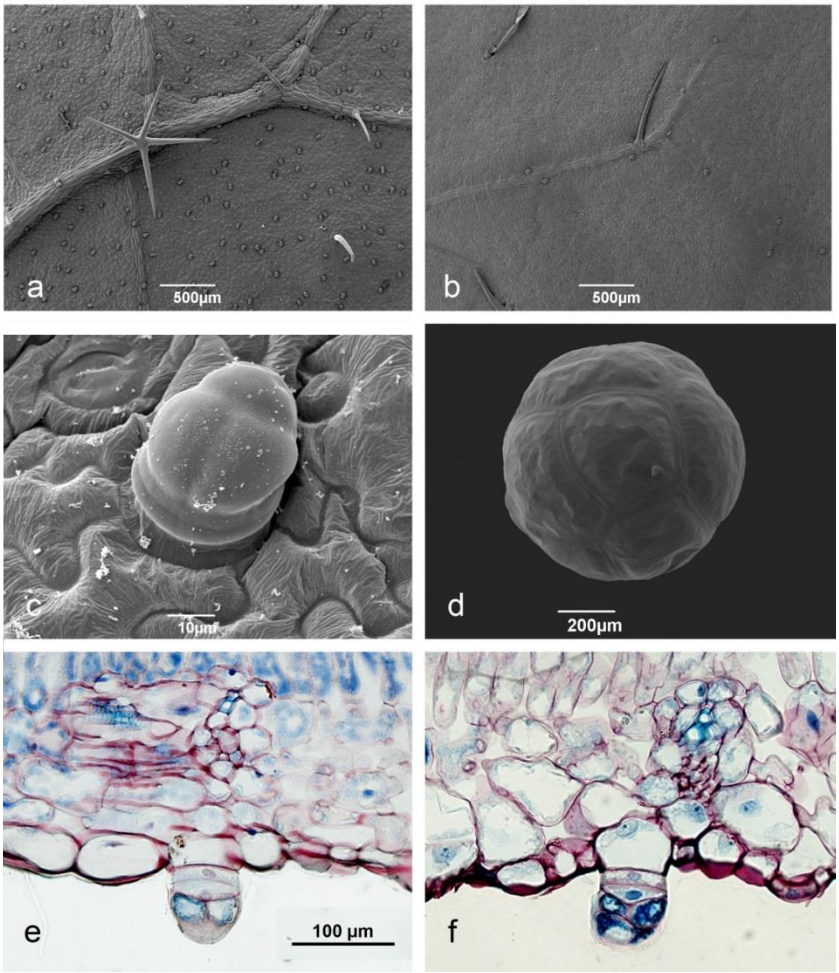

Figure 1: Pearl body structure in Okra varieties. (a, c) lower leaf surface of *Clemson* young leaf, (b) upper leaf surface *Clemson* young leaf, (d) mature pearl body from a *Hire* leaf, (e, f) longitudinal leaf sections of *Paysan* (e) and *Clemson* (f) with pearl bodies developing on the lower leaf surface. The initial epidermis cell, the two stalk cells and the apical cells dividing anticlinally can be seen.

1. Ant preference experiment (field experiment in Cameroon)

Ants started visiting the plants after an hour of putting the plants around the *Pheidole* sp. ant colonies. Several other ant species were also recorded on the plants such as *Anoplolepis taenella* Santschi*, Monomorium bicolor* Emery*, Tetramorium* spp.*, Lepisota* spp.*, Camponotus schoutodeni* Forel*, Camponotus avaeimenses* Mayr, *Monomorium gabrielense* Forel and *Myrmacaria opaciventris* Emery. Out of the total number of ants observed of all observations combined, 42% (354/833) were of genus *Pheidole,* 12% (97/833) of genus *Camponotus* and 46% (382/833) 'other' ants.

Total number of ants: The presence of aphids had a significant effect on the total number of ants on a plant ($F_{1,225}$=53.27, *P*<0.001) and more ants were recorded in aphid presence (4.9±0.87) than in aphid absence (0.9±0.18). We also recorded a significant interaction between aphid and PB treatment on total number of ants ($F_{1,225}$=23.17, *P*<0.001). Here, in PB kept plants, more ants were observed when aphids were also present than when aphids were absent and ants were never observed on plants with aphid absence and PBs removed (Figure SI 2). The total number of ants also differed across varieties ($F_{4,225}$=7.38, *P*<0.001) and on an average most ants were observed on *Caffeier* (7.5±1.97), followed by *Kirikou* (4.1±1.47), *Paysan* (3.5±1.12), *Hire* (2.9±0.55) and *Clemson* (1.4±0.67).

Total number of Pheidole, Camponotus and 'other' groups: Due to various different ants observed on plants we also tested to see if the number of different ant groups differed across our different treatments. Aphid treatment and pearl body treatment affected numbers of all different ant groups (Table 1).

Table 1: Effect of main explanatory variables on number of *Pheidole*, *Camponotus* and 'other' ant groups

	No. of *Pheidole* ants			**No. of *Camponotus* ants**			**No. of 'other' ants**		
	Df	**F**	**P**	**Df**	**F**	**P**	**Df**	**F**	**P**
Block	1,224	1.46	0.228	1,225	2.41	0.122	1,225	0.46	0.496
Field cover	1,225	3.32	0.070	**1,226**	**10.06**	**0.001**	**1,226**	**10.90**	**0.001**
Plant variety	**4,225**	**8.50**	**<0.001**	**4,226**	**5.93**	**0.001**	4,226	1.40	0.235
Aphid(Presence/Absence)	**1,225**	**9.53**	**0.002**	**1,226**	**10.83**	**0.002**	**1,226**	**58.13**	**<0.001**
PB(PB Kept/PB Removed)	**1,225**	**34.43**	**<0.001**	**1,226**	**17.05**	**<0.001**	**1,226**	**12.38**	**0.005**
Aphid × PB	**1,225**	**13.15**	**0.003**	-	-	-	-	-	-

Generalised linear models were used with quasipoisson distribution; all higher-order interaction terms were included in the maximal model and removed from the final model if not significant. '- 'indicates when a term was not significant

On all varieties, the numbers of 'other' ants were highest in aphid presence alone and these were not found on plants in aphid absence (Figure 2). *Camponotus* numbers were high in the presence of both pearl bodies and aphids and, *Pheidole* numbers were always higher in the presence of pearl bodies than in their absence (Figure 2). Plant variety only affected *Pheidole* and *Camponotus* ant numbers (Table 1) and their highest numbers were recorded on *Caffeier* and lowest on *Clemson* variety (Figure 2). *Pheidole* ($F_{1,111}$=7.39, P=0.007) and *Camponotus* ($F_{1,110}$=8.40, P=0.004) ant numbers were also higher on plants with higher initial PB count, whereas, initial PB count did not affect the numbers of ‘other’ ant species ($F_{1,109}$=0.04, P=0.846).

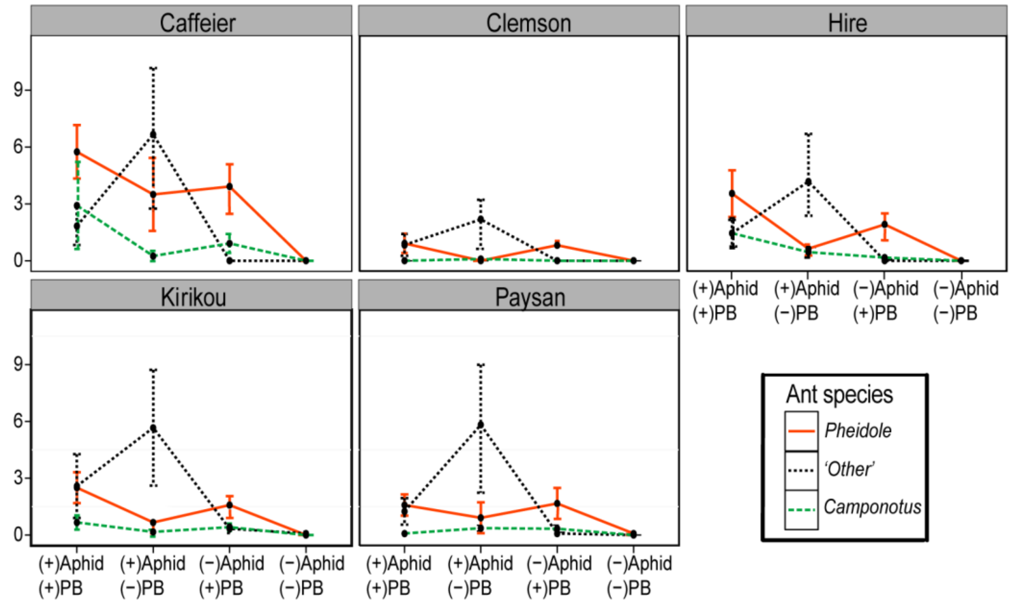

Figure 2: Average number of different ant groups found in different aphid and pearl body treatments. Error bars represent ± 1 SE.

Presence and absence of Pheidole, Camponotus and ‘other’ groups per plant: We recorded significant two way interactions between ant groups and aphid treatment (X^2=39.58, df=2, P<0.001), ant groups and PB treatment (X^2=12.08, df=2, P=0.001) and, aphid treatment and PB treatment (X^2=38.45, df=1, P<0.001) on ant presence on a plant; suggesting that different ant groups had different preferences for aphids and okra PB (Figure SI 3). This was similar to the trend seen for ant number, *Pheidole* ants were found more often in the presence of pearl bodies, ‘other’ ants in the presence of aphids and *Camponotus* ants in the presence of both pearl bodies and aphids (Figure SI 3).Overall, ant presence also varied across okra varieties (X^2=13.61, df=4, P=0.008) and it was highest on *Caffeier* and lowest on *Clemson* variety.

2. Short term pearl body production experiment (screenhouse experiment in Cameroon)

Final PB count (PB kept plants) only differed across varieties ($F_{4,43}$=11.05, P<0.001, Table SI 3) and there was no effect of aphids on final PB count ($F_{4,43}$=0.25, P=0.619). Ant presence was simulated in this experiment by artificial removal of pearl bodies at the beginning. Overall, PB replenishment rate was higher when pearl bodies were removed (9.3±0.90) in contrast to when they were kept (1.7±2.28) ($F_{1,89}$=5.28, P=0.024). In PB kept plants we recorded a two-way interaction between aphid treatment and plant variety on PB replenishment ($F_{4,40}$=2.61, P=0.049). Here, replenishment was higher on *Clemson* plants in aphid presence than in aphid absence, whereas, on *Kirikou* it was higher in aphid absence (Figure 3a and S4). Further, in aphid presence we found significantly higher PB replenishment for *Clemson* than for *Caffeier* (posthoc: t_{14}=2.57, P=0.011) and *Kirikou* plants (posthoc: t_{14}=2.39, P=0.016) (Figure SI 4). Hence, this variation of aphid effect across varieties could possibly explain why we saw no overall main effect of aphids on final PB count but did on PB replenishment (PB kept plants).

In PB removed plants, aphids had no effect on PB replenishment ($F_{1,42}$=1.36, P=0.250) and it only varied across plant varieties ($F_{4,42}$=4.31, P=0.006). Here, PB replenishment was highest on *Hire* (16.6±5.25), almost similar for *Caffeier* (8.7±2.75) and *Clemson* (8.2±2.59), then *Kirikou* (7.7±2.43) and, least for *Paysan* (5.2±1.64) (Figure 3a).

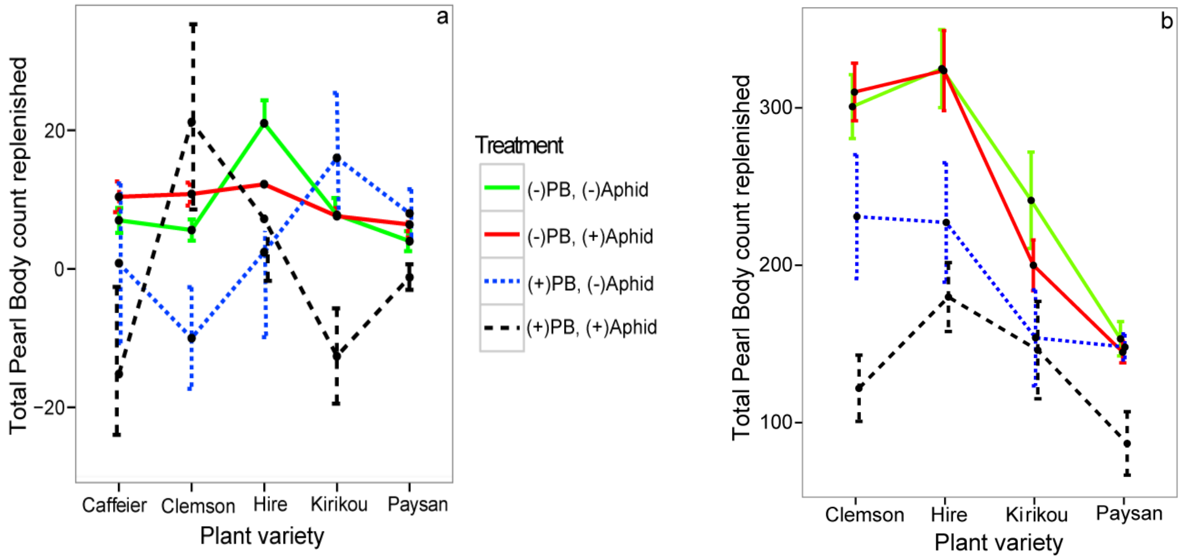

Figure 3: Variation across okra varieties in their total replenished pearl body count in (a) short term experiment (1 day) and (b) long term experiment (1 week). Pearl bodies were artifically removed at the beginning of both experiments only in (-)PB plants. Error bars represent ± 1 SE.

3. Long term pearl body production experiment (greenhouse experiment in Germany)

This experiment ran longer than the first two experiments and we also measured leaf area, final height and PB count of each individual leaf of all plants. We also repeated this experiment in 2016. All similar results are included in the secondary supplementary material (see supplementary material II). Effects of experimental variables on plant growth rate and leaf area differed in the year 2015 and 2016. These are mentioned and discussed below.

The final number of PBs on the plants, when not removed, varied across the plant varieties (Table 2) and it was highest on *Clemson* and lowest on *Paysan* variety (Table SI 3). PB number was also affected by aphids (Table 2) and it was lower in aphid presence (577.9±29.88) than in aphid absence (667.3±37.20). Additionally, it decreased with an increase in final height (Table 2).

Total PB replenishment and leaf PB replenishment/cm^2 differed across okra varieties (Table 2) and was highest for *Clemson* and lowest for *Paysan* (Figure SI 5). Variation across okra varieties in leaf and stem PB replenishment followed a different trend and the shortest

Kirikou variety had the highest stem PB replenishment/cm (Figure SI 5). This suggests that pearl body production is costly for plants. Similar to the screenhouse study in Cameroon, number of pearl bodies replenished was higher when PB was removed (248.9±10.5) than when PB was kept (169.5±11.57) and pearl body treatment had the most significant effect on all replenishment variables (Table 2), which were all higher when PB was removed than when PB was kept. Overall aphids reduced all replenishment variables (Table 2). We recorded a significant two-way interaction between aphid treatment and PB treatment on total PB replenishment (Table 2). Here, aphid reduction of PB replenishment was stronger in PB kept plants than in PB removed plants (Figure 4). In addition, total replenishment reduced with an increase in plant RGR (Table 2) i.e., more a plant grew lower was its replenishment rate

Table 2: Effect of main experimental variables and covariates

	Total final PB count (PB kept)	**Total PB replenishment**	**Leaf PB replenishment/ cm^2**	**Stem PB replenishment/ cm**
Final height	$F_{1,73}$=27.59, P<0.001	NA	NA	NA
Plant RGR	NA	$F_{1,150}$=23.32, *P*<0.001	NA	NA
Plant variety	$F_{3,73}$=41.96, *P*<0.001	$F_{3,150}$=13.58, *P*<0.001	$F_{3,151}$=28.8, *P*<0.001	$F_{3,149}$=10.89, *P*<0.001
Aphid treatment (Presence or absence)	$F_{1,73}$=13.71, P=0.004	$F_{1,150}$=5.31, *P*=0.023	$F_{1,151}$=4.57, *P*=0.030	$F_{1,149}$=14.13, *P*=0.002
PB treatment (Kept or Removed)	NA	$F_{1,150}$=45.07, *P*<0.001	$F_{1,151}$= 27.43, *P*<0.001	$F_{1,149}$=61.36, *P*<0.001
Aphid×PB treatment	NA	$F_{1,150}$=4.69, *P*=0.032	-	-
Plant variety×PB treatment	-	-	-	$F_{3,149}$=3.92, *P*=0.009

Linear models with normal distribution and generalised linear models with quasipoisson distribution were used; all higher-order interaction terms were included in the maximal model and removed from the final model if not significant. '-'indicates when a term was not significant. 'NA' indicated when a term was not included in the model.

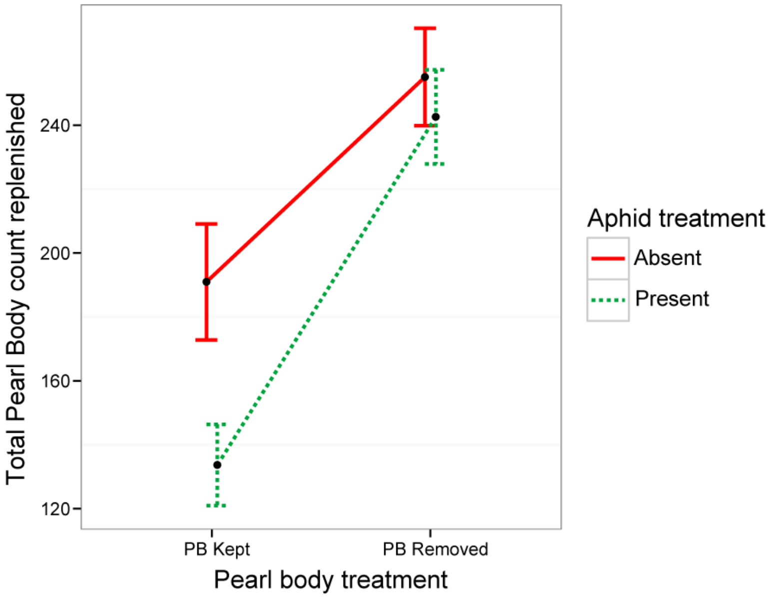

Figure 4: Total replenished pearl body count when pearl bodies are removed or kept, in the presence and absence of aphids. Error bars represent ± 1 SE.

As PB treatment had the most significant effect on PB replenishment we split the data between PB kept and PB removed plants. We found that in PB Kept plants total PB replenishment marginally varied across varieties, but stem PB/cm and leaf PB/cm^2 replenishment both differed in their trend across plant varieties. Additionally, in aphid presence replenished number of pearl bodies reduced, but only in PB kept plants (Table 3, Figure 3b). In PB removed plants, aphids did not affect any replenishment variable (Table3). Instead in PB removed plants, replenished PB number decreased with an increase in plant growth (Table 3) and varied across plant varieties (Table 3); it was highest for *Hire* and least for *Paysan* (Figure 3b).

Table 3: Effect of experimental variables on replenishment rates in PB kept and PB removed plants

	Total PB replenishment		**Leaf PB replenishment/cm2**		**Stem PB replenishment/cm**	
	PB Kept plants	**PB Removed plants**	**PB Kept plants**	**PB Removed plants**	**PB Kept plants**	**PB Removed plants**
Plant RGR	**$F_{1,73}$=8.44, *P*=0.029**	**$F_{1,73}$=25.44, *P*<0.001**	NA	NA	NA	NA
Plant variety	$F_{3,73}$=2.18, *P*=0.098	**$F_{3,73}$=26.42, *P*<0.001**	**$F_{3,74}$=6.98, *P*=0.003**	**$F_{3,74}$=61.11, *P*<0.001**	**$F_{3,74}$=7.53, *P*<0.001**	**$F_{3,74}$=17.31, *P*<0.001**
Aphid treatment (Presence or absence)	**$F_{1,73}$=7.09, *P*=0.009**	$F_{1,73}$=0.16, *P*=0.692	**$F_{1,74}$=4.59, *P*=0.035**	$F_{1,74}$=1.69, *P*=0.101	**$F_{1,74}$=10.21, *P*<0.001**	$F_{1,74}$=3.04, *P*=0.08

Linear models were used with normal distribution; all higher-order interaction terms were included in the maximal model and removed from the final model if not significant. 'NA' indicates a term not included in the model.

Plant relative growth rate (plant RGR) differed across okra varieties ($F_{3,148}$=17.99, *P*<0.001) and plants of *Paysan* variety grew the most followed by *Kirikou*, *Clemson* and *Hire*. All plants with aphid presence grew more than with aphid absence ($F_{1,148}$=14.67, *P*=0.001) and pearl body treatment did not affect the growth of plants ($F_{1,148}$=0.86, *P*=0.356). Additionally, plant growth also reduced with an increase in pearl body replenishment ($F_{1,148}$=21.37, *P*<0.001). We also recorded a significant two-way interaction between plant variety and aphid treatment on plant RGR ($F_{3,148}$=2.82, *P*=0.041), where aphid presence increased plant RGR for all varieties but the highest increase was observed for *Kirikou* variety (Figure SI 6). In 2016 too we recorded a significant effect of aphids on plant RGR, however, here aphid presence increased plant RGR on all varieties except *Hire* variety (Figure SI 6).

As mentioned above, total leaf area did vary across plant varieties (Table SI 1 and SI 4) and reduced with an increase in plant height ($F_{3,151}$=16.83, *P*<0.001). In the experiment conducted in 2015 we did not record a significant effect of aphids on leaf area ($F_{1,151}$=0.71, *P*=0.689), although, in 2016 we did observer total leaf area to marginally increase in aphid presence ($F_{1,149}$=8.29, P=0.005, Figure SI 7).

Discussion

Overall, our study shows that pearl body production differs across okra varieties and we also illustrate how this variation can mediate the ant-plant interactions in the field. Pearl body production was inducible by pearl body removal and was also costly for plants as plant growth reduced with an increase in pearl body replenishment. Our study depicts the context dependency of production of plant defensive compounds (pearl bodies) by describing the novel effect of aphid herbivores on pearl body production. Aphids reduced pearl body production only when these were not removed (i.e. simulated ant absence). When pearl bodies were removed (i.e. simulated ant presence) aphids had no direct effect on their replenishment. However, aphids indirectly affected pearl body production as plant growth increased when aphids were present, which was related to reduced number of replenished pearl bodies (Figure 5).

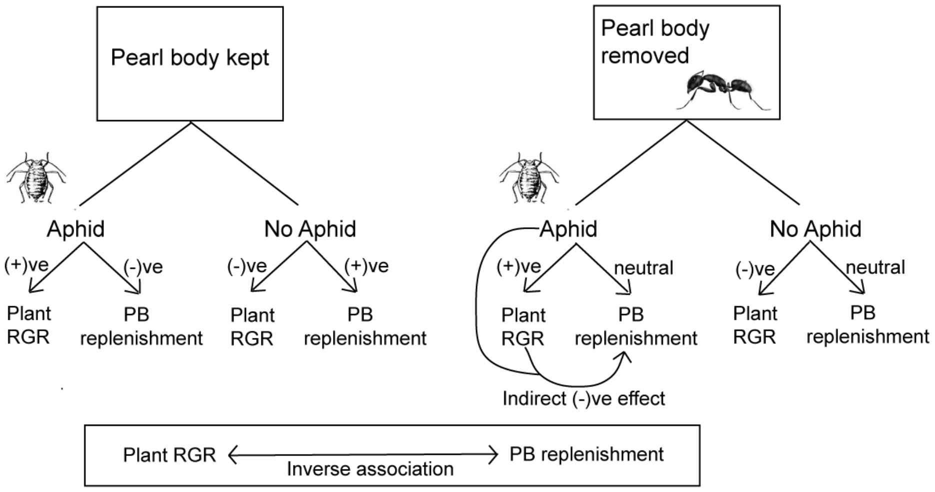

Figure 5: Indirect effect of aphid presence on pearl body (PB) replenishment in plants where pearl bodies were removed. Removal of pearl bodies simulates ant presence.

We found ant preference for aphid honeydew or okra pearl bodies to be species-specific; while *Pheidole* ants favoured okra pearl bodies, *Camponotus* ants showed no preference and all ‘other’ ants favoured aphid honeydew. Our results are in accordance with previous studies which have shown that preference of ants to sugars and amino acids is ant species-specific (Blüthgen & Fiedler, 2004). The chemical composition of aphid honeydew and pearl bodies is different, with honeydew largely composed of sugars (Völkl et al., 1999), whereas, lipids, proteins and amino acids are the primary constituents of pearl bodies (Fischer et al., 2002; Heil et al., 1998). Hence, variable preference of different ant species of different compounds can cause their variable preference of aphid honeydew or pearl bodies. Further, production of food bodies is usually linked to the attraction of specialist ants (Fiala et al., 1994). This may explain why okra pearl bodies do not attract all ant species equally and mostly attract ants of genus *Pheidole*. We also recorded ants of genus *Pheidole* to prefer the *Caffeier* variety of okra, which potentially occurred due to the highest initial pearl body count on this variety. Attraction of certain ant species to pearl bodies and to particular varieties can be beneficial for development of integrated pest control measures, specifically as several ant species are beneficial for agriculture (Offenberg, 2015).

The reduction of pearl bodies in our controlled studies in the presence of sap-sucking aphids (only when pearl bodies are not removed) may be due to nutrient drain. Studies in other pearl body bearing plants suggest that good nutrient supply seems to be crucial for pearl body production (Folgarait & Davidson, 1995; Heil & Baldwin, 2002; Paiva et al., 2009). This is contrary to many studies on extrafloral nectar (EFN), which have shown increased extrafloral nectar secretion to attract protective ants as a response to herbivory (Escalante-Pérez et al., 2012; Koptur, 1989; Ness, 2003). Interestingly, pearl body formation in all okra varieties correspond to EFN formation in some other members of the Malvaceae, eg *Hibiscus* or *Pavonia* (Pimentel et al., 2011; Rocha & Machado, 2009; Vogel, 2000). EFN as well as pearl bodies are trichomatous and are of epidermal original, ant attraction is reported for both. A big difference, however, may be the chemical content. EFN consists of large percentage of sugars (Bentley, 1977; Marazzi et al., 2013), whereas, pearl bodies have large amounts of lipids and proteins (Fischer et al., 2002; Heil et al., 1998; Webber et al., 2007).

Lipid and protein allocation may be more costly and one possible mechanism of pearl body reduction by aphids could be reallocation of the plant resources to plant growth than to pearl body production, in aphid presence. We did record aphid presence to increase plant growth and also to slightly increase leaf area. Plants are known to have various responses to herbivory and one of these is tolerance which is defined as capacity of plants to reduce negative effects of damage to fitness (Fornoni, 2011). Overcompensation in plants goes beyond tolerance and here plant fitness is actually increased following moderate herbivore damage and is higher than for non-attacked plants (Agrawal, 2000). Overcompensation can increase plant yield, seed set and plant biomass (Belsky, 1986; Olejniczak, 2011). This has been shown for agricultural crops; low infestation of the aphid *Toxoptera graminum* Rond. increased the dry leaf weight of wheat (Ortman & Painter, 1960) and, in a more recent study Poveda et al. (2010) showed a 2.5 times yield increase in potato tuber production after feeding by the larvae of Guatemalan potato moth. There are several suggested mechanisms for overcompensation such as resource allocation and activation of dormant meristem (Tiffin, 2000; Trumble et al., 1993). Allocation of stored resources states that distribution of available resources to a new site occurs at the expense of other metabolic centre (Trumble et al., 1993). As *A. gossypii* mostly feeds on leaves it may reduce leaf tissue resources, which may further cause utilization of stored plant resources for plant growth. Further, according to activation of dominant meristematic tissue mechanism (Tiffin, 2000) , aphid attack might have also caused okra plants dormant meristematic tissue to be activated, leading to an increase growth. This is further evident by our results where we observed a marginal reduction only on stem and no effect on leaf pearl body replenishment in aphid presence when pearl bodies were removed from the plants. This may suggest higher resource use for increase in stem length than in stem pearl body count. Hence, a certain level of aphid herbivory may be beneficial for okra plants.

Optimal defence theory suggests that plants allocate resources efficiently to defences and avoid redundant defences (Stamp, 2003). Possibly this is why pearl body production was increased only when it was removed (simulated ant presence). When pearl bodies were not removed the plant did not use resources for their production probably because first, there were pearl bodies present to attract ants and second, producing more pearl bodies is costly. However, when pearl bodies were removed, the plant starts to allocate more resources to their production to attract beneficial ants. Food body (pearl bodies are food bodies) production has been found to be inducible by ants (Risch & Rickson, 1981) and by their mechanical removal (Folgarait et al., 1994). Furthermore, food body production is also known to be costly for the plant and its production can reduce aboveground plant biomass (Heil et al., 1997) and also plant growth (Frederickson et al., 2012). Similarly, producing pearl bodies was also costly for okra plants as plant growth reduced with an increase in the number of replenished pearl bodies; the shortest plants of *Kirikou* variety had the highest stem pearl body replenishment/cm. It may be argued here that if aphids can increase plant fitness, then why does okra produce pearl bodies? It is possible that the positive effect of aphids on plants is reduced as aphid numbers increase and thus the plant invests in defensive compounds. As we had put only 30 aphids per plant at the beginning of the experiment, there numbers probably did not reach damaging levels in one week and hence we observed a positive effect.

Finally, we observed that for plants where pearl bodies were not removed, their initial response (short term experiment) to aphid presence was different to their later response (long term experiment) and this differed across varieties. On two of the varieties (*Clemson* and *Hire*) aphid presence increased and on the other three it reduced pearl body replenishment in the short term experiment (PB kept only). However, in the long term experiment aphid presence reduced replenishment for all plant varieties. As mentioned above, composition of plant defence compounds such as glucosinolates can vary across plant varieties and genotypes (Hopkins et al., 2009; Padilla et al., 2007). Furthermore, an induced plant response to herbivory can also vary across varieties (Agrawal et al., 2002; Underwood et al., 2000). Hence, it is not a surprise that we observed variation across okra varieties in their initial response to aphid presence with *Clemson* and *Hire* increasing pearl body production. However, as mentioned above, pearl body production is costly and in the absence of ants (simulated by non-removal of pearl bodies) these plant varieties reduce their pearl body production overtime. They would have specifically reduced it as aphid presence possibly lead reallocation of plant resources to plant growth.

Our study explores the possible tritrophic interactions involved in inducible defence and shows how inducible defences can be context dependent. We highlight how herbivory can induce multiple responses in a plant that can actually benefit the plant. Recruitment of *Pheidole* ants by pearl body production will be beneficial for okra plants as in our previous study we recorded *Pheidole dea* Santschi to predate upon aphids on *Caffeier* variety (Singh et al., 2016). Further, development of varieties like *Caffeier* which attracted higher *Pheidole* ant numbers can be advantageous for aphid control measures. Despite any potential positive effects on okra plants, control of aphids is still important as aphids can potentially transmit plant viruses (Katis et al., 2007). Pearl bodies are prevalent in many plant families and despite of their role in plant protection, they have largely been ignored. Further, work is needed to understand the potential role of pearl bodies in plant protection in different agricultural crops.

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Supplementary material I

Effect of ant-plant-aphid interactions on okra pearl body production and its variation across okra varieties

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Table S1: Effect of different varieties on initial plant height, leaf number, leaf area and initial pearl body count in all experiments

	Explanatory variable : Plant variety		
	Experiment 1	**Experiment 2**	**Experiment 3**
Initial plant height	$F_{4,228}=18.32$, $P<0.001$	$F_{4,95}=5.62$, $P=0.021$	$F_{3,154}=213.2$, $P<0.001$
Leaf number	$F_{4,228}=4.06$, $P=0.003$	$F_{4,95}=23.63$, $P<0.001$	$F_{3,154}=17.57$, $P<0.001$
Initial pearl body count	$F_{4,113}=21.32$, $P<0.001$	$F_{4,45}=26.45$, $P<0.001$	$F_{3,154}=67.81$, $P<0.001$
Leaf area	-	-	$F_{3,154}=21.3$, $P<0.001$

Table S2: Initial plant height and leaf number variation across okra varieties.

	Initial plant height (cm)			**Leaf number**		
Plant variety	**Expt. 1**	**Expt. 2**	**Expt. 3**	**Expt. 1**	**Expt. 2**	**Expt. 3**
Clemson	21.1±0.77	14.9±0.90	41.9±0.45	3.8±0.11	3.3±0.24	6.2±0.13
Hire	16.3±0.76	14.6±0.38	31.5±0.48	4.4±0.11	4.9±0.07	5.9±0.12
Kirikou	16.6±0.57	12.7±0.48	25.6±0.87	4.0±0.13	3.5±0.15	6.8±0.11
Paysan	23.9±0.68	16.4±0.41	44.6±0.51	4.2±0.10	4.8±0.09	7.1±0.09
Caffeier	18.9±0.90	18.7±1.11	-	4.4±0.09	4.7±0.18	-

Table S3: Initial pearl body count and final pearl body count variation across okra varieties.

	Initial pearl body count			**Final pearl body count**		
Plant variety	**Expt. 1**	**Expt. 2**	**Expt. 3**	**Expt. 1**	**Expt. 2**	**Expt. 3**
Clemson	112.3±9.81	86.7±12.52	564.2±19.41	-	92.3±16.16	751.2±37.38
Hire	137.7±13.25	146.1±22.81	540.1±11.75	-	150.9±26.91	741.0±24.40
Kirikou	152.1±12.28	100.1±6.32	437.5±28.94	-	101.8±10.05	652.3±41.87
Paysan	58.2±5.92	25.5±2.83	223.3±7.83	-	28.9±4.01	345.3±15.38
Caffeier	200.6±13.38	180.1±25.00	-	-	172.9±20.50	-

Table S4: Total leaf area, individual leaf size and final plant height of different plant varieties in the greenhouse experiment in Germany

Plant variety	Total leaf area (cm^2)	Individual leaf size (cm^2)	Pearl body count per cm^2	Average final plant height (cm)
Clemson	606.3±9.23	119.1±1.85	1.24	60.0±0.39
Hire	764.4±12.90	142.7±2.50	0.97	44.4±0.68
Kirikou	675.6±18.53	99.6±2.43	0.97	45.1±1.41
Paysan	665.8±13.68	99.3±2.26	0.52	65.2±0.83

Figure S1: Experimental set up in the field to test preference of different ant species of aphid honeydew or okra pearl bodies. The image shows set up of one replicate. The plants are placed in four groups around the ant colony; each group includes all five plant varieties. Group 1: (-)Aphid (+)PB, Group 2: (-)Aphid (-)PB, Group 3: (+)Aphid (+)PB, Group 4: (+)Aphid (-)PB.

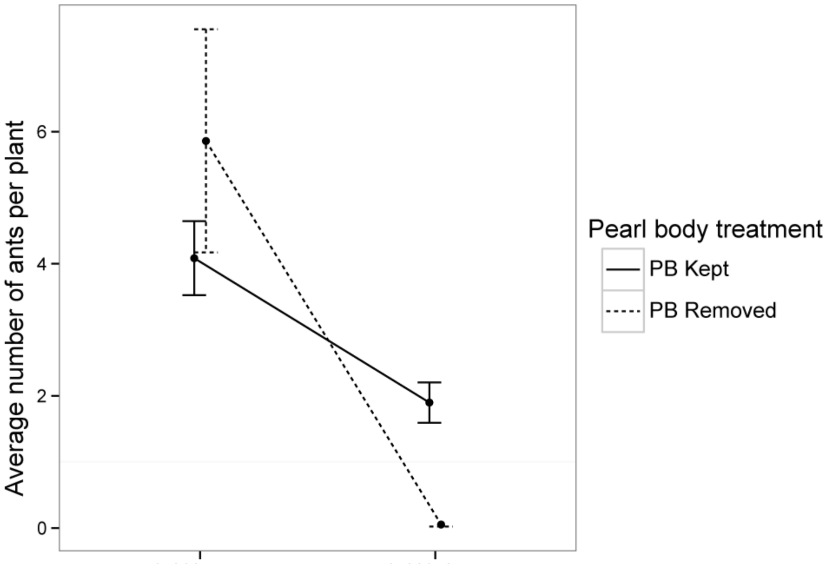

Figure S2: Average number of all ant species per plant recorded on plants with pearl bodies kept and removed, in the presence and absence of aphids. Error bar represent ±1 SE.

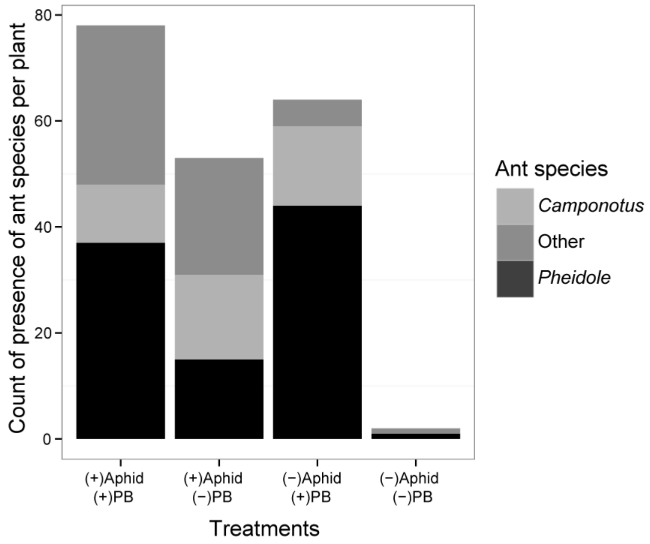

Figure S3: Count of presence of different ant species in different aphid and pearl body treatments. Each bar represents our aphid and pearl body treatments. The proportion of colour within a bar represents the total count of presence of a particular species within that treatment.

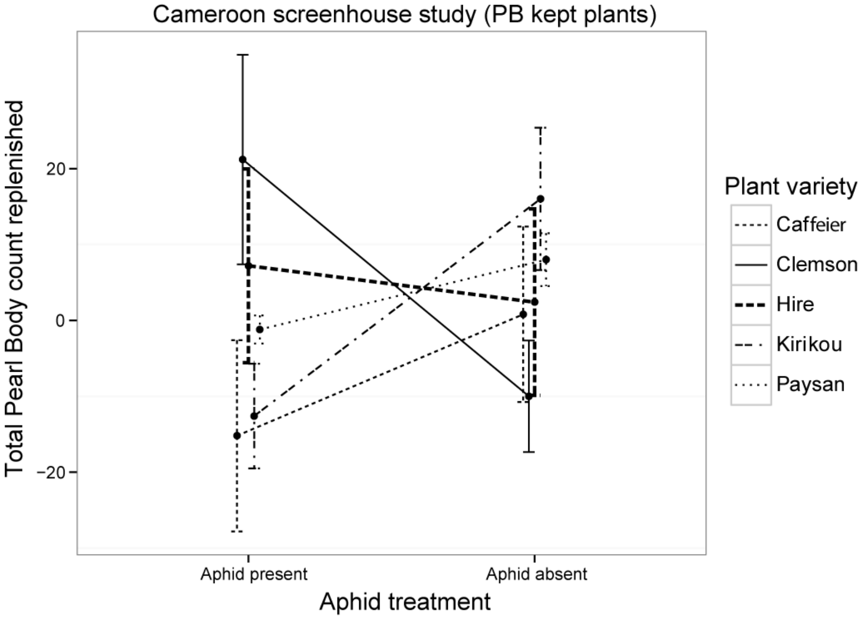

Figure S4: Variation across okra varieties in their total replenished pearl body count in PB kept plants, in the presence and absence of aphids. Error bar represent ±1 SE.

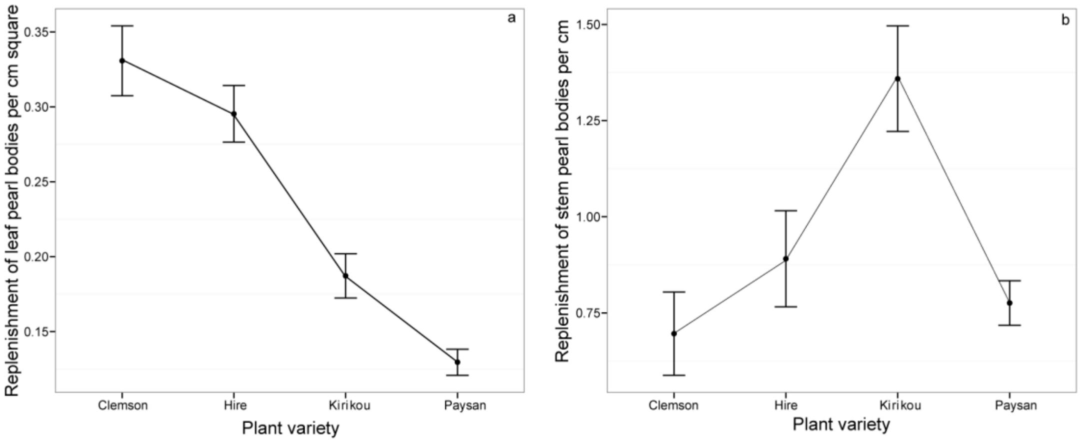

Figure S5: (a) Variation across okra varieties in leaf pearl body replenishment per cm^2, (b) Variation across okra varieties in stem pearl body replenishment per cm. Error bar represent ±1 SE.

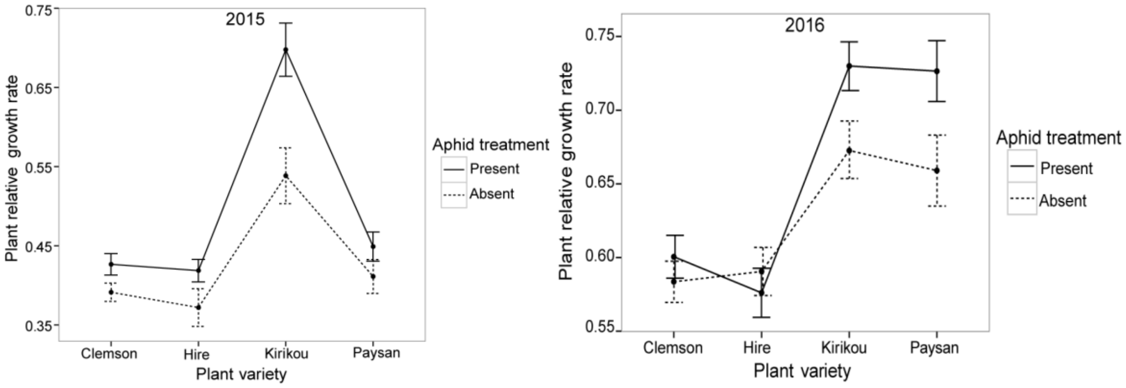

Figure S6: Variation across okra varieties in plant relative growth rate in one week, in aphid presence and absence. Error bar represent ±1 SE.

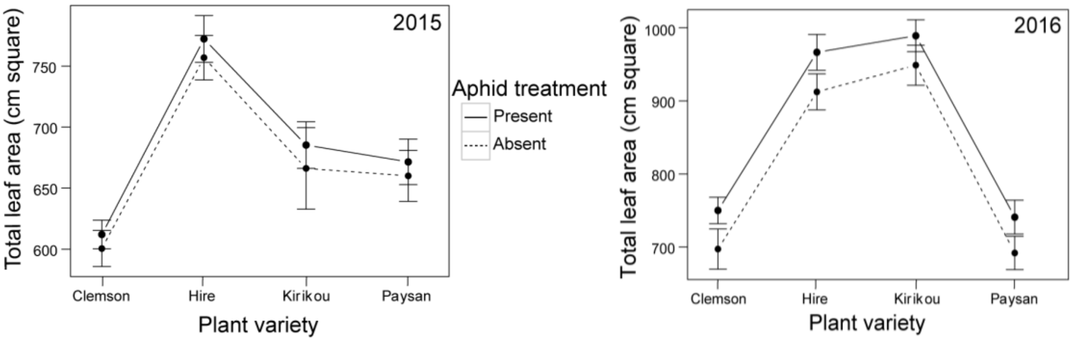

Figure S7: Variation across okra varieties in total final leaf area, in aphid presence and absence. Error bar represent ±1 SE.

Supplementary material II

Effect of ant-plant-aphid interactions on okra pearl body production and its variation across okra varieties

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Summary of results of the repeated long term pearl body production experiment (2nd June – 11th June, 2016)

1.1 Plant morphology and pearl body count variation across varieties

Similar to the results from 2015, plant variety significantly affected initial plant height ($F_{3,152}$=188.00, P<0.001), initial pearl body count ($F_{3,152}$=70.30, P<0.001) and total leaf area ($F_{3,149}$=17.09, P<0.001). Plants of *Clemson* and *Paysan* variety were found to be the tallest and of *Kirikou* the shortest (Table 1). There was lower difference (<1 leaf) recorded between varieties in their leaf numbers in the year 2016 than in the year 2015 (Table 1).

Similar to 2015, highest initial PB count and pearl body count per cm^2 was recorded for plants of *Clemson* variety and lowest PB count and the lowest PB count per cm^2 was recorded for plants of *Paysan* variety (Table 1). Pearl body count increased on all PB kept plants during the course of the experiments but the % increase was almost double in 2016 than in 2015. In 2016, final PB count of PB kept plants increased by 71.6%.The trend across varieties for final PB count was always similar as that for initial count in both years, with *Clemson* and *Hire* varieties having the highest and *Paysan* having the lowest pearl body count. For PB removed plants also the % final PB increase was higher in 2016. In 2016, PB removed plants replenished 116% of their initial PB count.

Leaf area variation across varieties (Table 2) followed an opposite trend than plant height in both years; *Hire* and *Kirikou* varieties with shorter plants had the largest and *Clemson* and *Paysan* varieties with taller plants had the smallest total leaf area (Table 2). Area of an individual leaf was also highest for *Hire* and *Kirikou* varieties (Table 2). In both years, Plants with largest leaf area did not have the highest PB count per cm^2 as *Clemson* plants with smallest total leaf area had the highest PB count per cm^2 (Table 1 and 2).

1.2 Effect of experimental variables on final pearl body counts (PB kept plants only)

In both years, we found similar overall effects of aphids, plant variety and final plant height on total final pearl body count (Table 3). Final PB count (PB kept plants) varied across varieties (Table 3) and it was highest on *Clemson* and *Hire* and lowest on *Paysan* variety (Table 1). Overall, final PB count was lower in aphid presence (713.5±44.79) than in aphid absence (827.7±53.19). Additionally, final PB count decreased with an increase in final height (Table 3).

1.3 Effect of experimental variables on pearl body replenishment in all plants

In both years we recorded significant effects of plant variety, aphid treatment and pearl body treatment on pearl body replenishment (Table 3). In both years, total PB replenishment and leaf PB replenishment/cm^2 was highest for *Clemson* and lowest for *Paysan* variety. Further, total replenishment rate was always recorded to be higher when PB was removed (620.8±38.16) than when PB was kept (401.4±32.88) and leaf replenishment/cm^2 and stem PB replenishment/cm (Table 3) were also higher when PB was removed than when PB was kept. Similar to previous year, we recorded a significant two-way interaction between aphid treatment and PB treatment in both years on total PB replenishment (Table 3). Here, aphid reduction of PB replenishment was stronger in PB kept plants than in PB removed plants (Figure 1). In addition, total replenishment reduced with an increase in plant RGR (Table 3) i.e., more a plant grew lower was its replenishment rate. Overall, aphids reduced both leaf replenishment/cm^2 and stem replenishment/cm (Table 3).

In 2016, we recorded a significant 2-way interaction between pearl body treatment and plant variety on all replenishment response variables (Table 3). On all plants total, and leaf replenishment per cm^2 was higher when pearl bodies were removed than when they were kept, except for *Paysan* variety (Figure 2). On this particular variety we recorded no difference in pearl body replenishment between PB removed and kept plants. Similar effects of pearl body treatments were observed in the year 2015 (Figure 2), despite no significant interaction.

1.4 Effect of experimental variables on pearl body replenishment in PB kept and PB removed plants

In both years, we consistently found that in aphid presence replenishment rate reduced, but only in PB kept plants (Table 4, Figure 3). In PB removed plants, aphids did not affect total, leaf/cm^2 PB replenishment or stem replenishment/cm (Table 4). Instead in PB removed plants, plant variety and relative plant growth mediated pearl body replenishment rate (Table 4).

Table 1: Variation across okra varieties in their initial and final plant height, initial leaf number, initial and final pearl body count and final leaf pearl body count/cm^2

Plant variety	Initial plant height	Initial leaf number	Final plant height	Initial pearl body count	Final pearl body count	Final leaf pearl body count/cm^2
Clemson	17.5±0.19	6.9±0.08	39.5±0.99	915.8±63.49	983.4±70.95	1.22
Hire	13.3±0.20	6.9±0.07	23.8±0.22	695.1±3.41	992.7±4.88	1.00
Kirikou	10.3±0.17	7.1±0.07	20.8±0.26	440.3±2.98	875.4±3.70	0.83
Paysan	15.0±0.29	6.9±0.06	29.9±0.28	163.8±1.49	223.7±1.84	0.23

Table 2: Variation across okra varieties in their total final leaf area and individual leaf size

Plant variety	Total final leaf area	Individual leaf size (cm^2)
Clemson	731.7±15.72	125.3±2.04
Hire	942.7±1.72	137.2±0.23
Kirikou	974.1±1.70	142.6±0.19
Paysan	717.6±1.65	92.3±0.19

Table 3: Effect of main experimental variables and covariates

	Total final PB count (PB kept)	**Total PB replenishment**	**Leaf PB replenishment /cm^2**	**Stem PB replenishment /cm**
Plant RGR	NA	$F_{1,145}$=4.435, *P*=0.037	NA	NA
Final height	↓$F_{1,70}$=4.85, P=0.031	NA	NA	NA
Plant variety	$F_{3,70}$=112.99 , *P*<0.001	$F_{3,145}$=65.31, *P*<0.001	$F_{3,144}$=51.66, *P*<0.001	$F_{3,147}$=2.05, *P*=0.109
Aphid treatment (Presence or absence)	$F_{1,70}$=9.32, *P*=0.003	$F_{1,145}$=15.85, *P*=0.001	$F_{1,144}$=17.62, *P*=0.001	$F_{,147}$=7.12, *P*=0.008
Pearl body treatment (Kept and Removed)	-	$F_{1,145}$=42.98, *P*<0.001	$F_{1,144}$=31.94, *P*<0.001	$F_{,147}$=14.81, *P*<0.001
Aphid×PB treatment	-	$F_{1,145}$=4.03, *P*=0.047	-	-
Plant variety×PB treatment	-	$F_{1,145}$=4.35, *P*=0.006	$F_{3,144}$=3.97, *P*=0.009	$F_{3,147}$=4.99, *P*=0.002
Plant variety×Aphid	-	-	$F_{3,144}$=3.10, *P*=0.028	-

Linear models with normal distribution and generalised linear models with quasipoisson distribution were used; all higher-order interaction terms were included in the maximal model and removed from the final model if not significant. '-'indicates when a term was not significant. 'NA' indicated when a term was not included in the model.

Table 4: Effect of experimental variables on replenishment rates in PB kept and PB removed plants

	Total PB Replenishment		Leaf PB replenishment/cm2		Stem PB replenishment/cm	
	PB Kept plants	**PB Removed plants**	**PB Kept plants**	**PB Removed plants**	**PB Kept plants**	**PB Removed plants**
Aphid GR	$F_{1,35}$=15.86, *P*=0.003	$F_{1,34}$=0.28, *P*=0.602	$F_{1,35}$=11.20, *P*<0.001	$F_{1,35}$=0.002, *P*=0.966	$F_{1,35}$=1.19, *P*=0.283	$F_{1,34}$=0.87, *P*=0.358
Plant RGR	$F_{1,70}$=0.589, *P*=0.589	$F_{1,74}$=23.07, *P*<0.001	NA	NA	NA	NA
Plant variety	$F_{3,71}$=15.12, *P*<0.001	$F_{3,74}$=43.17, *P*<0.001	$F_{3,71}$=11.20, *P*<0.001	$F_{3,75}$=207.99, *P*<0.001	$F_{3,71}$=2.23, *P*=0.092	$F_{3,75}$=4.81, *P*=0.004
Aphid treatment (Presence or absence)	$F_{1,71}$=13.03, *P*<0.001	$F_{1,74}$=2.77, *P*=0.101	$F_{1,71}$=11.27, *P*=0.001	$F_{1,75}$=1.16, *P*=0.163	$F_{1,71}$=6.95, *P*=0.010	$F_{1,75}$=1.19, *P*=0.279

Linear models with normal distribution and generalised linear models with quasipoisson distribution were used; the higher-order interaction term was included in the maximal model and removed from the final model as it was not significant.'NA' indicated when a term was not included in the model.

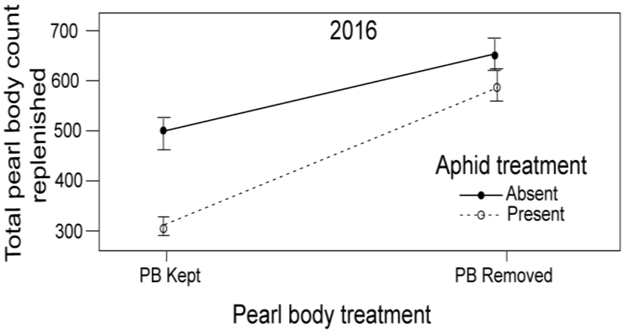

Figure 1: Total pearl body count replenished when pearl bodies are removed or kept, in aphid presence and absence

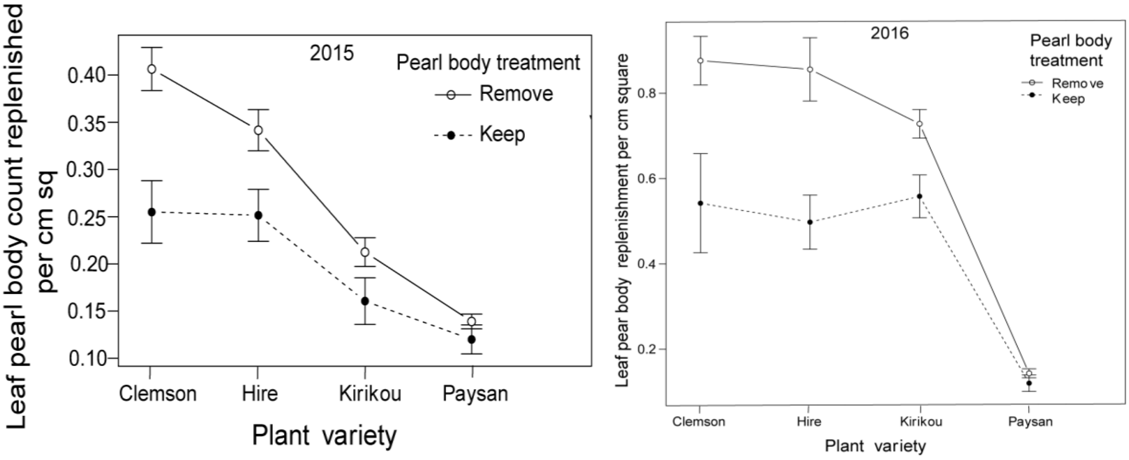

Figure 2: Variation across okra varieties in their leaf per body replenishment per cm^2 in plants where pearl bodies were removed and kept, in the experiments conducted in the year 2015 and 2016

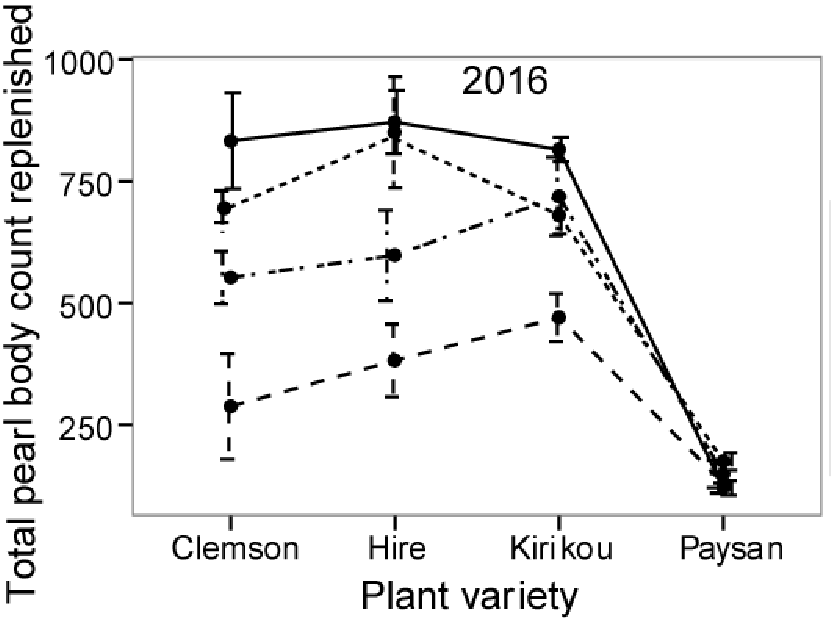

Figure 3: Total pearl body replenished in one week in different experimental treatments, in the experiments conducted in the year 2016

Manuscript IV

Plant genetic variation mediates an indirect ecological effect between belowground earthworms and aboveground aphids

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Plant genetic variation mediates an indirect ecological effect between belowground earthworms and aboveground aphids

Akanksha Singh, Julia Braun, Emilia Decker, Sarah Hans, Agnes Wagner, Wolfgang W Weisser and Sharon E Zytynska*

Abstract

Background: Interactions between aboveground and belowground terrestrial communities are often mediated by plants, with soil organisms interacting via the roots and aboveground organisms via the shoots and leaves. Many studies now show that plant genetics can drive changes in the structure of both above and belowground communities; however, the role of plant genetic variation in mediating aboveground-belowground interactions is still unclear. We used an earthworm-plant-aphid model system with two aphid species (*Aphis fabae* and *Acyrthosiphon pisum*) to test the effect of host-plant (*Vicia faba*) genetic variation on the indirect interaction between the belowground earthworms (*Eisenia veneta*) on the aboveground aphid populations.

Results: Our data shows that host-plant variety mediated an indirect ecological effect of earthworms on generalist black bean aphids (*A. fabae*), with earthworms increasing aphid growth rate in three plant varieties but decreasing it in another variety. We found no effect of earthworms on the second aphid species, the pea aphid (*A. pisum*), and no effect of competition between the aphid species. Plant biomass was increased when earthworms were present, and decreased when *A. pisum* was feeding on the plant (mediated by plant variety). Although *A. fabae* aphids were influenced by the plants and worms, they did not, in turn, alter plant biomass.

Conclusions: Previous work has shown inconsistent effects of earthworms on aphids, but we suggest these differences could be explained by plant genetic variation and variation among aphid species. This study demonstrates that the outcome of belowground-aboveground interactions can be mediated by genetic variation in the host-plant, but depends on the identity of the species involved.

Keywords: Aboveground-belowground interactions, *Aphis fabae*, *Acyrthosiphon pisum*, Genetic interactions, Plant genotype, *Vicia faba*

Background

There is increasing recognition that aboveground-belowground interactions are important drivers of community and ecosystem processes, e.g. nutrient cycling [1]. Investigating the link between aboveground and belowground species is therefore important not only to understand the various interactions, but can also benefit the conservation of ecosystems and the services they provide [2]. Interactions between belowground and aboveground communities are often mediated by the plants that connect them [3]. This can also be described as an indirect ecological effect (IEE) when the outcome of an interaction is mediated by the presence of a third species (e.g. plant) [4,5]. Plant-mediated indirect effects have now been shown for a variety of species interactions [6-9].

In an aboveground-belowground system, aboveground herbivores can positively influence soil communities by increasing soil nitrogen through returning organic matter as labile faecal material [10]; but, they can also have negative effects through impairment of net primary productivity via tissue removal [11], by reducing plant root growth and biomass [12,13] and through the induction of secondary

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defense compounds [14]. Similarly, soil organisms have varying influences on aboveground communities. Insect root herbivores can induce nutrient changes within the foliage of the host plant and have been found to increase fecundity of leaf miners [15,16] and aphids [15]. Belowground decomposers mobilize nutrients that increase plant quality and the fitness of aboveground herbivores [17,18], and they can also upregulate defensive compounds in the plant which may negatively influence aboveground herbivores [19]. Root herbivores have also been shown to influence seed predators and natural enemy trophic levels, via plant-mediated interactions [20].

The mechanisms that drive plant-mediated interactions include effects on resource quality [8] and the induction of plant defenses [9]. The outcome of belowground-aboveground indirect interactions can be positive for the organisms involved, when both components respond similarly, or negative/neutral, when each component responds to different abiotic constraints or resource quality outweighs the effects of resource heterogeneity [2,21]. Studies on plant-mediated indirect interactions have only rarely considered the role of plant genetic variation (but see [22,23]); however, it is known that genetically-based traits in a plant lead to variation amongst individuals (e.g. for plant structure, nutritional value or defense chemicals) and these differences possibly play a role in species interactions. Genetic variation in plants is already known to influence the community structure of invertebrates, fungi and plants living on and around the focal plant [24-26]. This means that ecological communities associated with different plant genotypes vary and this can lead to changes in the interaction networks; for example, through host-associated differentiation via trophic cascades [27]. Genetic variation in the host-plant can also lead to genotype-by-environment interactions where the plant genotype mediates the effect of the indirect interaction [28]. For example, the effect of rhizobacteria in the soil on aphids feeding on the plant, and their parasitoids, is dependent on the specific genotype of the host plant and further, the genotype of the aphid [22,23]. The study of the link between plant genetic variation and soil communities is still nascent and focuses on decomposer communities, but does show strong effects indicating potential strong linkages between these components [29].

Aphids are a good model species when studying aboveground-belowground interactions because they experience an intimate relationship with their plant hosts through feeding on the plant phloem-sap and thus are able to detect even slight changes in host quality [30]. Aphids often feed on only a few host plants but some are more polyphagous than others, which may lead them to be more susceptible to physiological changes in the plant than other more specialized aphids [31,32]. Furthermore, aphids exhibit preference and performance differences among host-plant species and genotypes indicating that changes in host quality can affect fitness and host-choice traits [33-35]. Another good model species for studying belowground-aboveground interactions are earthworms which are known decomposer ecosystem engineers [36]. The regulation of plant performance by earthworms has been documented in a number of studies showing that earthworms can alter plant nitrogen content by enhancing nitrogen availability in the soil [37-39]. This is generally beneficial for plants but can have an indirect disadvantage, for example, when it leads to increased herbivory [40,41]. Inconsistent effects of earthworms on aphids have been found; with positive, negative and no effects being published [40,42-46]. These studies did not consider plant genetic variation within the system and were predominantly carried out using *Myzus persicae* aphids. We suggest that these interactions may be mediated by plant genotype and vary across aphid species.

We conducted a greenhouse study to determine if belowground-aboveground interactions are mediated by plant variety, using a model system with composting earthworms, four broad bean plant varieties, and two species of aphid (Figure 1). The aphid species used are both common pests of bean plants, but differ in host-plant breadth (one feeding solely on legumes and the other is polyphagous, feeding on multiple plant families). We hypothesized that, earthworms would generally increase nitrogen availability in the soil, thus increasing plant biomass and also aphid density; although the magnitude of this effect would depend on the variety of the plant. In addition, we reared the two aphid species alone and together to see if competition between the aphid species would influence the effect of the earthworms on plants and aphids. The bean varieties used in our study are commercially sold broad bean varieties which are commonly grown by farmers.

Results

Aphid growth

Overall, the two aphid species differed in their reproductive performance ($F_{2,249} = 32.42$, $P < 0.001$; Table 1) with fewer *A. pisum* aphids than *A. fabae* aphids after two weeks growth ($t = 7.55$, $P < 0.001$). When there were only *A. pisum* we observed 206.9 ± 22.4 (mean ± SE) aphids at the end of the experiment, whereas in the pots with only *A. fabae* there were 353.5 ± 22.2 (mean ± SE) aphids, and when both aphid species (mixed) were present we observed an intermediate level with 304.4 ± 19.2 (mean ± SE) aphids. Further, the effect of the earthworm treatment on aphid numbers was dependent on the plant variety (worm x plant interaction: $F_{3,249} = 3.42$, $P = 0.018$). However, this interaction effect was not consistent across the two aphid species (Table 1; Figure 2). There was a significant

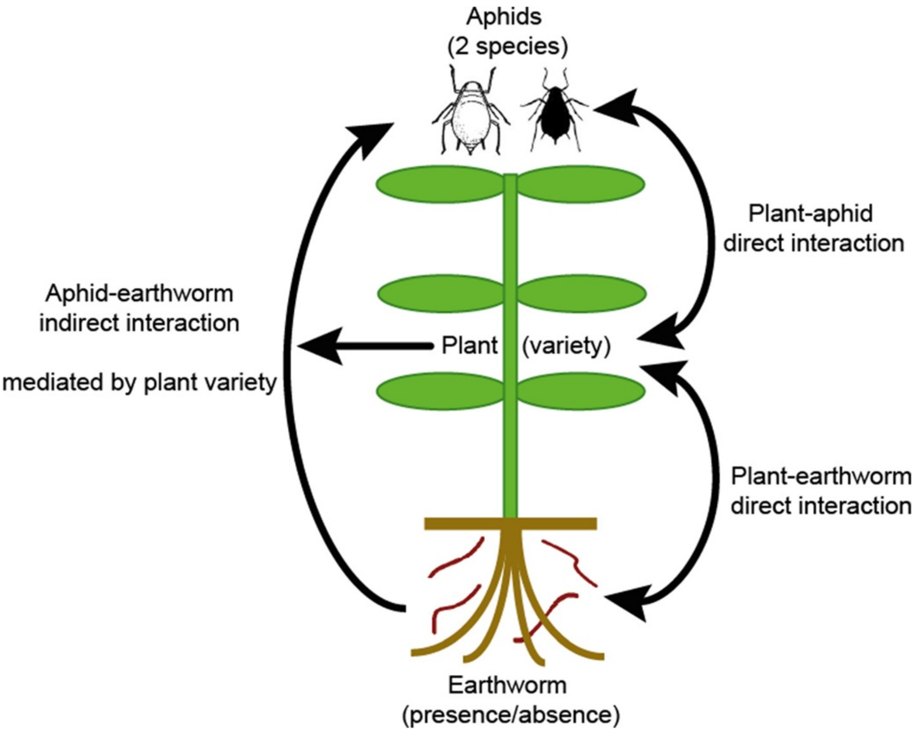

Figure 1 The model system used in this study consisted of four varieties of the broad bean plant (*Vicia faba*), with two aphid species (*Aphis fabae* and *Acyrthosiphon pisum*) and earthworms (presence or absence). Through this we were able to study direct and indirect effects of above-belowground interactions.

effect of the worm-by-plant variety interaction on the growth rate of *A. fabae* ($F_{3,170} = 4.51$, $P = 0.005$) but no effect on *A. pisum* ($F_{3,156} = 0.26$, $P = 0.856$).

For *A. fabae* we found that for three of our four plant varieties the presence of earthworms in the system increased the growth rate of the aphids, but for one variety (Hangdown) the growth rate decreased (t = 2.08, $P = 0.038$, Figure 2). There was no effect of competition between the two aphid species, with the growth rate of neither aphid species being affected by the presence of the other (*A. fabae*: $F_{1,169} = 0.24$, $P = 0.622$; *A. pisum*: $F_{1,162} = 0.06$, $P = 0.812$).

Plant biomass

The plant biomass varied across plant variety ($F_{3,339} = 36.69$, $P < 0.001$), with Hangdown producing the largest (t = 2.85, $P = 0.005$) and Piccola the smallest (t = 6.66, $P < 0.001$) plants. Earthworm presence in the soil increased plant biomass by 10.6% (across all plant varieties) ($F_{1,339} = 10.07$, $P = 0.002$). There was an effect of aphid treatment ($F_{3,339} = 5.44$, $P = 0.001$, Figure 3), with a reduced plant biomass when *A. pisum* was alone compared to the control plants with no aphids (t = 3.90, $P < 0.001$). There was no reduction in plant biomass when both aphids were present (t = 0.70, $P = 0.485$) or *A. fabae* alone (t = 0.12,

Table 1 Effects on aphid growth rate, for all aphids combined and each separate species

Response variable:	All aphids			*A. fabae*			*A. pisum*		
Aphid density	df	F	P	df	F	P	df	F	P
Block	3,249	11.85	<0.001	3,170	4.05	0.008	3,167	10.53	<0.001
Plant biomass	-	-	-	-	-	-	1,167	9.97	0.002
Plant variety	3,249	1.87	0.136	3,170	1.07	0.362	-	-	-
Aphid treatment	2,249	32.42	<0.001	-	-	-	-	-	-
Worm treatment	1,249	3.98	0.047	3,170	7.22	0.008	-	-	-
Plant variety x worm	3,249	3.42	0.018	3,170	4.51	0.005	-	-	-

Notes: '-'shows where a term was not retained in the minimal adequate model. Models used were linear models in R. All interactions between plant, aphid, and worm treatment were tested. Aphid treatment for combined shows the difference between the species and when each species is analysed separately it shows the effect of competition from being reared together with the other aphid.

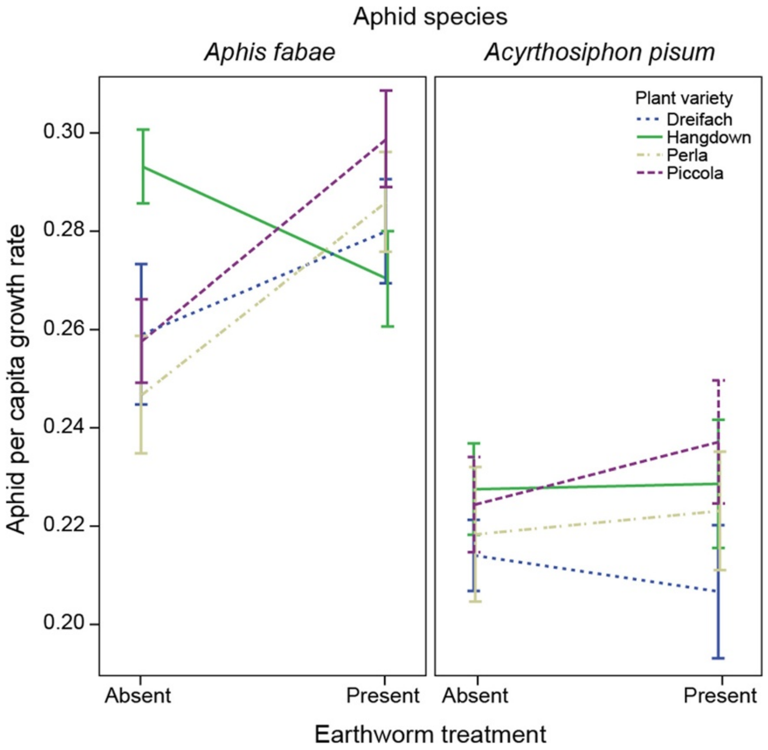

Figure 2 Aphid growth rate as a function of plant variety and earthworm presence in the experiment. Aphids were counted after 14 days and growth rate calculated separately for each species. There was a significant effect of the plant-by-earthworm interaction on the growth rate of *A. fabae*. Error bars represent ±1 SE.

P = 0.734). Growth rate of *A. pisum* also influenced plant biomass depending on the plant variety ($F_{3,163}$ = 9.40, *P* < 0.001); Hangdown experienced reduced biomass with increasing aphid growth rate (t = 2.35, *P* = 0.021) whereas the other plant varieties experienced no such effect.

Plant Carbon/Nitrogen ratio

Plant C:N did not influence aphid density (all aphids: $F_{1,136}$ = 0.007, P = 0.935) but it was itself influenced by a three-way interaction between plant variety, earthworm treatment and aphid treatment ($F_{9,163}$ = 2.64, P = 0.007; Figure 4). This means there was no overall negative or positive effect on plant C:N of the earthworms, with the outcome dependent on the combination of plant variety and aphid treatment (Figure 4). This interaction term was partly driven by differences in the Hangdown variety across the worm and aphid treatments (t = 2.24, P = 0.026), where the C:N was lower (increased nitrogen) when earthworms were present in control and *A. pisum* treatments, but the opposite was true for the *A. fabae* treatment (Figure 4). In addition, there was a higher C:N (reduced nitrogen) in Perla when aphids were present than when aphids were absent. Overall the plant varieties, Dreifach and Hangdown had lower C:N than Perla and Piccola.

Discussion

In this paper we have demonstrated an effect of belowground earthworms on aboveground aphid growth that was mediated by plant genetic variation and differed among aphid species. On three of the four plant varieties tested, the presence of earthworms increased the number of *A. fabae* aphids, whereas on one (Hangdown) there were more aphids when the earthworms were absent. These effects were only found for *A. fabae* and not for *A. pisum* aphids. *Aphis fabae* aphids had no reciprocal effect on the plant biomass whereas the presence of *A. pisum* aphids was found to reduce plant biomass; this was most apparent in the variety Hangdown, which in addition to the effect of *A. pisum* presence also showed a decrease in plant biomass with increasing aphid growth rate. Plant C:N had no influence on aphid growth rate, but it was itself influenced by the combination of all treatments in the experiment, showing that interactions between the above- and belowground communities can alter plant chemistry.

The interaction outcome between *A. fabae* and earthworms on the plant variety Hangdown differed compared to the other plant varieties for aphid number and also for plant C:N. The control plants and those with *A. pisum* had lower C:N (increased nitrogen) in the presence of worms, which is expected since earthworms can enhance

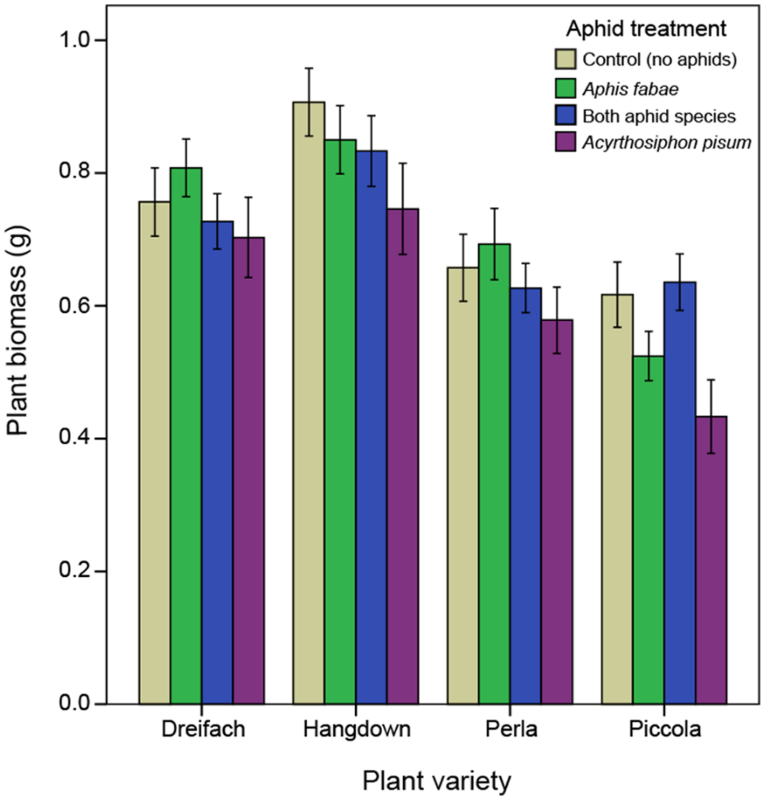

Figure 3 Effect of aphid treatment on plant aboveground biomass, as a function of plant variety, after 14 days. Plant biomass was only significantly reduced from the control (average over all varieties) when *A. pisum* was alone. Error bars represent ±1 SE.

nitrogen availability in the soil [37-39]. When *A. fabae* aphids were on the Hangdown plants with earthworms, the C:N was increased (reduced nitrogen) and this corresponded to a reduction in aphid growth rate when earthworms were present; however, as there was no significant direct effect of plant C:N on *A. fabae* aphid growth rate other genetically-based traits must also be involved, e.g. plant-defense chemicals [9]. This work suggests that earthworm mediated changes in plant nutrients are to some extent involved in these interactions, but it is not a simple effect of changing resource availability. Since this interaction effect was only detected for the polyphagous *A. fabae* aphids, this may support the findings in other herbivores that generalists are more susceptible to changes in the plant than specialists [31,32].

In our study, plant biomass increased in the presence of earthworms; this has also been found in previous studies, where earthworm presence increased the nitrogen content of plant roots and shoots leading to increased overall plant biomass [44,45,47]. The effect of earthworms on the plant chemistry in our study was found to be dependent on the plant variety and aphid treatment, such that the level of nitrogen in the plant was not consistently increased in the presence of earthworms. We also found that an increasing growth rate of *A. pisum* aphids reduced plant biomass in the Hangdown variety with no such influence for the other varieties. This was not driven by plant C:N since the ratios were similar between *A. pisum* and no aphid controls in this plant variety. *Aphis fabae* did not affect plant biomass at the densities reached in our experiment. It is expected that future aphid growth would have resulted in further detrimental effects on the plant, especially as in our experiment the aphids were unable to disperse from the experimental environment [48,49].

In our experiments we used two different aphid species and we found that there was no effect of competition between the two species on the growth rate of aphids on the plants. It is thought that competition between closely related phytophagous species would be higher than between unrelated species due to similar resource usage [50]. Predominantly, we observed *A. fabae* on the plant stem and *A. pisum* on the leaves indicating that spatial separation may reduce competition between these aphid species. The lack of competition effects also shows that there was little resource limitation during the experiment possibly due to the use of nutrient-rich potting substrate. Our study used a legume plant grown in potting substrate that is high in nutrients and it is possible that in less

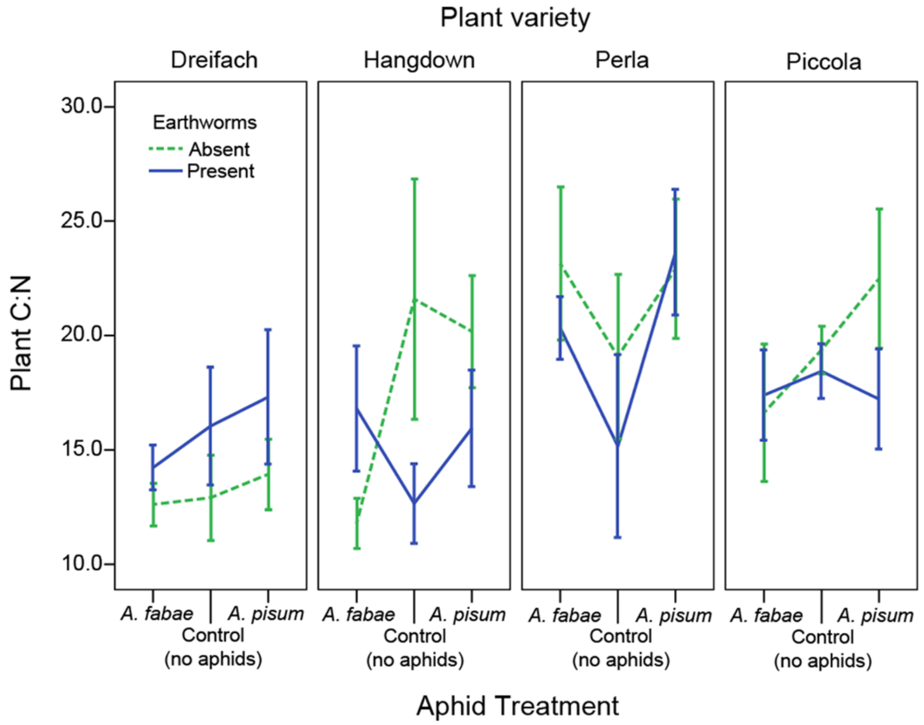

Figure 4 The effect of plant variety, worm presence/absence and aphid treatment on plant C:N ratio. There was a significant 3-way interaction between all factors on plant C:N. Error bars represent ±1 SE.

nutrient-rich soils we would detect a stronger effect of the earthworms on the plants and aphids [43]. Nevertheless, earthworms did mobilize additional resources that resulted in better plant and aphid growth. Furthermore, *V. fabae* is a rhizobial species and studies have shown the nature of this legume-rhizobium mutualism to depend on factors such as abiotic nitrogen and genotype of interacting partners [51,52]. Herbivores, earthworms and rhizobia can all influence ecological interactions involving their host plant and indirectly effect the performance of one another, due to their influence on plant resources [53]. Similarly, in our system, rhizobial associations may have differed amongst *V. fabae* varieties, which further resulted in the varied effect of earthworms on *A. fabae* on different varieties.

Previous work has found that different species of earthworms and plants have varying effects on aphid populations. Research on the combined effect of Collembola and earthworms on the development of aphids, with plants (*Poa annua* and *Trifolium repens*) grown in nitrogen-limited soil, found the outcome to vary across time periods [43]; one period in their experiment showed a 70% increase in aphids but in others there was no effect. Additionally, earthworms were found to increase aphid growth rate on *Cardamine hirsuta* in a study investigating the effect of earthworms in soils with contrasting nitrogen content on plant-aphid-parasitoid interactions [44]. However, the same lead author [45] found varying effects across different plant species when investigating the combined effects of earthworms and litter distribution of plants of different functional groups; here, earthworms reduced the number of aphids on *Plantago lanceolata* (forb) but had no effect on *Lolium perenne* (grass) or *T. repens* (legume). It was assumed that the reduced growth rate of aphids here could be the result of earthworm enhancement of defense-related secondary compounds via increased nitrogen availability to the plant [45]. These studies were all conducted with the aphid *Myzus persicae*, involved endogeic soil-feeding earthworm species (*Aporrectodea caliginosa* and *Octolasion tyrtaeum*) and were conducted on only one plant variety in each species. In studies on other species of aphid, no effect of *A. caliginosa* earthworms on *Sitobion avenae* aphids [40], and a decline in *Rhopalosiphum padi* under drought conditions [42], were detected. Thus, the mechanism driving any indirect effect between earthworms and aphids is far from simple and will likely depend on many other interacting abiotic (e.g. water availability; [42]) and biotic factors (e.g. presence of other soil organisms; [46]). It can also be influenced by differences amongst plant varieties and potentially mediated by plant chemistry as we have shown here.

Conclusions

We found that plant genetic variation can mediate interactions between aboveground and belowground communities. It is accepted that plants mediate aboveground-belowground interactions; however, our study emphasizes the significant role plant genotypes could play in regulating these interactions. More so, these effects are complex and species dependent. Our study showed how plant variety mediated the earthworm effect on only one of the aphid species *A. fabae*, whereas, it was the other aphid species, *A. pisum*, that reduced plant biomass. Our work adds the knowledge of how aboveground-belowground interactions are an important driver of species interactions and ecosystem processes.

Methods

Study system

Our system consisted of composting earthworms (*Eisenia veneta* (Rosa) formally *Dendrobaena veneta*; Lumbricidae), broad bean plants (*Vicia faba* L., Fabaceae) and two species of aphid, the legume specialist pea aphid (*Acyrthosiphon pisum* (Harris), Homoptera: Aphididae, clone FS_PA1, collected in Freising, Germany) and the polyphagous black bean aphid (*Aphis fabae* Scop. Homoptera: Aphididae, clone JAF1 originally collected in Jena, Germany). The compost earthworm *E. veneta* prefers warm and moist environments, and can rapidly consume a wide variety of compost material. The aphid species used both readily feed on the host plant (a common agricultural plant) and other legumes, although *A. fabae* is a more generalist feeder than *A. pisum*.

The earthworms were purchased from Wurmwelten, Germany (www.wurmwelten.de) and maintained in plastic boxes (7.5 cm × 15 cm) with air holes. Prior to the experimental setup, we cleared the gut contents of the worms to avoid contamination from packing soil by washing the earthworms with tap water and placing them in clean plastic boxes containing only moistened tissue for 24–48 hours at room temperature. Then the worms were sorted by size (small, medium and large) and placed into new boxes containing the experimental soil (Floragard product Floradur Topfsubstrat, pH 5.6, salinity 1.2 g/l). We used similar, 'medium' sized earthworms (0.2-0.4 g per worm) in the experiment.

The four plant varieties in the experiment (dreifach Weiße, Piccola, Hangdown and Perla) were purchased from Garten Schlueter, Germany (www.garten-schlueter.de). The seeds were germinated in experimental soil in pots (11 cm diameter), one seed per pot, and grown for three weeks in a greenhouse at 23/18°C (day/night) 16:8 hours (light:dark) watering daily with tap water. Experimental plants were selected by similar height and number of leaves (within variety) and kept in the same pots as the seeds were germinated in.

The experimental aphids were reared on *Vicia faba* variety *'The Sutton'* from Nickerson Zwaan, UK (www.hazera.com) in a climate chamber at 21°C 16:8 hours (light:dark) prior to use in the experiment. The aphids used have been maintained as clonal lines at Dürnast Experimental Station since 2011.

Experimental design

We used a fully factorial randomized block experimental design with two earthworm treatments (presence and absence), four plant varieties (*Dreifach Weiße*, *Piccola*, *Hangdown* and *Perla*) and four aphid treatments – two single aphid treatments (*A. pisum* or *A. fabae* alone), a paired treatment (*A. pisum* + *A. fabae* together) and a no-aphid control. This produced 32 treatments and we made 12 repeats per treatment (384 pots) over four treatment blocks, each containing three repeats. Within a block the treatments were fully randomized. Each experimental block was separated by time and all were conducted between October 2012 and January 2013 in a greenhouse with 18°C 16:8 hours (light:dark) at Dürnast Experimental Station, Technische Universität München, Freising, Germany.

Experimental setup

For the earthworm present treatment we added seven earthworms into the soil (placed into a small hole and covered with soil) and for the aphid present treatments, we added six 4th instar or adult aphids to the bottom leaf of a plant using a fine paintbrush. To maintain the same density of aphids we added six of the same species for the single aphid treatments, or three of each species for the paired aphid treatments (substitutive design). Each plant was then covered with an air-permeable transparent plastic bag (18 cm × 30 cm; UNIPACK, Hamburg, Germany) secured by a rubber band around the pot, to stop aphid movement between plants. The pot bases were also covered using a fine mesh material and secured with a rubber band to stop earthworm movement between pots, whilst allowing for watering at the base of the plant. The pots were placed into trays (12 per tray) and watered every two days by flooding the tray; each tray had drainage holes to ensure the soil was not waterlogged. Plant height (cm) was measured before the worms were added, from the top of the seed (below the soil surface) to the terminal bud. After 14 days, the number of aphids on each plant was counted using a tally counter - we started counting (separately for each species) from the bottom of the plant, and moved upwards up to the top. The height of the plant (cm) was again recorded (top of seed, under soil surface, to terminal bud) and the plant shoot was harvested and dried at 60°C for five days, in a paper bag, after which the dry biomass was measured. The number of remaining earthworms was also counted by breaking open the root structure of the plant to recover the earthworms; they were mainly found in the dense root section of the pots.

To determine if any effect of the worm treatment on the aphids could be explained by carbon (C) and nitrogen (N) changes in the plant we analysed a subset of the samples for C and N levels. After all biomass was recorded, 198 plants were analysed with 6–8 repeats from each treatment. A 5 cm section of the dried stem was collected and ground to a fine powder using a mixer mill (MM 300; Retsch). To aid grinding, the stem tissue was manually cut into pieces using a scalpel blade (cleaned with 70% ethanol between samples) and the stem tissue was frozen at −20°C for one hour. A 2 mg sample of the ground powder from each plant sample was weighed in a tin container and then loaded into a CNH analyzer (EuroEA3000 element analyser purchased from HEKAtech GmbH).

Data analysis

We removed 27 pots from the analyses due to no worms remaining in the worm present pots and for a couple of pots where there was contamination of aphids across aphid treatments. An average of five earthworms was recovered at the end from each pot, with no effect of the manipulated variables on the number of earthworms recovered. This means our sample size was 350 with 8–12 replicates remaining per treatment. We calculated the aphid per capita growth rate by calculating the difference between the natural log of the number of aphids at the end of the experiment with the natural log of the number of aphids at the start, and then dividing by the number of days the aphids were on the plant. We used this method because we started with either three or six aphids, per plant genotype, depending on the aphid treatment (single or paired) and in block one the aphids were grown for 15 days whereas they were grown for 14 days in all other blocks.

The data were analysed using linear models in R v2.15.2 using R-studio v 0.97.314. Our dependent variables were aphid growth rate (one model each for all aphids, and then each species separately) and plant biomass. The independent fixed effects were earthworm presence/absence, plant variety and aphid treatment (*A. fabae*, *A. pisum*, both or none). We also added block to the model and plant biomass as a covariate for the aphid models. For the plant biomass, we also ran a model with *A. pisum* aphid growth rate, plant variety and earthworm treatment due to the results of the previous model. For the subset of data with plant CN ratios, we added these to the above models and ran another model with CN ratio as the dependent and earthworm, plant and aphid treatments as fixed effects with block (factor) and plant biomass (covariate). The minimal adequate models are presented in the results. Briefly, we first fit a full model will all main and interaction effects, and then we simplify the model by removing the non-significant terms (starting with the highest interaction term), testing to see if model fit has significantly changed. If model fit was significantly changed then the term was re-added into the model. If an interaction term was significant the corresponding main effects remained in the model, whether or not they were significant. Treatment levels were compared in R using post-hoc contrasts.

Availability of supporting data

The data set supporting the results of this article is available in the Labarchives repository DOI:10.6070/H42N507N. https://mynotebook.labarchives.com/share/Sharon%2520Zytynska/MjAuOHw1ODUwNy8xNi9UcmVlTm9kZS8yNjY3ODg4ODQxfDUyLjg=.

Abbreviations

IEE: Indirect ecological effect; C:N: Ratio of carbon to nitrogen.

Competing interests

The author declares that they have no competing interests.

Authors' contributions

SZ designed the experiment, with AS, SZ, JB, ED, SH, AW collecting the data. Analysis and interpretation was done by AS, SZ and WW. AS, JB, ED, SH, AW all contributed to the first draft, completed by AS and commented on by SZ and WW. All authors read and approved the final manuscript.

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