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The role of allelopathy in *Reynoutria japonica* Houtt. ecological dominance

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The role of allelopathy in *Reynoutria japonica* Houtt. ecological dominance - Abstract

Impacts of humanity on earth systems are tied to its dramatic technological improvements in the last centuries. Taking advantage of subsequent globalization processes, invasive species are nowadays one of the main causes of biodiversity loss worldwide. Among those, invasive Japanese knotweed (*Reynoutria japonica* Houtt.) is known as one of the worst invasive species in the world. While numerous hypotheses account for invasive plant species success in their invaded range, this work focuses on one of them that might explain *Reynoutria japonica* rampant invasion of Europe and North America: The Novel Weapons Hypothesis (NWH). The NWH states that invasive plant species are highly successful in their invaded range because of the new set of chemicals they bring with them, against which native species would be defenseless. Allelopathy is the process by which a plant (donor) inhibits the growth of another plant (target) via the release in the environment of chemical compounds and provides a theoretical frame to the NWH. This work aims at observing suggested allelopathic patterns of *R. japonica* in an ecologically meaningful setting to try and account for the role of allelopathy in this plant's successful invasions. Methods used were based on theorical and experimental frames of phytotoxicity and allelopathy and on previous shortcomings identified in similar studies. A study site was selected in Ottignies (Belgium) in which Japanese knotweed was identified using indumentum criteria. Bioassays were performed on *Zea mays* and *Trifolium incarnatum* and involved tissue extracts along with invaded soil of *R. japonica*. Greenhouse growth experiments used a target-neighbor design with varying *T. incarnatum* (neighbor) densities in natural, artificial, and nutrient deficient substrates. Tissue mineral and photosynthetic pigments contents of crimson clover were also assessed. Despite identifying phytotoxic effects of tissue extracts on test species' radicle length, we could not find strong supplementary evidence for *R. japonica* allelopathy in both growth experiments and bioassays, which contradicted our hypotheses. Germination of test species was not affected by tissue extracts. Biomass production and physiological parameters results of *T. incarnatum* plants in growth experiments were mainly explained by substrate's abiotic conditions. We did not observe any optimal density patterns linked to Japanese knotweed phytotoxicity. Absence of observed effects could be due to resource competition, soil mediated processes or facilitation among clovers. Overall, this study did not provide arguments in favor of an ecologically important allelopathic process and of NWH in the case of *R. japonica*. Rather, our results support the integration of such effects in a set of other dominant processes.

Le rôle de l'allélopathie dans la dominance écologique de *Reynoutria japonica* Houtt. – Résumé

Les impacts de l'humanité sur les systèmes de la planète sont liés aux importants développements technologiques de ces derniers siècles. Prenant avantage de la globalisation qui en découle, les espèces invasives sont aujourd'hui une des causes majeures de perte de la biodiversité dans le monde. La renouée du Japon (*Reynoutria japonica* Houtt.) est connue pour être l'une des pires espèces invasives au monde. Tandis que de nombreuses hypothèses tendent à expliquer le succès des plantes invasives, ce travail se focalise sur l'une d'entre elles qui pourrait expliquer l'invasion fulgurante de *R. japonica* en Europe et en Amérique du Nord : la Novel Weapons Hypothesis (NWH). Celle-ci stipule que les plantes invasives sont particulièrement prospères dans leur aire d'invasion parce qu'elles apportent avec elles de nouveaux composés actifs contre lesquels les espèces natives ne peuvent pas se défendre. L'allélopathie est le procédé suivant lequel une plante inhibe la croissance d'une autre en relâchant des composés dans l'environnement et fournit un cadre théorique à la NWH. Ce travail vise à observer les schémas supposés d'allélopathie de *R. japonica* dans un contexte écologiquement valable pour essayer de comprendre quel est le rôle de l'allélopathie dans le succès invasif de cette plante. Les méthodes utilisées se basent sur les cadres théoriques et expérimentaux de la phytotoxicité et de l'allélopathie, ainsi que sur les manquements relevés dans des études similaires. Un site d'étude a été sélectionné à Ottignies (Belgique) sur lequel la renouée du Japon a été identifiée en utilisant les critères relatifs aux trichomes. Des tests biologiques sur *Zea mays* et *Trifolium incarnatum* employant des extraits de tissus et de sols contaminés de *R. japonica* ont été réalisés. Les expériences de croissance en serre ont utilisé un design target-neighbor avec différentes densités de *T. incarnatum*. Le contenu en éléments minéraux et en pigments photosynthétiques des trèfles a aussi été analysé. Nous avons trouvé des effets phytotoxiques des extraits de tissu sur la croissance radiculaire mais pas sur la germination des espèces testées. Cependant, ni les tests biologiques ni les expériences de croissances n'ont permis d'obtenir des preuves solides de l'allélopathie de la renouée du Japon, ce qui contredit nos hypothèses. Les effets observés sur la biomasse et les paramètres physiologiques du trèfle incarnat étaient principalement dus aux caractéristiques des substrats utilisés, et aucun schéma d'optimum de croissance lié à l'allélopathie n'a pu être observé. Ceci peut être expliqué par des effets masquant de la compétition aux ressources, du microbiote du sol, ou de la facilitation des trèfles entre eux. En définitive, ce travail suggère l'intégration de l'allélopathie de *R. japonica* dans une gamme de procédés dominants et remet en cause son importance écologique ainsi que l'applicabilité de la NWH dans ce cas.

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1.Introduction

1.1 The problem of invasive species

1.1.1 General context

Recent history of the planet's systems is deeply tied to the dramatic increase in humanity's demography and its technological improvement (Crutzen & Stoermer, 2000; Ruddiman, 2013). Impacts of humanity on earth's biological, geological, and atmospheric systems are so important that Crutzen and Stoermer (2000) coined the term '*Anthropocene*' to describe the geological epoch corresponding to the sharp development of humanity (Ruddiman, 2013). Despite the start of this epoch being disputed in the scientific community, effects of humanity on earth systems such as oceanic acidification, depletion of groundwater resources, increase in greenhouse gas emissions as well as fragmentation of ecosystems are widely recognized (Crutzen & Stoermer, 2000; Ruddiman, 2013).

One of the numerous impacts of human actions is this mankind has on ecosystems and global biodiversity (Watson et *al.*, 2005). Following the Millennium Ecosystems Assessment's Biodiversity Synthesis findings, biodiversity changes in the past 50 years have reached a faster pace than ever before in human history and are projected to continue if not accelerate (Watson et *al.*, 2005). Five main human-caused drivers of biodiversity loss and ecosystems changes were identified in this Synthesis: habitat/land use change, anthropogenic climate change, overexploitation, pollution and finally invasive alien species (Watson et *al.*, 2005).

1.1.2 Invasive Alien Species and biological invasions

Invasive Alien Species (or IAS) and biological invasions are a key feature of the current era, the Anthropocene (Kumschick et *al.*, 2015). This is due to the globalization of trade, which connected parts of the world in terms of people and goods exchanges and inevitably lead to the transport of species outside their native range, whether such movements are voluntary or not (Keller et *al.*, 2011; Pyšek et *al.*, 2010; Kumschick et *al.*, 2015, Olson, 2006; Watson et *al.*, 2005). Because of the global scale of the biological invasions' process, IAS have major often negative impacts on biodiversity and native ecosystems, which through ecosystem services impair global economy and human well-being (Pyšek et *al.*, 2010; Keller et *al.*, 2011; Olson, 2006; Watson et *al.*, 2005). Biological invasions are thus important processes that have a multi-disciplinary dimension.

Impacts of IAS can be estimated in monetary terms with varying reliability depending on the information available, with numerous countries spending billions each year to cope with their

invasion situation (Olson, 2006). The United States of America spend an estimate of US$120 billion per year due to all costs related to the invasive species present on its territory (Pyšek et *al*., 2010). In Europe, such costs were estimated at €3.7 billion yearly for invasive terrestrial plants only (Keller et *al.*, 2011). Focusing on their ecological negative effects, it was shown that IAS can deplete native species habitats, richness (through extinction or competition often in the case of a predatory or competitor invasive species, respectively) and community assemblages (notably through disruption of the species interactions necessary to plant reproduction), or yet decrease native genetic diversity through hybridization events with closely related native species (Keller et *al.*, 2011; Pyšek et *al*., 2010). Such impacts lead to biological homogenization and loss of native flora's uniqueness (Pyšek et *al*., 2010, Watson et *al.*, 2005). In addition to this, IAS have negative impacts on socioeconomic and cultural frames, human health, security, social relations and even freedom of choice and action (Pyšek et *al*., 2010; Watson et *al.*, 2005). Adding to the indirect effects, examples exist in which people's well-being and financial situation were directly impaired by biological invasions (Watson et *al.*, 2005).

The biological invasions problem's global scale and increasing extent led to a sharp development of the field of invasion biology in the last decades (Pyšek et *al*., 2010; Crystal-Ornelas & Lockwood, 2020). With it, a tremendous bulk of 30 hypothesis were put forward to explain invasion process, success and impacts (Crystal-Ornelas & Lockwood, 2020). Despite a highly variable set of standpoints when it comes to explaining invasions, an integrative work conducted by Catford et *al.* (2009) identified the three key concepts around which all theories revolve: Propagule pressure, Abiotic characteristics and Biotic characteristics (MacDougall et *al.*, 2009; Crystal-Ornelas & Lockwood, 2020). Propagule pressure describes the frequency and quantity of invading propagules arriving in a native ecosystem; Abiotic characteristics include resource availability changes, ecosystem disturbances and their interaction; Biotic characteristics include all species interactions susceptible of facilitating or impeding biological invasions, with characterization of ecosystems as being prone to undergo invasions and description of trait sets of IAS associated with successful invasion (Keller et *al.*, 2011, MacDougall et *al*., 2009, Catford et *al.*, 2009; Kaushik et *al.*, 2022). These three concepts are mediated to some extent by human activity and interact with the position in space and time when predicting for an invasion (Catford et *al.*, 2009).

As mentioned previously, biological invasion is a staged process occurring through determined pathways (invading routes) and the theoretical framework of Catford et *al*. (2009) applies to all

stages of this process (Pyšek et *al*., 2010; Keller et *al.*, 2011). Introduction pathways can be classified in three categories (importation as or with a commodity, arrival with a transport vector and dispersal by the species themselves) and comprise for example water of ship's ballast in the case of marine or freshwater species or yet ornamental plants trading routes in the case of invasive terrestrial plants (Keller et *al.*, 2011). Depending on the author, invasion stages are between 3 and 5 (6) with the more widely used terms of *introduced* (the species arrives in a new region outside its native range), *established* (the species escapes in the native ecosystem and start reproducing without human help), and *invasive* (the widely spread species has considerable environmental and economic impacts) to describe the invasion situation (Pyšek et *al*., 2010; Keller et *al.*, 2011). The 'tens rule' is a rule of thumb describing how a limited amount of introduced species go through each invasion stage: out of all introduced species, 10% will become established and out of those yet 10% will become invasive and have negative impact on the invaded environment (Keller et *al.*, 2011). Initially developed for terrestrial plants, this rule doesn't effectively match invasion processes of all taxa and serves as a general guideline (Keller et *al.*, 2011). Given the diversity of invasion routes and despite the tens rule, biological invasions are increasing worldwide as the rates and extent to which species becomes invasive are building up (Pyšek et *al*., 2010). This together with the fact that IAS are a major cause of global biodiversity loss make them a hot topic when it comes to biodiversity conservation and restoration of ecosystems (Pyšek et *al*., 2010).

1.1.3 Challenges and shortcomings in managing biological invasions

Managing biological invasions is one of the major challenges that stakeholders must address in the near future (Watson et *al.*, 2005; Pyšek et *al*., 2010; Keller et *al.*, 2011). Despite being highly context-specific, such management efforts are possible thanks to the increasing amount of information available about IAS and the tools developed in that aim. Those tools are risk assessments, pathway and vector management, early detection and rapid response, eradication and mitigation and restoration. The most efficient of them being those allowing for a prevention of introduction, which was showed to be the most cost-effective approach (Pyšek et *al*., 2010; Keller et *al.*, 2011). Several regions and countries in the world have already launched a series of management programs using such tools to mitigate impacts of IAS on their territory, resulting in often important costs as seen above (Pyšek et *al*., 2010, Keller et *al.*, 2011). Nationwide and international policies are also available but still need to be improved as current guideline gaps and the tendency that stakeholders have to overlook them severely hinders their efficiency (Pyšek et *al*., 2010, Keller et *al.*, 2011). In Europe, it is the Convention on Biological Diversity

(CBD, https://www.cbd.int/invasive/about.shtml) that sets the standards for IAS 'introduction, spread and export' prevention, although application of it have been varying among European nations so far (Keller et *al.*, 2011).

Furthermore, managing biological invasions has been difficult due to the extent to which they create new interactions with native species. Straightforward eradication of IAS was proven to have unpredictable effects on native ecosystems and to be hazardous for such communities in some cases. This is because the role of invasive species as key-functional species in native ecosystems only becomes more important with the increase in their presence (Pyšek et *al.*, 2010). We speak of IAS that 'infiltrate' native species networks, and this asks the question of redefining the invaded ecological frame through an extensive study of invasive-native species interaction and coexistence dynamics (Pyšek et *al.*, 2010).

As put in the Millennium Ecosystem Assessment: "*Science can help ensure that decisions are made with the best available information, but ultimately the future of biodiversity will be determined by society*" (Watson et *al.*, 2005). Even though lots of effort are needed in order to convert available information on IAS into effective policies, it is known that the study of invasive species suffer from strong bias and research gaps that should be addressed to increase the quality of this information (Crystal-Ornelas & Lockwood, 2020; Kumschick et *al.*, 2015). Lack of information on IAS include their impacts' quantification metrics, standardized evaluation protocol and predictive patterns elucidation; while bias in the field of biological invasions translates to research highly skewed towards diversity and/or fitness metrics at population and community biological scales, shorter timescales and in a narrow range of taxa and ecosystems (Crystal-Ornelas & Lockwood, 2020; Kumschick et *al.*, 2015).

Studying plant chemical interactions at an ecophysiological scale in the frame of a biological invasion, this work will help investigate coexistence dynamics of a 'novel ecosystem' consequent to IAS establishment and provide further insights in potential eradication, mitigation and restoration tools usable in this study case while addressing some of the bias hindering invasion ecology research.

1.2 Allelopathy and biological invasions

1.2.1 Definition of allelopathy and precision of this work's view

Allelopathy as a word was coined by Molisch in 1938, after the Austrian plant physiologist observed how ethylene could influence the ripening of fruits. It comes from the Greek 'allelon'

meaning “mutual” and ‘pathos’ meaning “harm” or yet “affection” (Inderjit & Duke, 2003). In its initial sense, Molisch saw it as “the phenomenon following which one plant can influence another”, this influence being either positive or negative, taking place in the environment through the release of chemical compounds and being rather direct (from plant to plant; Inderjit & Weiner, 2001; Inderjit & Duke, 2003; Ens et. *al*, 2009). However, this definition evolved from then to now and progressively took different meanings oscillating between the broad and narrow sense.

In 1983, Rice described the phenomenon as all effects of plants (including microorganisms) on the growth of neighboring plants through the release of chemical compounds into the environment (Inderjit & Duke, 2003; Inderjit & Weiner, 2001). This definition yielding to scientists being able to label a vast ensemble of interactions as allelopathy, it was criticized for its troublesome broadness (Inderjit & Duke, 2003; Inderjit & Weiner, 2001). This led to the emergence of a narrower definition, stated by Lambers in 1998: “[Allelopathy is the] suppression of growth of one plant species by another due to the release of toxic substances” (Inderjit & Duke, 2003; Inderjit & Weiner, 2001). This definition, only recognizing the negative effects of a ‘donor’ plant (the plant emitting the chemical substances in the environment) towards the ‘target’ plant (the plant undergoing the inhibition of the donor plant) and intending a given notion of ‘direct’ interaction, is the most widely used and accepted definition in literature nowadays (Inderjit & Weiner, 2001; Inderjit & Duke, 2003; Ens et. *al*, 2009; Inderjit & Callaway, 2003). However, some papers and institutions, such as the International Allelopathy Society, still use the definition of allelopathy as including both negative and stimulating effects and all-level of complexity (IAS website, About – International Allelopathy Society (osupytheas.fr)).

The latest broadly accepted definition is still a debated topic among specialists today due to the “direct” aspect of the inhibition from the donor to the target plant. It appears that researchers emphasized the investigation and characterization of direct allelopathy (the inhibition of target plants by a donor plant solely via the release in the environment of chemicals directly acting on the target plant without any other process involved), as it was described originally, even going up to the description by them of exhaustive and difficult protocols based on Koch’s postulate and through which it would be possible to correctly identify allelopathy (Inderjit & Weiner, 2001; Inderjit & Duke, 2003; Ens et *al.*, 2009). Other researchers argue that allelopathy, due to its very nature, must be considered in its ecological context in which a set of indirect allelopathy

Table 1: Classification of the allelochemical effects and their definition (from Inderjit & Weiner, 2001).

Box 1. Classification of potential allelopathic effects, broadly defined.

I. Direct plant-plant allelopathic interference (allelopathy in the narrow sense)
Plant A produces Compound X, which interferes with Plant B

II. Indirect soil ecological interactions (indirect allelopathic effects)

A. Indirect allelopathy

1. Decomposition-mediated plant-plant allelopathy
Plant A produces Compound X which is degraded or otherwise transformed by Microorganism C into Compound Y, which interferes with Plant B

2. Induced allelopathy
Plant A produces Compound X which is released and induces Organism D to produce Compound Z which interferes with Plant B

B. Indirect toxicity
Compound X interacts with soil ecosystem and causes generation of Compound Z (which is not a breakdown product of Compound X), which interferes with Plant B

C. Indirect environmental effects
Compound X causes a change in the soil environment, which affects the nutrient status of the soil, thus reducing the growth, survival or reproductive output of Plant B, without toxic effects

processes would be relatively more important regarding occurrence and amplitude than the direct allelopathy ones, and criticize this former view (Inderjit & Weiner, 2001; Inderjit & Duke, 2003; Ens et *al.* 2009). Inderjit & Weiner (2001), endorsing the more ecologically based view of allelopathy, established a classification of these allelopathic effects, as shown in Table 1.

The I. entry of direct plant-plant allelopathic interference is the case of the original representation of the concept of allelopathy, which was inspired by the example of the *Juglans* litter described by Massey (1925) and Davis (1928) in the beginning of the 20th century (Inderjit & Weiner, 2001; Inderjit & Keating, 1999).

In this work, the allelopathic effect that will be investigated is defined as follows: "**The inhibition of growth of one plant by another via the release of chemicals in the environment, whether this inhibition takes place <u>indirectly</u> in the ecological context of the plant or <u>directly</u> from a plant to another.**", thus considering the 'negative effect only' of Lambers' definition, along with the aim to replace allelopathy in its ecological context defended by Inderjit & Weiner (2001). This definition is also widely accepted and used by the scientific community (Murrell et *al.*, 2011; Dommanget et *al.*, 2014).

1.2.2 Ecophysiological considerations of allelopathy

Here the ecophysiological aspects of allelopathy will be discussed in more detail. This work is however not aiming at exposing an exhaustive review of this subject but will rather limit the explanations to the points that are important to stress in order to understand the methods used.

Fisrt, labelling a compound as an allelochemical can be tricky due to the various natures allelochemicals as molecules can have (see Table 2). Despite this, they are released into the environment via a limited number of means (Inderjit & Duke, 2003). Mainly, those means include root exudation of compounds in the soil, foliar leaching of molecules by rain or snow contact (washout of the leaves by water that then drips down to the ground), volatilization of molecules in the atmosphere, decomposition of residues by microbiota and plant debris incorporation in the soil (Inderjit & Duke, 2003; Inderjit & Keating, 1999; Inderjit & Weiner, 2001). In this study, we will focus mainly on root exudation and debris incorporation into soil

Table 2: List of allelochemicals as described in Kato-Noguchi (2021) and respective chemical classes. This is a part of a table from Kato-Noguchi (2021).

Phytochemical Class	Compound
Quinone	Emodin (**1**)
	Physcion (**2**)
	Emorin-1-*O*-β-D-glucoside (**3**)
	Physcion-1-*O*-β-D-glucoside (**4**)
	Emodin dianthrone (**5**)
	Fallopion (**6**)
	Physcion dianthron (**7**)
	Torachrysone glucoside (**8**)
Stilbene	Resvertrol (**9**)
	Piceid (**10**)
	Resveratroloside (**11**)
	Piceatannol glucoside (**12**)
Flavanoid	(-)-Catechin (**13**)
	(-)-Epicatechin (**14**)
	Procyanidin B_3 (**15**)
Phenylpropanoid	Vanicoside B (**16**)
Indole	Tryptphan (**17**)

(Murell et *al.*, 2011; Dommanget et *al.*, 2014; Mincheva et *al.*, 2016; Parepa & Bossdorf, 2016; Moravcova et *al*., 2011).

As seen when discussing the definition of allelopathy as well as in the Table 1 above, the allelopathy process can take various forms. The most distinctive of them would be whether allelopathy is direct (I case) or indirect (II case), even though this distinction is hard to establish experimentally (Inderjit & Weiner, 2001). As seen in Table 1, direct allelopathy and indirect allelopathy differ in that the former involves the simple inhibition of the target plant by the allelochemical that was released by the donor plant, while indirect allelopathy involves the target and donor plant, as well as other biotic or abiotic factors through which the initially released allelochemical will exert its inhibitory effect (Inderjit & Weiner, 2001; Inderjit & Duke, 2003).

This study makes use of another important aspect of allelopathy's ecophysiology: the fact that allelopathy is an inducible phenomenon (Dommanget et *al.,* 2014; Inderjit & Weiner, 2001; Inderjit & Duke, 2003). It is known that whether or not a plant will produce the compounds necessary for it to make the allelopathy process happen is subject to a complex regulation integrating the cues of many factors such as soil type, pH, mineral nutrition of the donor plant, temperature, season, light, humidity or yet herbivory (Dommanget et *al.*, 2014; Inderjit & Weiner, 2001). Following this logic, it was also demonstrated that nutrient deficiency such as the absence of inorganic ions in the substrate might lead a plant to apply an allelopathic effect to its neighbors (Inderjit & Weiner, 2001; Inderjit & Duke, 2003). As an example, it was found that the shikimate pathway, producing phenolic compounds which are suggested to be responsible for many allelopathy effects, is triggered when the plant perceives a Boron and/or Phosphorous deficiency in the substrate (Inderjit & Weiner, 2001; Inderjit & Duke, 2003).

1.2.3 Explaining biological invasions: the Novel Weapons Hypothesis

As mentioned above, dozens of hypotheses were proposed to elucidate biological invasions' success and numerous impacts (Crystal-Ornelas & Lockwood, 2020; Catford et *al.*, 2009). However, this work will focus on investigating the role of allelopathy in the ecological dominance of an invasive species basing its rationale on a single one of these hypotheses: the Novel Weapons Hypothesis. Thoroughly described and supported by Callaway & Ridenour (2004), this hypothesis states that invading species increase their competitive ability and chances of successful invasion through the release of biochemicals (subsequently termed allelochemicals) that are inhibitory to native species, which didn't have time to develop defenses (Catford et *al.*, 2009; Hierro et *al.*, 2005). Arguments in favor of the Novel Weapons

Hypothesis (NWH) involve studies showing that invasive plant species inhibited germination and development of their new neighbors (present in the IAS's invaded range) to a greater extent than their old neighbors (present in the IAS's native range), with some providing evidence for specific molecules responsible of this effect (Callaway & Ridenour, 2004; Hierro et *al.*, 2005).

Finally, as argued by Callaway & Ridenour (2004) and supported by the same evidence, invasive plant traits falling in the NWH might be subject to a strong selection pressure in their invaded range due to the advantage those provide. This would make such traits prone to evolve towards their own reinforcement and lead to stronger allelopathic effects when comparing with source populations, thus improving the chances to observe allelopathy empirically when studying those IAS.

Although a wide range of plant taxa might be concerned by interspecific chemical interactions, the NWH and arguments above support the view that IAS, studied in their invaded range, are suitable models for the study of allelopathic effects.

1.3 The study of allelopathy

1.3.1 The practical and fundamental importance of allelopathy for modern science

Allelopathy as a concept received increasing interest over the past decades (Macías et *al.*, 2019). It is true that, while some scientists tend to overlook the study of this concept's causes and consequences, mounting evidence for such phytotoxic phenomena made it progressively clear that allelopathy is a process of which the impacts cannot be ignored (Ens et *al.*, 2009; Inderjit & Weiner, 2001). The importance and pertinence of allelopathy for modern science is mainly twofold: fundamental and practical.

The fundamental basis describing plant-plant, plant-microbial, plant-environment relationships, community assembly or yet ecological succession is currently overlooking the intricate potential relationships that allelopathy as a concept would help uncover and is based on theories and pattern involving e. g. resource competition, nutrient dynamics or yet mycorrhizal ecology (Inderjit & Weiner, 2001). However, as mentioned by Inderjit & Weiner (2001), all these processes were already shown to be able to interact with allelopathy and be influenced by it (Weidenhammer, 2006, Inderjit & Nishimura, 1999). Furthermore, it is relevant to suggest an effect of plant-plant interactions on community composition: a situation in which some species are inhibited in growth by a small number of others and where a neighbor preference takes place is enough for community dynamics and species coexistence to be influenced (Inderjit &

Callaway, 2003; Weidenhammer, 2006). The importance of the concept of allelopathy thus lies in part in the fact that so many different fields of plant ecology and ecosystem processes are potentially influenced by it (Inderjit & Callaway, 2003; Weidenhammer, 2006; Inderjit & Weiner, 2001). The study of such a concept is thus of incredible importance given how its full understanding and implementation could potentially lead to a revolution of the plant ecology field. In other words, as Muller had already put it in 1966 : "Traditional theories of competition, reaction, biomass proportions, energy flow, mineral cycling, and ecosystem organization are all liable to reevaluation where allelopathy is demonstrable." (Muller, 1966)

Practically, allelopathy has also been raising interest regarding its potential applications in agriculture (Weidenhammer, 2006; Einhellig & Leather, 1988; Macías, 2019). As the biochemical process responsible for an allelopathic effect became clearer, it appeared that agriculture could take great advantage of some of its mechanisms (Weidenhammer, 2006; Einhellig & Leather, 1988; Macías, 2019). As a matter of fact, scientists wanted to improve the knowledge about and exploit the process, along with the use of other techniques such as cover crops or cropping rotations, in order to develop sustainable means to control weeds and/or pests (including parasitic plants) by developing allelopathic crop varieties or yet using allelopathic compounds as clean herbicides, and thus optimize the yield of crop cultures (Weidenhammer, 2006; Einhellig & Leather, 1988; Macías, 2019). We can cite here the example of sorghum (*Sorghum bicolor*) cultures, this crop presenting weed control properties thanks to the long persistence of sorgoleone, one of the allelopathic molecules it produces, in the soil and thus shows deeply valuable features for the future of agriculture (Einhellig & Leather, 1988; Macías, 2019).

1.3.2 Pertinence of the methods used in addressing the shortcomings identified in previous studies demonstrating an allelopathic effect

Historically, the study of allelopathy has always been challenging (Blair et *al.*, 2009; Inderjit & Callaway, 2003; Ens et *al.*, 2009; Thijs et *al.*, 1994). The very process of allelopathy is complex in terms of causes, impacts on the target plant or on the environment, and interactions with other factors of plant and soil ecology (Thijs et *al.*, 1994 ; Weidenhammer, 2006). The great research flaw that scientists willing to investigate the behavior of allelopathic interactions face is to fail incorporating the complexity of this concept by misrepresenting one of the components of the natural system in their experimental design (Weidenhammer, 2006). Studies on the subject published in the past decades got criticized over one or another of the numerous component

interacting with the concept of allelopathy being misrepresented or overlooked, and over the years relevant methods for identifying an allelopathic effect or compounds responsible for it were described and refined (Thijs et *al*., 1994 ; Weidenhammer, 2006; Inderjit & Callaway, 2003).

Nevertheless, such critiques led some researcher to propose complex protocols based on Koch's postulate to identify an allelopathic interaction, but these protocols quickly showed their limits when it comes to experimental investigation, given the difficulty that their implementation represents (Thijs et *al*., 1994; Inderjit & Weiner, 2001; Inderjit & Duke, 2003; Ens et *al.*, 2009). As exemplified by Inderjit & Weiner (2001), a good analogy for these high-standard requirement in producing a proof of allelopathy would be this of the effect of smoking on cancer: tobacco corporations indeed asked scientists to demonstrate the exact mechanism through which smoking would lead to cancer before to officially accept that there is a causal link. The previously mentioned protocols offer a similar example of such a flawed reasoning. While their application would indeed greatly improve our knowledge of the subject, the difficulties of this application are not necessary to produce good science (Inderjit & Weiner, 2001). Instead, one can envision to lower such a proof burden by designing "exploratory allelopathy studies", which usually use simple experimental designs to gain knowledge about a general aspect of a given allelopathic interaction (Ens et *al.*, 2009). In this study, we will attempt to acknowledge the previously described shortcomings by using relevant methods for the investigation of allelopathy, while keeping a rather simple experimental design. In this section below, shortcomings and the way we intend to address them are described.

These shortcomings could be summarized in one consideration bigger in scale: there is a lack of extrapolation imputable either to laboratory studies or field experiments (Dommanget, 2014; Ens et *al.*, 2009). In laboratory studies, it is easy to realize a bioassay using plant extracts and conclude to an actual allelopathic interaction in the eventuality that a phytotoxic effect is found. Such studies are biased in the distinction between phytotoxicity and allelopathy, and loose in significance due to the misrepresentation of natural conditions in the experiments (Ens et *al.*, 2009). In this study, such a misrepresentation is avoided by incorporating soil media extracts in the bioassay, along with the leaves and rhizomes extracts, since plants should not be separated from their substrate when it comes to allelopathy and soil mediated interactions (Ens et *al.*, 2009; Inderjit & Callaway, 2003). In the case of the growth experiments, addressing such a bias linked to the misrepresentation of field relevant substrate occurs using natural soils over artificial ones in the greenhouse (Parepa & Bossdorf, 2016; Inderjit & Callaway, 2003;

Dommanget et *al*., 2014). This precaution is based on the consideration that natural conditions should be represented as closely as possible (Inderjit & Callaway, 2003; Dommanget et *al*., 2014), but also more precisely on the study of Parepa & Bossdorf (2016) that highlights the great advantage that artificial soils gives to *R. japonica* (our study species) in terms of competitive potential, this being probably due to the physicochemical properties of it (an improved aeration, water retention, porosity and a decreased capacity to adsorb allelopathic compounds of artificial soils compared to natural ones all benefiting to *Reynoutria japonica*). By implementing those methods, field relevance is restored for both the laboratory and growth experiments, and extrapolation from their results is easier to justify.

In field studies, the risk is to have experimental conditions so close to the natural ones that extrapolation from results becomes hard due to the intricate network of processes that can interact with allelopathy and that are not controlled or yet identifiable (Dommanget, 2014; Weidenhammer, 2006; Thijs et *al*., 1994). To avoid this specific case, it is necessary to at least use methods that allow control on known processes in order to clearly identify which observation is the result of an allelopathic effect and which is not. While this work does not include a field study of *Reynoutria japonica* populations, even though this kind of study was incentivized by Inderjit & Callaway (2003), it does contain a greenhouse growth experiment of which the experimental design intends the use of natural soil. Natural soil contains population of macro- and microinvertebrates, mycorrhizae, and bacteria, and is prone to present the chemical signatures of living organisms that were in contact with it. Since this work focuses on investigating the effect of living *R. japonica* on neighboring plants, it is intended that the natural soil comes from a non-invaded site. Other processes such as the presence of mycorrhizae or the influence of soil bacteria can influence the outcome of the study but will not be addressed by the experimental design, even though they will be discussed along with the results of this work. Nevertheless, an important process influencing allelopathy will be addressed by the chosen experimental design: resource competition (Weidenhammer, 2006; Thijs et *al*., 1994; Inderjit & Callaway, 2003).

Since they have similar observable effects on plant growth, allelopathy and resource competition are crucial to tell apart from each other (Weidenhammer, 2006; Thijs et *al*., 1994; Inderjit & Callaway, 2003). This can be done via several reliable methods: the use of Activated charcoal to eliminate the effect of a putative allelopathic compound directly in the substrate, the use of given amounts of an identified allelopathic compound to aggravate allelopathic effects, or yet exploitation of the phytotoxicity's density-dependent principle (Weidenhammer,

2006; Thijs et *al*., 1994; Inderjit & Callaway, 2003). Activated carbon is not an ideal tool for studying allelopathy, since it was reported that, despite being a good way to eliminate toxicity, its incorporation in substrate could influence plant biomass and microbial activity through the addition of inorganic nutrients or modification of other physicochemical properties of the soil such as pH or water retention (Thijs et *al*., 1994; Inderjit & Callaway, 2003). Addition of a suggested allelopathic molecule in order to amplify the effect could have been done in the frame of this study, as well as an experimental design using both activated carbon and a putative allelopathic molecule but wasn't for practical reasons. Rather, this work will focus on disentangling resource competition form allelopathy using the phytotoxicity's density-dependent principle (Weidenhammer, 2006; Thijs et *al*., 1994; Goldberg & Werner, 1983).

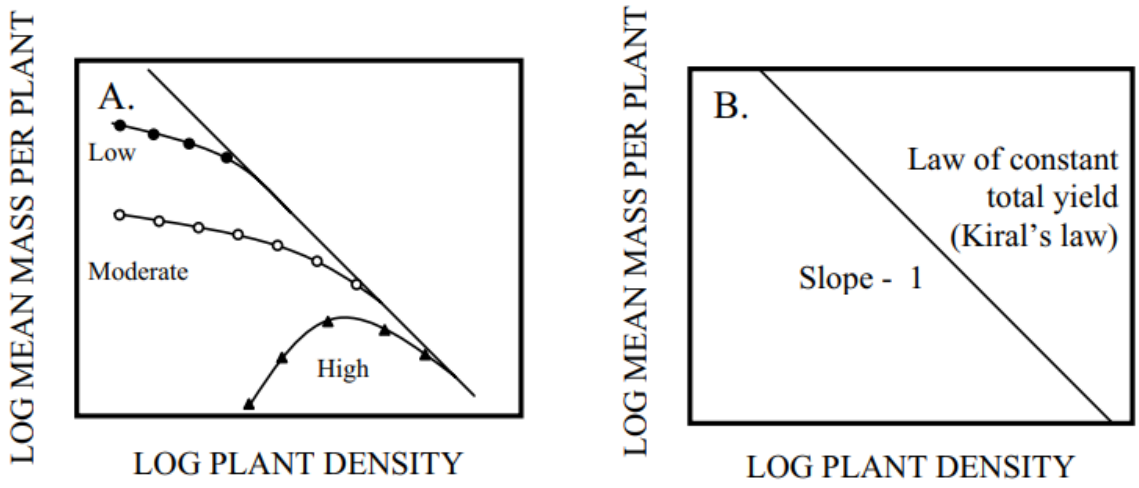


Figure 1: Representation of theorical expectations of the behavior of Law of total constant yield A. when plants are exposed to increasing concentrations of toxins and B. in an ideal case of optimal resource use. From Weidenhammer (2006).

The theorical frame of this method was described by Weidenhammer (2006) and Thijs et *al.* (1994) gave a good example of its application. The theorical core of the method is using a deviation of the plant ecology law of constant total yield (or Kiral's law) stating that in an ideal crop field using maximum of resources (in which total yield is constant), the expected relationship between mean mass per plant and plant density should be this of a trade-off, as shown in the Figure 1 B. (Weidenhammer, 2006).

It appears that in a system exposing plants to a phytotoxin, the expected curve will change and, at highest densities, be such that plants will present a decreased growth at both low (due to the amount of phytotoxin present in the ground) and high densities (due to the intense competition between the plants), as seen in Figure 1 A. (Weindenhammer, 2006). This deviation of the curve

is due to the density-dependent behavior of phytotoxicity: at lower densities, due to the intense presence of phytotoxic compounds in the substrate, plant growth will be severely inhibited, but this effect will be diminished with increasing densities of plants since more and more plants will share the same amount of toxin (Weidenhammer, 2006; Thijs et *al*., 1994). Once the number of plants becomes high enough, the mean mass per plant curve goes back down due to competition for resources.

This principle was already used as a method by Thijs et *al*. (1994), which used atrazine to study the behavior of soybean and corn plants grown together when exposed to an external toxin. In this study, the authors used an experimental design as depicted in Figure 2, where a 'target' species was surrounded in the same pot by increasing numbers of 'neighbor' species (Thijs et *al*., 1994). The most striking result of this study is shown in Figure 3, in which we can see the curves of the relationship between Soybean dry mass and soybean (neighbor) density at increasing concentrations of atrazine (Thijs et *al*., 1994). In Figure 3, we can clearly see the slope of dry mass by density being reversed, since more plants share the same amounts of toxin, as predicted by Weidenhammer (2006). However, such a behavior of the curve is completely in opposition to what a classic resource competition scenario would predict, and these results show with strong evidence the presence of an involved toxin (Weidenhammer, 2006; Thijs et *al*., 1994).

With this in mind, this work will use the modified version of a protocol of Hegazy et *al.* (2001) that was criticized by Weidenhammer (2006) and of which the rationale is to use a well-known problematic plant (*Nymphaea lotus* L. in the case of Hegazy et *al.* (2001), *Reynoutria japonica* in our case) as the source of toxin, instead of an artificial one. To our knowledge, this study will be the first one to use such a method to disentangle resource competition from allelopathy while comparing stressed plants with non-stressed ones and trying to tell apart indirect from direct allelopathy.

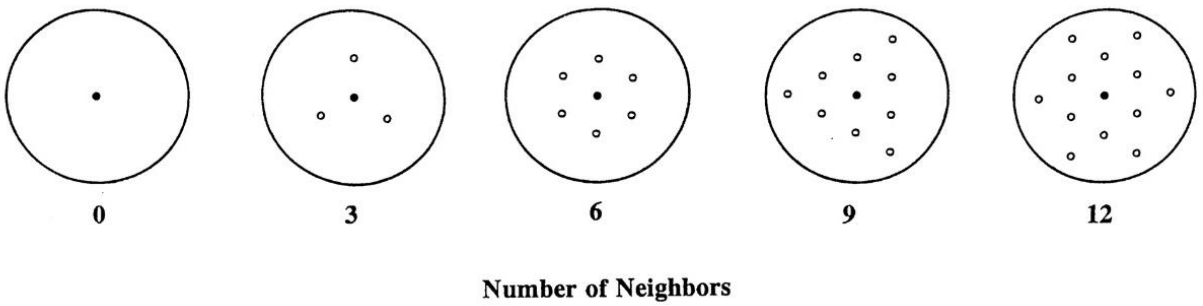


Figure 2: Schematic representation of the experimental design used by Thijs et al. *(1994). In the center is represented the target species (black dot) and surrounding it are the neighbor plants (white dots). From Thijs et* al. *(1994).*

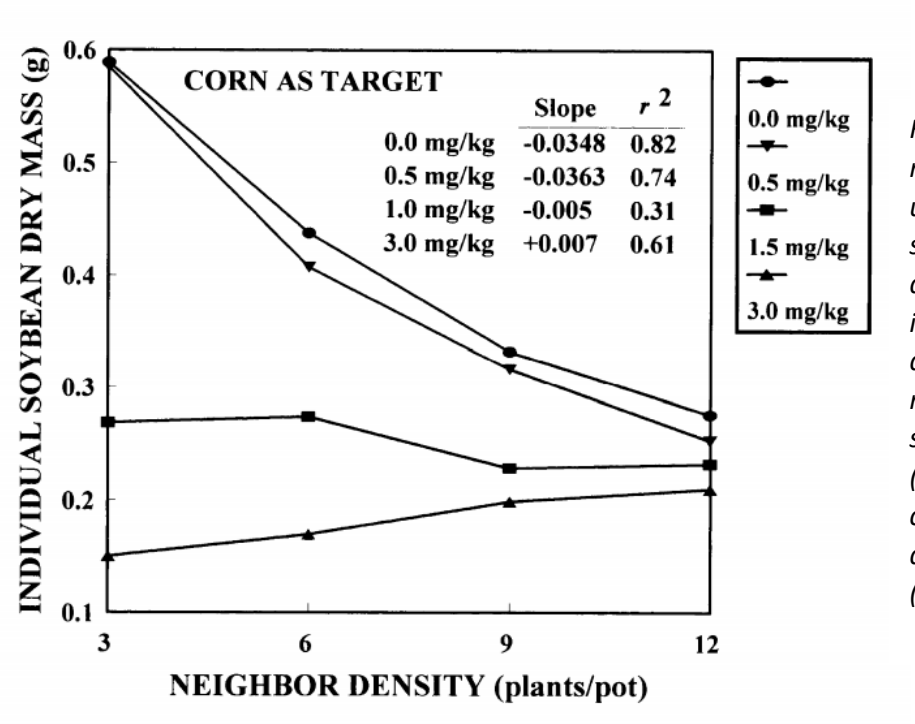


Figure 3: Results from a target-neighbor growth experiment using corn as target and soybean as neighbor. Each density treatment is exposed to increasing concentrations of atrazine. The graph shows the relation between mean soybean dry mass and soybean (neighbor) density at increasing concentrations of applied atrazine. From Thijs et al. *(1994).*

1.4 Allelopathy and Reynoutria japonica

1.4.1 Reynoutria japoninca Houtt.

1.4.1.1 Morphological description and development cycle

Reynoutria japonica is a rhizomatous perennial geophyte (Mincheva et *al.*, 2016; Lavoie, 2017, Kato-Noguchi, 2021). Its round stems are up to 4cm diameter, visibly divided into hollow segments, and grouped in shoot clumps (Fig. 4). It can reach two to three meters in height and forms large stands with a dense canopy. Leaves are 7-15 cm long, truncated at base and with an acute tip, usually as long as they are wide and glabrous on both sides (see material and methods section). *R. japonica* blooms between August and October and produces small three-winged white flowers (Fig. 4; Dumé et *al.*, 2018, Kato-Noguchi, 2021).

The Japanese knotweed, as the other knotweed species, are gynodioecious, meaning that in its native range, both female and hermaphrodite plants can be found (Bailey et *al.*, 2009). However, the main reproduction mean of *R. japonica* in its invaded range is through displacement of fragmented rhizomes via water current (e.g. during floods), or human transport (e.g. industrial or commercial transportation, earthmoving construction machinery, etc.), thus through asexual reproduction (Bailey et *al.*, 2009). It appears that the *Reynoutria japonica* clone that spread asexually all over its invasion range is male-sterile, and therefore cannot ensure sexual reproduction mean with itself (Bailey et *al.*, 2009).

1.4.1.2 Taxonomy

A key characteristic of knotweeds is their ability to hybridize (Lavoie, 2017; Bailey et *al.*, 2009). There are three main species of knotweeds present in Europe and America: *Reynoutria japonica* Houtt., *Reynoutria sachaliensis* (F. Schmidt) Nakai (synonyms : *Fallopia sachalinensis* (F. Schmidt) Ronse Decraene, *Polygonum sachalinense* F. Schmidt), and the hybrid between the two former species, *Reynoutria x bohemica* Chrtek et Chrtková (synonyms : *Fallopia x bohemica* (Chrtek et Chrtková) J. P. Bailey, *Polygonum x bohemicum* (Chrtek et Chrtková) P. F. Zika et A. L. Jacobson) (Bailey et *al.*, 2009; Dumé et *al.*, 2018). While most *R. japonica* individuals spread across Europe are male-sterile because of predominance of asexual reproduction through rhizome fragmentation and displacement, there are places in the invaded range of *R. japonica* where it coexists with *R. sachaliensis*, which presents hermaphrodite individuals (Bailey et *al.*, 2009). This case of coexistence eventually led to pollen from *R. sachaliensis* to fecundate flowers of *R. japonica*, which in turn led to the apparition of a hybrid between those two species: *R. x bohemica* (Bailey et *al.*, 2009). This hybrid was suggested to be much more threatening to native ecosystems and much more difficult to eradicate, in accordance with the Hybrid Vigor hypothesis, and due to its increased

Figure 4: Reynoutria japonica *Houtt.* ***a****: large stand,* ***b****: inflorescence,* ***c****: stems grouped in shoot clumps,* ***d****: shoot clumps base, rhizomes and roots,* ***e****: leaves. All pictures were taken between the 24th of December 2020 and the 14th of September 2021 at study site (50°40'15.1"N 4°34'17.7"E, see material and methods section) except for plant material in picture* ***d*** *that was collected in Liège (50°37'23.8"N 5°32'53.8"E). All pictures are original (Augustin Baussay).*

genetic diversity compared to its parents (Bailey et *al.*, 2009; Murrell et *al.*, 2011). Beyond this hybridization, knotweed species cited above present a much more complex crosses network, with for example the crossing occurring between *R. japonica* and *R. baldschaunica* and includes all backrosses and F2 cases of *R. x bohemica* (Bailey et *al.*, 2009).

1.4.1.3 Ecology

Reynoutria japonica is native to China, Japan, Korea, and Taiwan and was introduced in the 19th century as an ornamental plant in Europe (1825) and North America. Since then, it widely spread to the entire European continent and North America and is found in numerous additional countries in the world today (Dumé et *al.*, 2018; Kato-Noguchi, 2021).

R. japonica is developing in a wide set of conditions, including basic to slightly acidic soils, nutrient poor to nitrogen rich, and dry to very humid substrates (Dumé et *al.*, 2018; Kato-Noguchi, 2021). It is heliophilous and a pioneer species, found colonizing harsh volcanic substrates at high altitudes in its native range (Bailey et al., 2009; Kato-Noguchi, 2021). However, it demonstrates a strong preference for anthropogenic habitats and is commonly found invading riverbanks and shores, roadsides, railways or yet construction sites by (Mincheva et *al.*, 2016; Lavoie, 2017).

1.4.2 *Reynoutria japonica* Houtt. is a problematic invasive plant species

Reynoutria japonica is one of the worst invasive species in the world (Mincheva 2016, Lavoie 2017, Dommanget et *al.*, 2014, Murrell et *al*., 2011). *R. japonica* (synonyms: *Fallopia japonica* (Houtt.) Ronse Decraene, *Polygonum cuspidatum* Siebold & Zucc., (Dumé et *al.*, 2018; Bailey et *al.*, 2009), referred to below as *R. japonica* or *Rj*, is extremely problematic in Europe, North America and other parts of the world due to its impact on native plants survival and on chemical characteristics of the invaded soil (Mincheva et *al.*, 2016; Lavoie 2017; Dommanget et *al.*, 2014; Beerling et *al*., 1994). Its greatly successful invasion led to an always increasing distribution of the plant in the aforementioned areas, with great difficulties in removal of the plant from invaded sites because of its deeply-buried rhizomes (Mincheva et *al*., 2016; Lavoie, 2017; Bailey et *al.*, 2009; Beerling et *al*., 1994). The plant figures on the "100 of the world's worst invasive alien species" list of the Invasive Species Specialist Group (ISSG), and on the Black List of invasive species of Belgium (A3 category: widespread with a high impact on native ecosystems), where this study takes place (Simberloff & Rejmanek, 2019; Invasive Alien Species in Belgium: http://ias.biodiversity.be/).

As described in the review of Lavoie (2017), the impacts of knotweeds on the native ecosystems are various. It was found that *R. japonica* can be responsible for favoring fungi and amoebae over bacterial populations, reducing native plant species richness or cover, or yet lower the biomass and diversity of macroinvertebrates in some cases (Lavoie, 2017). A negative impact on frog populations due to the lack of prey in knotweed stands, and conflicting results on birds were found although only few studies focusing on the impacts of knotweed species on vertebrates were conducted (Lavoie, 2017). Nevertheless, some organisms, such as large detritivorous riparian invertebrates, were shown to benefit from the presence of knotweeds, even if overall they represent a minority of studied organisms (Lavoie, 2017). Even though this effect was suggested to strongly depend on the native plant communities that they would replace, knotweeds were also found to durably impact the chemical properties of the soil and thus ecosystem processes, through acidification of the soil, lowering of K and mineral N content, or yet increasing mineral nutrient and C presence in the substrate (Lavoie, 2017).

Given its impact on native environments, knotweeds are a kind of "worst enemy" alien invasive plant, with millions and billions spent in management plans aiming at its eradication (260 million $US/year in the UK, with an estimated price of £ 1.6 billion for its total removal from the country, while the annual price for its management is estimated to € 32 million per year in Germany (Dommanget et *al.*, 2014; Murell et *al*. 2011). However, recent studies improve our knowledge about the knotweeds' biology and with the help of their promising results, restauration schemes are successfully conducted nowadays (Lavoie, 2017; Dommanget, 2014).

1.4.3 Relevance of the study of allelopathy in the case of Reynoutria japonica Houtt.

Hypothesis regarding causes of alien species' remarkable invasiveness exist and *Reynoutria japonica* Houtt. case fit with some of them (Catford et *al.*, 2009). It was for example suggested that *R. japonica* Houtt. is successful in invaded ecosystems because of the absence of their natural predators or competitors which would cancel top-down regulation of the invasive plant populations (Enemy Release, regulatory hypothesis) and/or increase the amount of energy used by the invasive plant in growth and reproduction (Enemy Release, compensatory hypothesis; Mincheva 2016; Catford et *al*., 2009). Great support in literature can be also found, in the case of *R. japonica*, for the Novel Weapons hypothesis described above (Callaway & Ridenour, 2004; Dommanget et *al*., 2014; Fan et *al.*, 2010; Lavoie, 2017; Moravcová et *al.*, 2011; Murrell et *al.*, 2011; Parepa et *al.*, 2012).

Even though this latter hypothesis is strongly claimed in literature in the case of *R. japonica*, Parepa and Bossdorf (2016) argued that previous results concerning *R. japonica* phytotoxic behavior are biased because of a fundamental error of methodology: the use of artificial soils and not natural ones. The former substrate is inherently favorable to *R. japonica* growth regarding its physicochemical properties (Parepa & Bossdorf, 2016). Results of those studies are such that in a 2017 review, Lavoie deemed *R. japonica*'s allelopathic potential as "*minor because of lack of field evidence and potential experimental problems*" (Lavoie 2017). There is thus now a gap in knowledge regarding reliable results of *R. japonica* allelopathic behavior that this study aims to address. Furthermore, this same review by Lavoie (2017) points out a lack of scientific studies of the impact of *R. japonica* on native ecosystems and incentivize to conduct research on this species.

Although strong support of the NWH in the case of *Reynoutria japonica*, and given the major experimental flaw pointed out by Parepa & Bossdorf (2016), it is necessary to describe here what is the literature position on the subject. Some studies employing reliable methods following Inderjit & Callaway (2003), concluded to an allelopathic effect of *R. japonica* on other neighboring plants (Dommanget et *al.*, 2014; Moravcová et *al.*, 2011; Fan et *al*., 2010). Even though opposite arguments are formulated (Parepa & Bossdorf, 2016; Mincheva et *al*., 2016) and that one could question the method used in these studies, it is still interesting to note that researchers found an effect strong enough to conclude that *Rj* does allelopathy, and to suggest a phytotoxic effect of ecologically important molecules produced by it (Dommanget et *al.*, 2014; Moravcova et *al.*, 2011; Fan et *al*., 2010). Additionally, previous studies concluded to allelopathy (still using debatable methods) effects in species related to *R. japonica* (Murell, 2011; Inderjit & Weiner, 2001; Inderjit & Nishimura, 1999). Murell et *al*. (2011) indeed demonstrated that *Reynoutria xbohemica* can exert an allelopathic effect on native plants. As for Inderjit & Nishimura (1999) who investigated the deleterious effects of emodin and physcion, two allelochemicals found to be produced by *P. sachalinense*. All these previous findings, although debatable and not subject to a consensus, are still highly suggestive and reinforce the pertinence in investigating for allelopathy in the case of *R. japonica*.

1.4.4 Ecophysiological relevance of methods used in demonstrating *Reynoutria japonica* allelopathic potential

While direct allelopathy is a possible explanation in the case of *Reynoutria japonica* invasion, key case 'II. A.' (Table 1) of so-called indirect allelopathy also seems to be a good candidate since it was already suggested by Dommanget et *al*. (2014) and Dassonville et *al.* (2011) that

R. japonica has an impact on soil nitrogen composition by affecting microbial communities responsible of denitrification. As mentioned above, it is particularly challenging to distinguish direct from indirect allelopathy. However, this work will follow an empirical mean to do so, and this for its seed germination bioassay using both *Rj* extracts (rhizomes and leaves) and soil extracts, based on the protocol of determination between phytotoxicity, direct allelopathy and indirect allelopathy that was described in Ens et *al.* (2009). This protocol is based on the fact that soil phytotoxicity on seed germination should always be observed to determine allelopathy (Ens et *al.*, 2009). Following this logic, the case of a phytotoxicity only observable for soil extracts (and not of the plant material) corresponds to an indirect allelopathy based on how likely it is that a third factor (either biotic or abiotic) is involved in that case (Ens et *al*. 2009). Phytotoxicity of both plant material and soil would be representative of a direct allelopathy case, while phytotoxicity of plant material alone does not prove allelopathy (Ens et *al*. 2009).

In addition, the experimental design of the growing experiments of this work will take advantage of phytotoxicity density-dependent principle by exposing *R. japonica* plants to increasing levels of stress in order to try and observe an effect of *Rj* allelopathy. This feature of allelopathy regulation was mentioned by Dommanget et *al.* (2014) since the authors were trying to avoid such an effect, which could have led to "over-estimating the impact of *F. japonica* leachate on target species.". Such an over-estimation is not problematic in the case of this study since the experimental design of growing experiments allows for comparison between stressed and non-stressed plants.

1.5 Aims and hypotheses

1.5.1 Study aim and main hypothesis

The proposed hypothesis regarding *Reynoutria japonica* allelopathy, falling in line with the literature, states the following: *R. japonica* does allelopathy, but its phytotoxic effects cannot be separated from all other characteristics of the plant, meaning that *R. japonica'*s ecological dominance must come from a combination of factors (high growth rate, optimal resource use, lack of enemies in native ecosystems, etc.) rather than from a single feature. This leads us to suggest that the allelopathy of *R. japonica* exists and is observable but is rather weak, even under stress conditions.

Using seed germination and radicle length bioassays involving *R. japonica* leaf, rhizomes and soil extracts, plant growth experiments exploiting the density-dependent principle of phytotoxicity as well as physiological measurements of plant status and soil composition

analyses, this work aims at empirically observing the allelopathic effects of *Rj* in an ecologically meaningful context. This investigation is based on the supported assumption that allelopathic effects of *R. japonica* are observable empirically and focuses on two distinct allelochemicals release pathways: root exudation and debris incorporation into soil, with a methodological emphasis on the former one (see Moravcová et *al.* (2011) for an example of study suggesting allelopathic potential using leaves extracts).

1.5.2 Experimental aims and hypotheses

With greenhouse growth experiments, this study tries to show the relative role of allelopathy and resource competition in driving competitive dynamics in the case of *Reynoutria japonica* invasion in conditions close to this of field ones. Since a weak allelopathic effect of *Rj* on other plants is hypothesized, we expect the neighbor plants of growth experiments to present a deviation in the law of constant total yield when grown along with *R. japonica* as a target plant or in soil with direct history of *R. japonica* invasion. This implies that mean mass per plant of neighbors will increase when incrementing densities (particularly true for the lower densities), and this with stronger amplitude as the plants are exposed to nutrient deficiency stress.

Physiological measurements and soil analyses complement the growth experiment and respectively aim at studying native and invader plant status in the frame of a biological invasion set up and clarifying the causes of observed growth patterns. Specifically, we expect to observe a clear physiological stress in terms of mineral nutrient uptake and chlorophyl production for plants exposed to allelopathic stress (lowest densities of neighbor species growing with *Reynoutria japonica*) and/or nutrient deficiency stress (reduced quantity of available nutrients in potting substrate), with possible interaction. Soil characteristics are expected to be highly similar between infected soil taken beneath *R. japonica* canopy and non-infected/natural soil taken on the same site but that wasn't exposed to *R. japonica*'s presence. Finally, we hypothesize that both site soil types used in the experiments present high carbonic content and high levels of available nutrients, thus accounting for a potential weaker observable allelopathic effect in their respective treatments.

A germination bioassay will also be useful to support the growth experiments in determining whether, following Ens et *al.* (2009) empirical definition of the following concepts, *Reynoutria japonica* exert phytotoxicity, direct or indirect allelopathy. More precisely, we hypothesize that *Rj* does direct or indirect allelopathy and that bioassay results will thus include phytotoxic effects of both plant material and soil extracts or solely of soil extracts, this latter case providing evidence for indirect allelopathy (Inderjit & Weiner, 2001).

In conclusion, this study tries a new method to provide new arguments in favor or against *R. japonica* allelopathic effects while coping with the shortcomings that were addressed to previous studies trying to demonstrate allelopathy and aims at substantiating previous results.

2.Material and Methods

2.1 Study site and plant material identification

2.1.1 Study site selection

Plants were sampled in the region of Ottignies-Louvain-La-Neuve, in central Belgium. To find a study site of the right size and accessibility, several field outings were conducted between early February and mid-June 2021 in the Region around Louvain-La-Neuve and Wavre (Walloon Brabant, central Belgium). In total, 61 sites in which invasive *Reynoutria* spp. occur were found, mainly along the Dyle's shore (Fig. 5a), of which 50 were reported on iNaturalist. For each site, population size was assessed using four distinct categories. If only a few stems/shoot clumps were developed, the stand was labeled 'Small'; if 5 to 20m² of soil surface was covered by the stand, the stand was labeled 'Medium'; if the stand covered 20 to 30m², it was labeled 'Large'; and if around 30m² of soil or more was covered by it, the stand was labeled 'Extra large'.

Site selection depended on three main factors: size, accessibility, and correct identification of *Reynoutria japonica* on site (see below). For the purpose of this study, the *Reynoutria* stand that would be used in the experiments needed to be large and contain sufficient plant material to perform the experiment. The word "stand" refers here to a compact colony of knotweed stems at a given site, regardless of whether those stems belong to different *Reynoutria* species (Mereda et *al.*, 2018). The two largest stands were found along the Dyle river, and only one of them was outside of any Natura 2000 zone (site 16 (Limelette), see Fig. 5b, is included in the Natura 2000 site number BE31006: Vallée de la Dyle à Ottignies, which spans over the river Dyle as do three other Natura 2000 zones, see Fig. 5a).

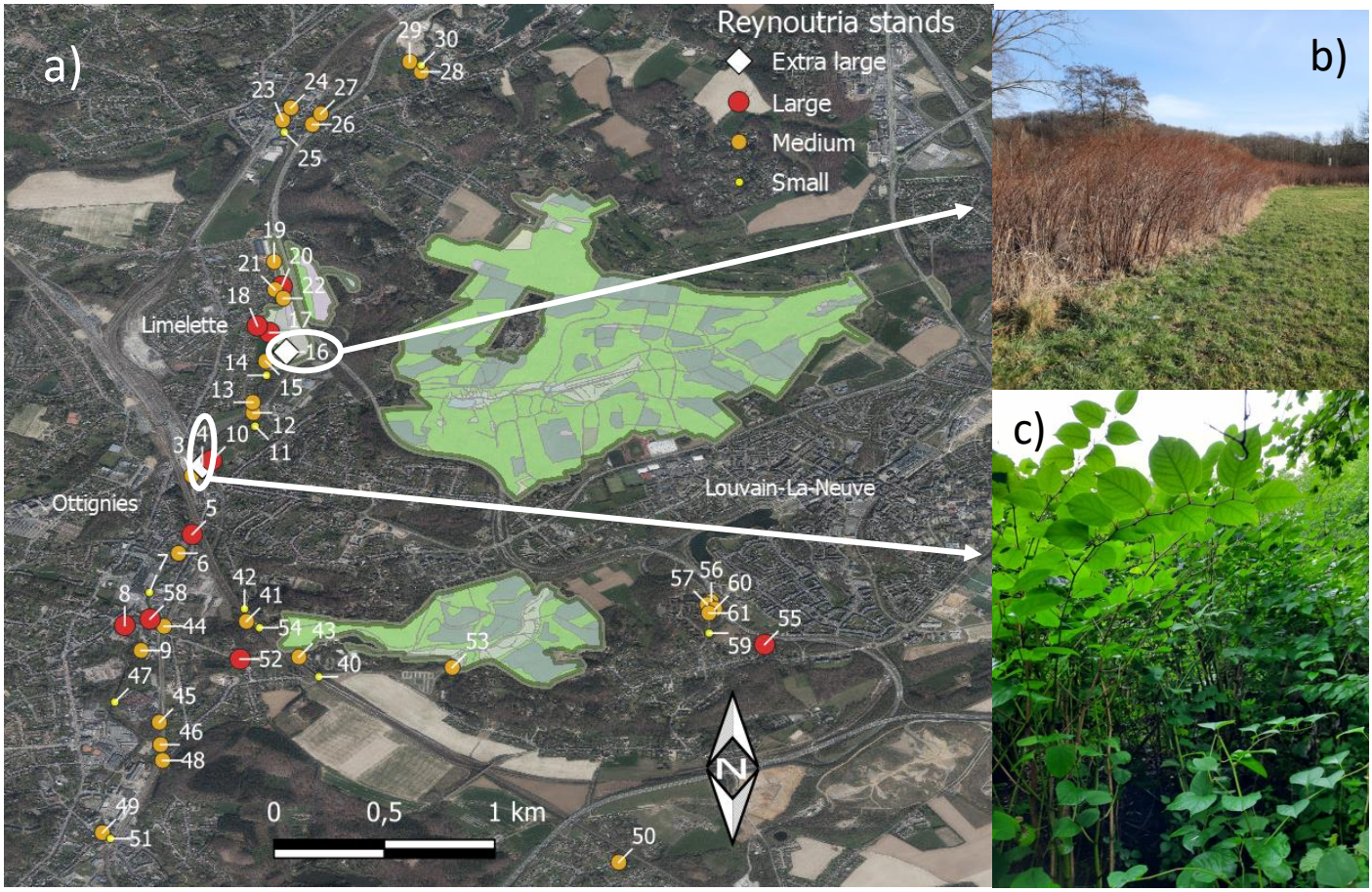


Figure 5: Overview of the study area and representative photographs of the sites visited between early February and mid-June 2021. ***a*** *is a map of region between Ottignies and Louvain-La-Neuve on which 50 out of the 61 recorded sites are represented. The size of each site is given by the point's color and size as shown in the legend in the upper right corner. Small, medium, large and extra-large sizes correspond to stands consisting of a few shoot clumps, covering 5 to 20m², 20 to 30m², and 30m² or more, respectively. Areas highlighted in green and grey represent Natura 2000 zones. Sites 15 to 22 are located in the BE31006 zone. The map was made using QGIS software (QGIS Desktop 3.16.14).* ***b*** *and* ***c*** *are photographs of extra-large sites 16 and 4, respectively. Geographic coordinates of all sites can be found in Annex M3.*

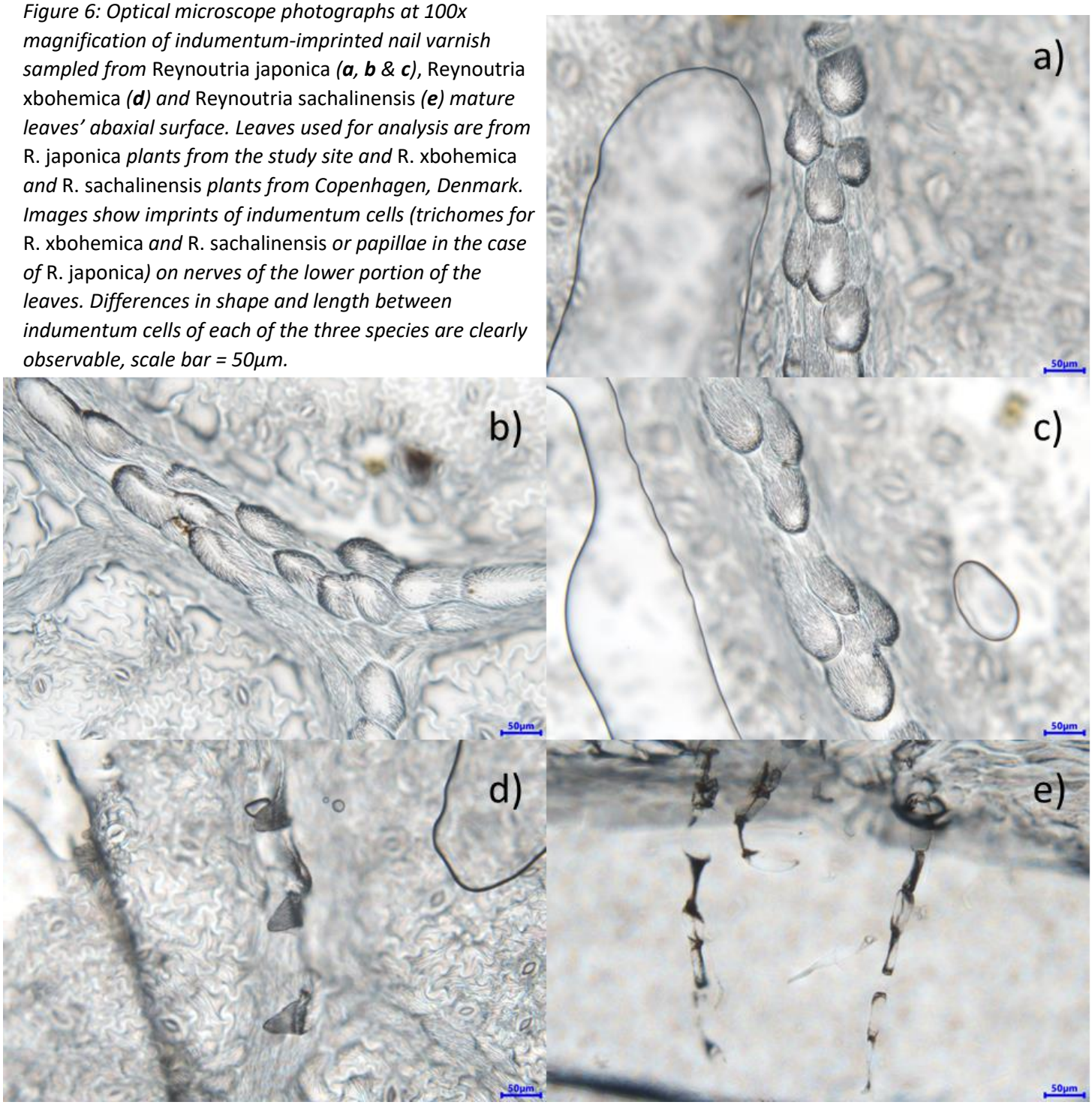
The selected site is site 4 on Fig. 5a. It consists of a highly developed *Reynoutria japonica* stand (most likely homogenous for this species) of about 30m² right next to the river Dyle and is surrounded by a woody vegetation. This site is located near the city center of Ottignies (Geographic coordinates: 50°40'15.1"N 4°34'17.7"E) and is contiguous to the Dyle (NW), Railways (uphill, NE), the Paul Delvaux avenue (SW), and a forest patch (SE) itself juxtaposed to a parking lot. The site is referred to as a habitable zone in the land register and belongs to the city of Ottignies-Louvain-La-Neuve. Authorization to collect soil and plant material was given by the city's administration on the 24th of June 2021, and an iNaturalist observation was created and is available through this link: Reynoutria japonica (Japanese Knotweed) from 1340 Ottignies-Louvain-la-Neuve, Belgique on August 17, 2021 at 10:07 AM by Augustin Baussay · iNaturalist.

2.1.2 Reynoutria japonica identification

Identification of the knotweeds at each site was first carried out via the use of local flower guides (Flore Forestière française, 2018; Flore écologique de Belgique, 2018) and first only considered the identification criteria of those guides. Whereas the Flore écologique de Belgique (2018) only mentions leaf shape and size along with inflorescence and fruit shape to tell apart between the three invasive knotweed species present in Europe, *Reynoutria japonica*, *R. sachalinensis*, and their hybrid *R. xbohemica*, the Flore Forestière française (2018) uses more precise and reliable criteria such as the presence and structure of mature leaves' abaxial indumentum. This latter criterion, found to be the most reliable by Mereďa et *al.* (2018), was investigated on sites 4 and 16 using a 10x magnifying glass, and later on site 4 plant material using nail varnish imprinting technique (Fig. 6).

Site selection procedure thus relied on the identification of *R. japonica* via those two guides' criteria which gave an idea of knotweed distribution and abundance at visited sites (Fig. 5a). However, as mentioned in Mereďa et *al.* (2018), efficient distinction between *Fallopia* sect. *Reynoutria* taxa is highly biased given that the existence of *R. xbohemica* itself is often overlooked and that most of the guides in the world either don't cite this species or do but don't give the proper criteria to distinguish it from *Reynoutira japonica*, to which it is morphologically closer related than previously expected (Mereďa et al., 2018). To address this shortcoming in identification, light-microscopy analyses of indumentum were performed on nail polish imprinted with the abaxial surface of fresh mature leaves collected on site (Fig. 6). The nail polish technique consists of applying three layers of nail polish on the leaf's lower surface before to delicately remove the imprint formed and mount it on a slide. Imprints of *Reynoutria x bohemica* and *Reynoutria sachalinensis* leaf abaxial surface were analyzed for comparison with the study material (Fig. 6). These latter species were found in Copenhagen, Denmark, in May and June 2022 and were identified using indumentum and morphological criteria (*R. sachalinensis*: 55°40'21.1"N 12°36'06.0"E, *R. xbohemica*: 55°41'20.2"N 12°35'43.7"E). Additional analyses were conducted on plants grown from rhizomes collected on study site and growing in the greenhouse along with the plant material used in the experiments. Annex M1 contains a picture of nail polish imprinted with abaxial leaf surface epidermis of these plants and optical microscopy images of the abaxial surface of their leaves.

Figure 6: Optical microscope photographs at 100x magnification of indumentum-imprinted nail varnish sampled from Reynoutria japonica ***(a, b & c)***, Reynoutria xbohemica ***(d)*** *and* Reynoutria sachalinensis ***(e)*** *mature leaves' abaxial surface. Leaves used for analysis are from* R. japonica *plants from the study site and* R. xbohemica *and* R. sachalinensis *plants from Copenhagen, Denmark. Images show imprints of indumentum cells (trichomes for* R. xbohemica *and* R. sachalinensis *or papillae in the case of* R. japonica*) on nerves of the lower portion of the leaves. Differences in shape and length between indumentum cells of each of the three species are clearly observable, scale bar = 50µm.*



Analysis of these data yielded clear results in favor of *Reynoutria japonica* identification. Because of its precision and reliability, the species identification process was based on the abaxial surface leaf indumentum criteria and used morphological criteria to confirm the findings (Bailey, 2009; Mereďa, 2018). Papillae (cells presenting a hollow of the cell wall) present on veins of the abaxial surface of the knotweeds sampled on study site clearly correspond to the single-celled 'bumps' of *R. japonica* as described by Mereďa et *al.* (2018) and Bailey et *al.* (2009). In fact, each indumentum cell visible in Fig. 6 matched specific criteria listed in the key

developed by Mereďa (2018). Leaves of identified *R. japonica* presented one-celled papillae 0.3–0.9(−1.7) times longer than wide (Fig. 6a-c), while those of *R. xbohemica* and *R. sachalinensis* had hairs constituted of 1 to 4 cells and 4 to 12 cells, and (0.7–)1–5 times and 12-25 times longer than wide, respectively. Such identifications and personal communication with M. Pavol Mereďa lead us to conclude that *Reynoutria japonica* was the plant present on the study site and used as plant material in this study.

2.2 Growth experiments

2.2.1 Plant and soil material preparation

Reynoutria japonica rhizomes and natural non-colonized soil needed for the first growth experiment (GroE1) were collected on field on the 24th of August 2021 in sufficient quantity. For the second growth experiment (GroE2), both colonized (soil that is in direct contact with *R. japonica* rhizomes, referred to below as 'infected') and non-colonized (soil collected on the same site but in a location where *R. japonica* is absent, referred to as 'non-infected') soils were collected on the 29th of September 2021, in smaller quantities than for GroE1.

In both experiments, *Trifolium incarnatum* was used as the neighbor species following the target-neighbor model of Thijs et *al.* (1994) (see Figure 7) and given that several authors pointed out that forbes and legumes species are more sensitive to allelopathy than grasses (Inderjit & Keating, 1999; Parepa et al, 2013; Murell et al, 2011), and that a negative effect of *Reynoutria japonica* on a representative of the *Trifolium* genus was already pointed out by Mincheva et *al.* (2016). *Trifolium incarnatum* seeds were bought from a local producer of wildflower seeds:

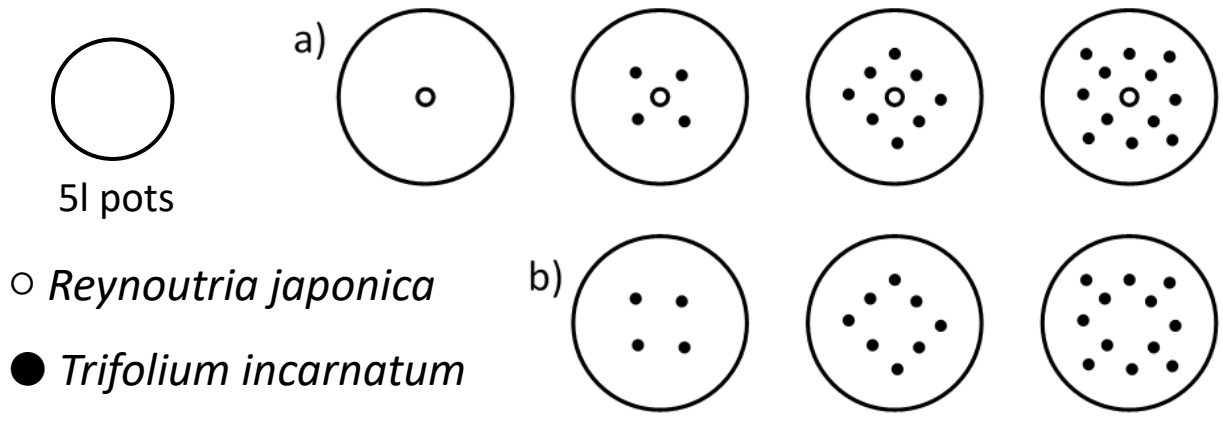


*Figure 7: Schematic representation of the experimental design used for the growth experiments. The design uses increasing densities of neighbor species (*Trifolium incarnatum*, black dots) around a 'target' species (*Reynoutria japonica*, white dots, a : GroE1) or without the target species (b: GroE2). Three densities of* T. incarnatum *were used in this case: 4,8, and 12. A fourth density consisting of a single* R. japonica *plant was added in the first growing experiment.*

ECOSEM sprl, in June 2021. The same seeds were used for both growth and germination experiments (see below).

Rhizomes of *Reynoutria japonica* were carefully washed from soil and cut to fit a standard length of 5 to 15cm with 2 or 3 intact nodes before they were put in soil trays for germination. *T. incarnatum* seeds and cut *R. japonica* rhizomes were placed in separate trays for 8 days on the 24th of August for GroE1. For GroE2, *T. incarnatum* seeds were put to germinate for 9 days on the 4th of October 2021.

In both experiments, collected soil was let to dry (8 days for GroE1, 14 days for GroE2) and then sieved with a 0.65 cm mesh sieve before potting. Natural non-infected soil was used in the first growth experiment in order to recreate the natural conditions of the site without implementing putative allelopathic effects that would occur through *R. japonica* rhizome exposure. Natural infected soil was used in GroE2 to investigate this putative allelopathic effect of the soil linked to its invasion history while recreating natural conditions. To test whether substrate nature and nutrient intake had an effect on growth of the studied plants, a 50-50 Perlite Vermiculite mix was used as inert substratum. Pots filled with this substrate were either watered with nutritive solutions following Hoagland protocol (Hoagland full, 'P/V Nutritive' treatment) or with a 5-times diluted Hoagland solution (Hoagland diluted 5 times, 'P/V Deficient' treatment). This allowed us to assess plant growth in a natural soil, in an artificial soil with all nutrients availability (to potentially corroborate the findings of Parepa & Bossdorf (2016) about the advantage that such soils give to *Reynoutria japonica*), and in an artificial soil with nutrients deficiencies (test the hypothesis of deficiency-induced allelopathy, as mentioned by Inderjit & Weiner, 2001 and Inderjit & Duke, 2003).

2.2.2 Experimental design

The two growth experiments (GroE1 & GroE2) both lasted around 6 weeks, but took place at different times, with GroE1 starting on the 1st of September 2021 and GroE2 on the 13th of October 2021. 5-Liter pots were used in both experiments and solar radiation, ambient temperature and hygrometry were assumed to be stable through the experiments (see Annex M2). Their respective experimental designs are best visualized in Figures 8 & 9.

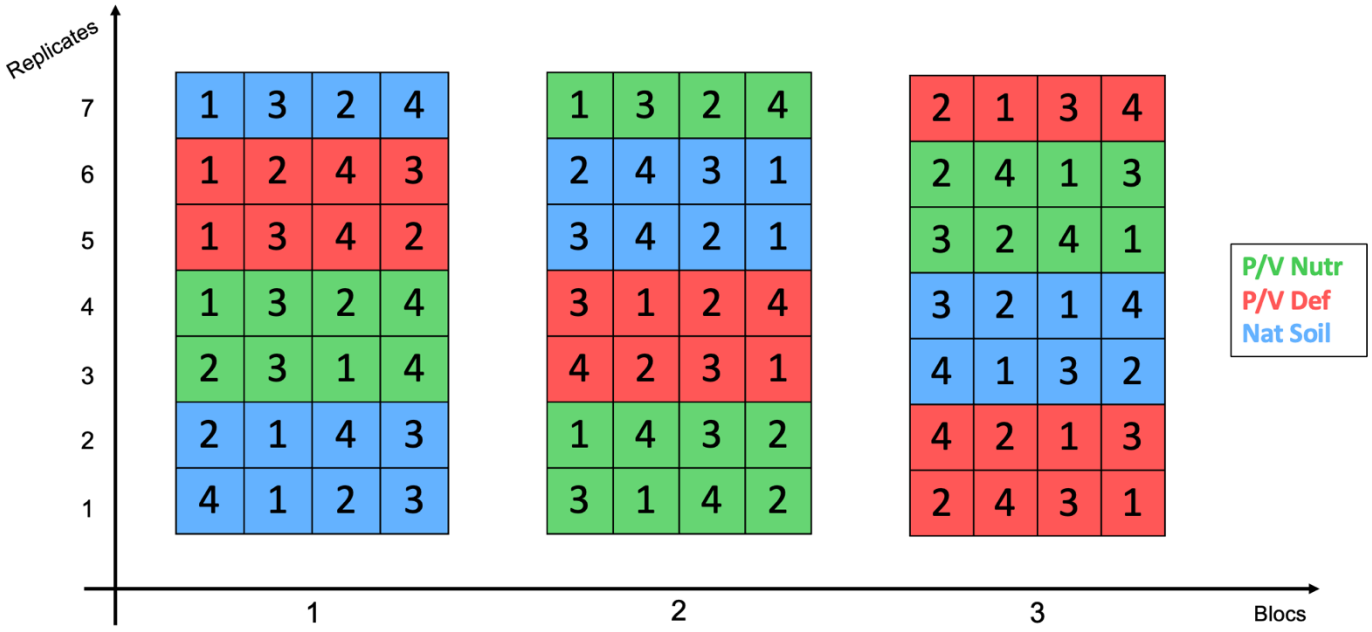


Figure 8: Experimental design of growth experiment 1. This experiment involved seven biological replicates and three blocks containing each treatment at least twice. Each of the 84 pots is represented by a square colored by treatment and contains one Reynoutria japonica *rhizome.* Trifolium incarnatum *density in each pot is represented by a number from 1 to 4; 1 indicating a density of 0* T. incarnatum*, 2 a density of 4, 3 a density of 8, and 4 a density of 12. P/V Nutr = P/V Nutritive treatment (watered with complete Hoagland solution), P/V Def = P/V Deficient (watered with 5-times diluted Hoagland solution), and Nat Soil = Natural soil (non-infected soil from study site).*

The first growth experiment consisted of a real target-neighbor design with *Reynoutria japonica* plants growing surrounded by *Trifolium incarnatum* neighbor plants (see Figure 7a, inspired by Thijs et *al.*, 1994), it used three soil treatments, four neighbor species densities, and had seven replicates for a total of 84 pots. Soil treatments comprised Natural Soil (non-infected), P/V Nutritive, and P/V Deficient. The four neighbor densities were of either 0 (*R. japonica* growing alone), 4, 8, or 12 *T. incarnatum* individuals growing together with one Japanese knotweed plant (see Figure 7a, in Figure 8, densities are shown as numbers from 1 to 4, 1 representing a density of 0 *T.incarnatum*, 2 a density of 4, 3 a density of 8, and 4 a density of 12). As mentioned before, those densities were used to investigate the putative allelopathic effect of *R. japonica* on *T. incarnatum* via the use of the density-dependent phytotoxicity principle. The seven replicates were arranged in the greenhouse as shown in the Figure 8. The 84 pots of the seven replicates were arranged in three blocks to test for homogeneity of abiotic conditions in the greenhouse.

On the 1st of September 2021, 84 5-Liters pots were filled with respective substrate, and one *R. japonica* rhizome with sprout(s) was planted in the center of the pot along with sprouts of *T. incarnatum* as presented in Figure 7a (four densities for each soil treatment, three soil

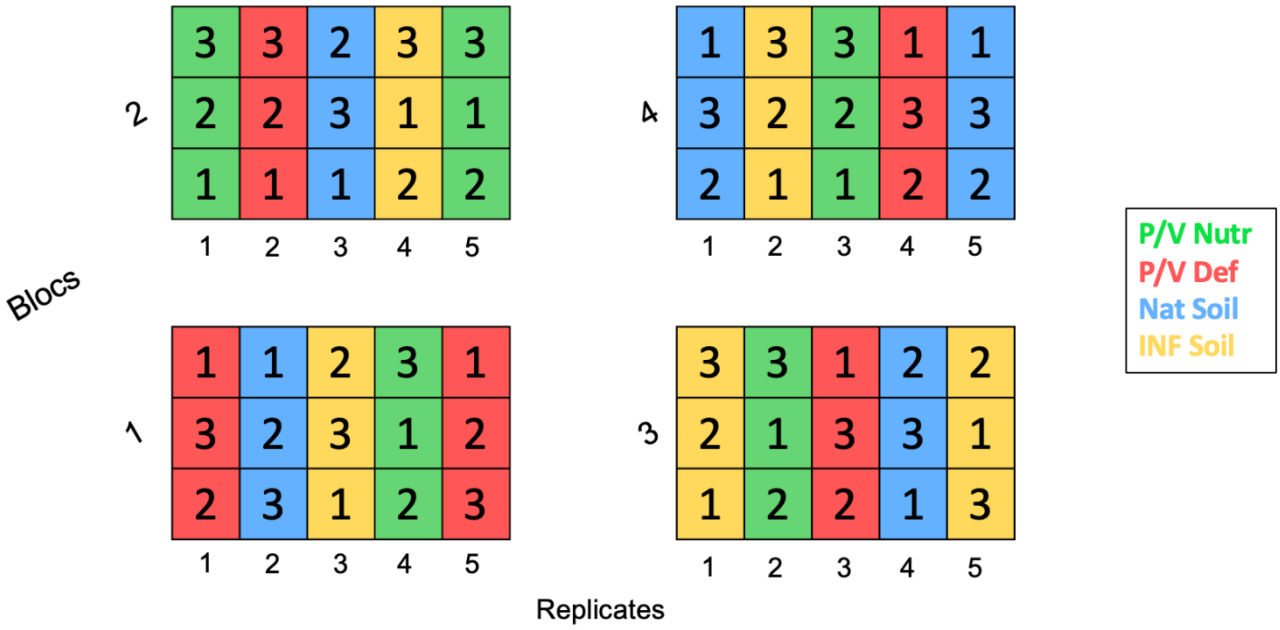


Figure 9: Experimental design of growth experiment 2, as arranged in the greenhouse. This experiment involved five biological replicates and four blocks containing each treatment at least once. Each of the 60 pots is represented by a square colored by treatment and does not contain any Reynoutria japonica *rhizome.* Trifolium incarnatum *density in each pot is represented by a number from 1 to 3; 1 indicating a density of 4* T. incarnatum *plants, 2 a density of 8 plants, and 3 a density of 12 plants. Treatment names are the same as the ones in Figure 7, except for INF Soil = Infected Soil (Natural infected soil from study site).*

treatments per replicate). For each rhizome, initial longest stem length above substrate level was monitored as well as the initial number of shoots that started growing on the rhizome. Watering of each pots was first carried out on the potting day, which was counted as the first day of experiment. Plants were watered every 2 to 3 days for three weeks and then every 5 days or depending on the plants need. During the experiment, wild plant species started to develop in the natural soil treatments. They were systematically removed and the number of them per pot was monitored. Harvest of the plants took place at the end of the six weeks of growth, on the 14th of October 2021. Data collection comprised weighing of total fresh *R. japonica* and *T. incarnatum* material. The root system and aerial parts were weighed separately for *R.j.*, while only aerial parts of all clovers in a pot were weighed simultaneously. Length of *R.j.* longest stem and shoot number were also measured. All *Trifolium* stems per pot and 4th and 5th youngest leaves (starting to count from the apex) of *R. japonica* plants were chopped with scissors prior to storage in a -80°C freezer in prevision of chlorophyll content analysis. After sampling and storage at -80°C of around 150mg of aerial parts fresh material of both species of all pots and replicates, this fresh material was placed to dry in an oven at 70°C for 3 days after which total dry mass of the aerial parts was determined. *R. japonica* root parts were directly put to dry after the harvest and their total dry mass monitored after 3 days.

The second growth experiment consisted of a control for *Trifolium* growth without *Reynoutria japonica* interference with the added feature of natural infected soil as a fouth soil treatment, thus allowing to test for the sole effect of a soil with *R. japonica* invasion history. No *R. japonica* plants were thus used in this experiment. This experiment used four soil treatments, three neighbor species densities, and had five replicates for a total of 60 pots. Soil treatments comprised natural soil (non-infected), infected soil, P/V Nutritive, and P/V Deficient. The three 'neighbor' densities were of either 4, 8, or 12 *T. incarnatum* individuals growing together without any Japanese knotweed plant (see Fig. 7b, and 9). In this case, those densities are used to get an idea of the effect of resource competition on the growth of *T. incarnatum* in order to compare those results with those of the GroE1 and are also useful to investigate the putative allelopathic effect of *R. japonica* infected soil on *T. incarnatum* via the use of the density-dependent phytotoxicity principle. The five replicates were arranged in the greenhouse as shown in the Figure 9. As represented in this latter Figure, the 60 pots of the 5 replicates were arranged in four Blocs to test for homogeneity of abiotic conditions in the greenhouse.

On the 13th of October 2021, 60 5-Liters pots were filled with respective substrate and sprouts of *T. incarnatum* were planted as presented in Figure 7b (three densities for each soil treatment, four soil treatments per replicate). Watering of each pot was first carried out on the potting day, which was counted as the first day of experiment. Plants were watered every 2 to 4 days for the first two weeks and then every 5 days. During the experiment, wild plant species started to develop in the natural soil treatments and were systematically removed. Harvest of the plants took place at the end of the 6 weeks of growth, on the 25th of November 2021. Data collection comprised weighing of total fresh *T. incarnatum* material (only aerial parts of all clovers in a pot weighed simultaneously), homogenization (fresh material chopped with scissors) of all *Trifolium* stems, and sampling of around 150mg of aerial parts fresh material for each pots in all replicates that were then stored in a -80°C freezer in prevision of chlorophyll content analysis. Fresh material was then placed to dry in an oven at 70°C for 5 days after which total dry mass of the aerial parts was determined.

2.2.3 Chlorophyll content analysis

To get an insight into the general health of the plants used in our experiments, chlorophyll a and b content as well as in carotenoid (xanthophylls and β-carotene) content were determined for each species in all pots of three of the biological replicates of each experiment. Fresh material conserved at -80°C was first grinded to obtain around 100mg of fresh material powder.

Acetone (80%, v/v) was then added to each sample to get a volume of 10ml. Each sample was then vortexed before to be put to centrifuge (Model Sigma 3-30KS, Osterode am Harz, Germany) at 3000rmp and 4°C for 10 minutes. The supernatant of each sample was collected and read at wavelengths of 663.2, 646.8 and 470 nm using a professional spectrophotometer (Model Shimadzu UV-1800 Spectrophotometer, 's-Hertogenbosch, the Netherlands). Data collected were absorbance of each sample at each wavelength. Concentration values for chlorophyll a and b and carotenoids were obtained by replacing these absorbance values in the Lichtenthaler's equations for Acetone (80%, v/v) as mentioned in Lichtenthaler (1987). Lichtenthaler's simultaneous equations were chosen over Armon ones given the non-negligible inaccuracies these introduce in concentration values (Porra, 2002).

2.2.4 Mineral content analysis

To investigate to what extent mineral intake differed between treatments of the growth experiments, mineral content of each of the two species in both experiments was analyzed. Potassium (K), Iron (Fe), Zinc (Zn), calcium (Ca) and magnesium (Mg) were quantified in aerial parts of *R. japonica* (4^{th} and 5^{th} youngest leaves) and *T. incarnatum* (all aerial parts). Material was oven-dried at 70 °C for 72 h and 50 to 100 mg dry weight were weighted and digested in 4 mL of warm 68% (v/v) HNO3. After complete dissolution, minerals were dissolved in aqua regia (HCl 37%: HNO3 68% 3:1), filtered (Whatman, 11 µm) and quantified by flame atomic absorption spectrophotometry (ICE 3300, Thermo Scientific, Waltham, MA) using suitable standards (Spectracer-CPACHEM; accredited through ISO/IEC17025). Quantification was done on three biological replicates per experiment, and results consisted of absorbance and concentration data for each mineral analyzed in all samples.

2.3 Germination experiments

2.3.1 Plant material and extracts preparation

Two crop species were used in our bioassays: seeds of maize (*Zea mays*) and crimson clover (*Trifolium incarnatum*) were bought from a local producer (ECOSEM sprl) in June 2021 (see above). These were selected to test previous findings suggesting that forbs are more sensitive to knotweed presence than monocotyledon species (Parepa et *al.*, 2012; Murell et *al.* 2011; Mincheva et *al.*, 2016).

Prior to germination, all seeds were sterilized using a 1% bleach solution (1ml bleach adjusted to 100ml with demineralized water, first Germination experiment (GerE1), 2/07/2021) or using 1% for *T. incarnatum* and 5% bleach solution for *Z. mays* (second germination experiment

(GerE2), 23/09/2021). Seeds were immerged in agitated respective solutions for five minutes, and then were rinsed three times for ten minutes in agitated demineralized water. Seeds were finally dried on paper before experiments started.

Differences in bleach solutions used to sterilize maize seeds between GerE1 and GerE2 aimed at preventing molding in the petri dishes, which occurred in the GerE1 more importantly on *Z. mays* and seemingly without affecting its germination rate. Preliminary germination tests conducted on *Z. mays* and *T. incarnatum* seeds sterilized with solutions containing increasing bleach concentrations did not allow us to conclude that increased bleach exposure had a significant effect on germination of *Z. mays*, whereas a negative effect on germination of *T. incarnatum* seeds was observed (data not shown).

Plant material used to make extracts for GerE1 was harvested form the study site on the 24th of June 2021 and stored in -80°C freezer until use. Soil samples used to make soil extracts for GerE2 were gathered from the study site and a nearby field (The field is located in Chaumont-Gistoux, 50°40'15.6"N 4°38'01.7"E) on the 31st of August 2021 and stored at 6°C until use. Plant material was then grinded using liquid nitrogen, while soil samples were not treated before extraction. Three solvents were initially considered for extraction: demineralized water (DW), methanol (Meth) and dichloromethane (DCM). However, due to technical issues, results obtained with DCM were not taken into account in GerE1, and DCM wasn't used in GerE2.

Grinded leaves and rhizomes were added to each solvent in a 2g plant material/10ml solvent proportion and were agitated for 2 hours before to be centrifugated at 2000g for 10 minutes and to be filtered. Soil samples were added to DW and Methanol in a 20g soil/80ml proportion and agitated for 30min before to be filtered directly. Before experiments started, all demineralized water used for future watering as well as manipulating tools and filter papers used in the experiments were autoclaved.

2.3.2 Experimental design

GerE1 and GerE2 were conducted on the 2nd of July and the 23rd of September 2021, respectively and both experiments lasted for 5 days in total. In both experiments, each plastic Petri dish (8.85cm diameter) contained 30 seeds of *Z. mays* or *T. incarnatum* and a filter paper (Whatman Grade 1, 85mm diameter) previously soaked with each treatment's solution and let to dry, on which the seeds were placed. These experiments aimed at investigating the impact of extract on seed germination and subsequent development. Sterilized demineralized water and

Hoagland (full) nutritive solution were used to water the petri dishes depending on the seeds' need. Germination experiments took place in a germination room with closable shelves to prevent *Trifolium* seeds to be exposed to light as they are sensible to it (de Lespinay et *al.*, 2010).

GerE1 focused on the effects on seed germination and sprout development of *R. japonica* extracts, particularly these obtained from leaves and rhizomes of the plant. This experiment consisted of twelve treatments in total: with three extract types (leaves, rhizomes and control), two solvent used (demineralized water and methanol) and two test species (*Z. mays* and *T. incarnatum*). Each treatment had 3 biological replicates (three Petri dishes per condition) for a total of 36 Petri dishes. On the start of the experiment, 5ml of each extract was added to a filter paper placed in each Petri dish, which were then let to dry to prevent seeds to enter in contact with solvents. Controls consisted of adding 5ml of pure demineralized water and methanol on the filter paper (testing the effect of solvent alone). After adding seeds, 6 and 4ml of sterilized demineralized water were respectively added to Petri dishes treated with methanol extracts and filled with *Z. mays* and *T. incarnatum*. Final count of germinated seeds was performed on the fourth day of the experiment. A seed was considered as germinated when radicle protrusion was observed. Estimation of radicle length was done on the fifth day of experiment and consisted of the measurement of five radicles of sprouts picked up randomly in each Petri dish.

GerE2 focused on the effects of *R. japonica* contaminated and non-contaminated soil extracts on seed germination and sprout development, using field soil as a comparison. This experiment consisted of 24 treatments in total. A first part of the experiment used soil extracts and involved four soil treatments (infected soil, non-infected soil, agricultural soil and control), two solvents (demineralized water and methanol) and two test species (*Z. mays* and *T. incarnatum*), yielding a total of 16 treatments. In addition, a 'full soil' treatment was added, which consisted of the placement of around 15g of each three studied soils in Petri dishes. This treatment thus comprised three soil treatments (infected, non-infected and agricultural) and two test species (*Z. mays* and *T. incarnatum*), yielding a total of 6 treatment combinations. All treatments were biologically replicated three times for a total of 72 Petri dishes.

At the start of the experiment, 5ml of each extract were added on a filter paper in Petri dishes, which was dried as seen above. After adding seeds, 5ml of demineralized water was added to the 'full soil' treatments. In Petri dishes containing a methanol treatment (control and extracts), 5 ml (*Z. mays*) or 2 ml (*T. incarnatum*) of demineralized water was added. No additional amount of demineralized water were added in Petri dishes treated with demineralized water (control

and extracts). Finally, the number of germinated seeds and radicle length were determined as previously described.

For GerE2, we also assessed germination kinetics as another trait on which exposure to infected soil could have an impact. Germination counts for each of the 72 Petri dish was performed every 12 hours from the start of the experiment until the end (all seeds counted 8 times in total). These data will allow us to investigate whether exposure of seeds to different kind of soils or soil extracts will impact the speed at which radicle development occurs.

2.4 Soil samples preparation

Soil samples analysis was carried out on infected, non-infected and agricultural soils. Samples were collected on the 20th of December 2021 at above mentioned locations. Soil samples consisted of several kilograms of the top 10cm layer of each soil type. Each soil sample was homogenized before preparation of three replicates of 100g that were stored at 6°C for two months. Sample analysis was conducted at ASBL Centre de Michamps and comprised measurements of carbon and humus content, pH, and content in magnesium, zinc, iron, phosphorous, calcium, copper, potassium and manganese.

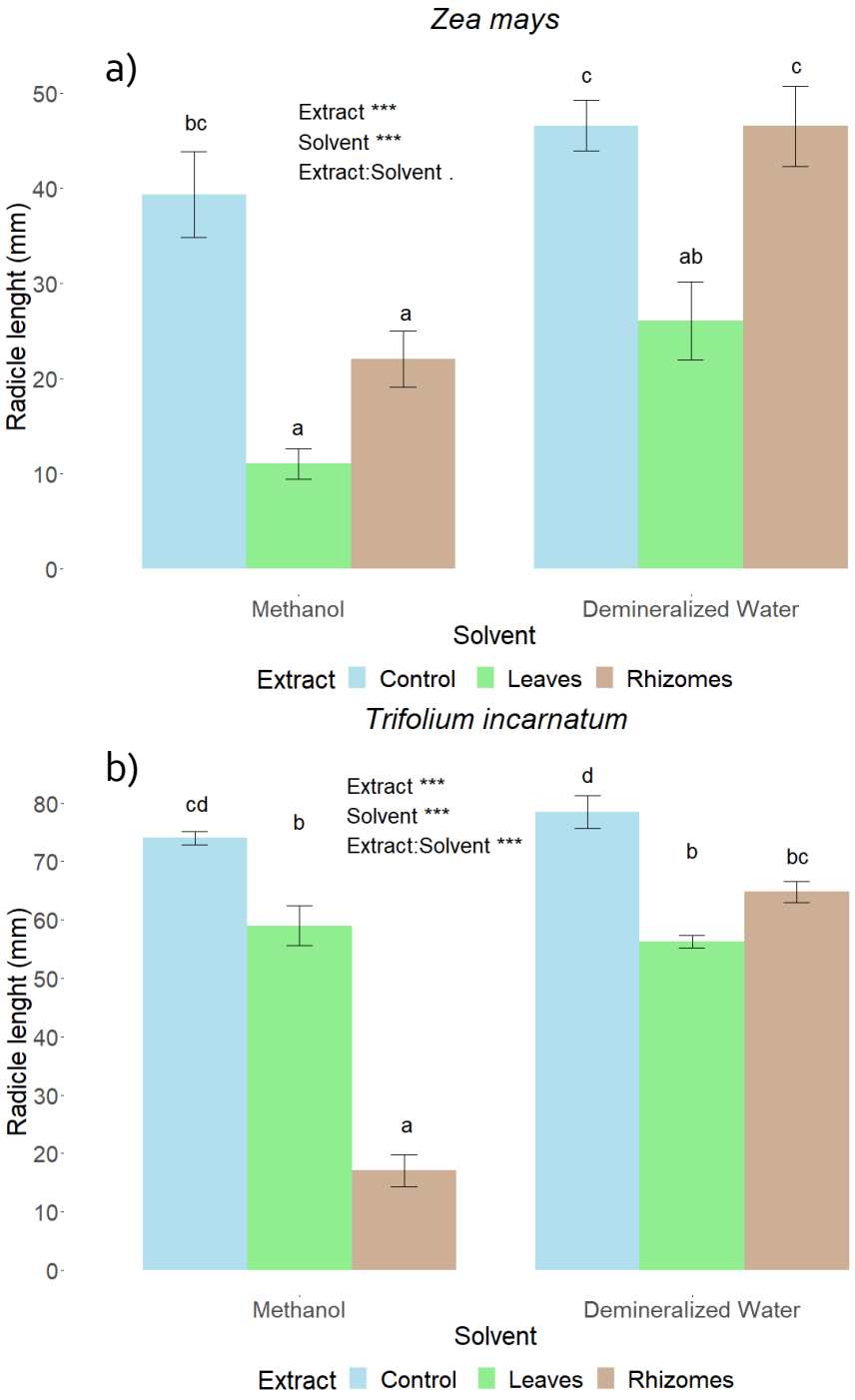
2.5 Statistical analysis

LM were used to analyze germination and radicle length data of bioassays as well as abiotic variables of soil sample analysis. LMM using blocs of the growth experiments as random factors were used to analyze clover aboveground biomass and physiological measurements data. For the physiological measurements, LMM aiming at comparing both growth experiments at once were created. All data was checked for normality and homoscedasticity using Levene, Bartlett and Shapiro tests. When not matching the application requirements, data was log-transformed as specified in each test table. PCA analysis were performed on clover tissue mineral content and soil samples abiotic variables. Correlation tests of allometry measurements were performed using Pearson correlation test. All statistical tests and plots were produced using RStudio (RStudio desktop v2022.2.3.492 “Prairie Trillium”) software.

3. Results

3.1 Bioassays

Germination rate was overall not significantly impacted by solvent, plant material, soil type and exposure type treatments or their interactions in any of the bioassays (Table 3). Exceptions to this included the case of interaction between solvent and plant material used on *Trifolium incarnatum* in the *Reynoutria japonica* tissue extracts experiment and the exposure type on *Zea mays* in the soil and soil extracts experiment.



Conversely, radicle length of test species was significantly responding to all treatments in both bioassays apart from the interaction between solvent and plant material used on *Zea mays* in the *R. japonica* extracts bioassay and the interaction between soil type and exposure type on *Trifolium incarnatum* in the Soil and soil extracts experiment.

In all cases, homoscedasticity and normality of the data were tested statistically and checked

Figure 10: bar plots of the mean radicle length of Zea mays *(a) and* Trifolium incarnatum *(b) by Solvent and* R. japonica *extract groups. Groups not sharing a letter are statistically different. Significance of associated Linear Model is reminded above the graphs. The following significance codes are applied: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

*Table 3: Statistical data of the bioassays. Anova tests were performed on Linear Models for both radicle length and germination rates. Responses variables not matching the application conditions were log-transformed as indicated. Values in bold are statistically significant (p-value < 0.05). The following significance codes are applied: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

***Reynoutria japonica* tissue extracts experiment**			
Zea mays			
Response variable	Explanatory variables	Df	p-value
Germination rate (/1)	Solvent	1	0.06783
	Plant material	2	0.65618
	Solvent : Plant material	2	0.26041
Radicle length (mm)	Solvent	1	**0.0001454*** **
	Plant material	2	**5.2e-05*** **
	Solvent : Plant material	2	0.0855845.
Trifolium incarnatum			
Response variable	Explanatory variables	Df	p-value
Germination rate (/1)	Solvent	1	0.941487
	Plant material	2	0.061263.
	Solvent : Plant material	2	**0.002007** **
Log10(Radicle length (mm))	Solvent	1	**3.779e-06*** **
	Plant material	2	**1.669e-07*** **
	Solvent : Plant material	2	**4.555e-07*** **
Soil and soil extracts exposure experiment			
Zea mays			
Response variable	Explanatory variables	Df	p-value
Germination rate (/1)	Soil type	3	0.22169
	Exposure type	2	**0.04189***
	Soil type : Exposure type	5	0.39700
Radicle length (mm)	Soil type	3	**0.01961***
	Exposure type	2	**7.764e-06*** **
	Soil type : Exposure type	5	**0.01703***
Trifolium incarnatum			
Response variable	Explanatory variables	Df	p-value
Germination rate (/1)	Soil type	3	0.6744
	Exposure type	2	0.6534
	Soil type : Exposure type	5	0.4326
Radicle length (mm)	Soil type	3	**0.0031574** **
	Exposure type	2	**0.0004008*** **
	Soil type : Exposure type	5	0.0928164.

visually. When those application conditions were not satisfied, a $\log_{10}$ transformation was applied to the response variable which often failed to mitigate heteroscedasticity and non-normality. Linear Models were used as both radicle length and germination rates were considered as continuous variables. Each of the eight models comprised two explanatory variables as detailed in Table 3.

3.1.1 *Reynoutria japonica* extracts experiment

Tukey post hoc tests were performed on the above-mentioned models to determine statistical significance of differences between each group of factor combinations. Only data for radicle length is represented for this bioassay.

In Figure 10, significant differences are observed between control and treatment groups in all cases, consistently with the statistical tests.

Zea mays' radicle length was decreased by all extracts except the water extract of *R. japonica* rhizomes. The greatest effect was recorded for methanol extracts of *R. japonica*'s leaves. Overall, methanol extracts had greater effects than water ones (Figure 10a). Radicle length of *Trifolium incarnatum* was decreased in response to all solvent and extract treatments with the

most dramatic effect recorded in the bioassay for rhizome methanol extracts. Overall effects of methanol extracts were more important (Figure 10b).

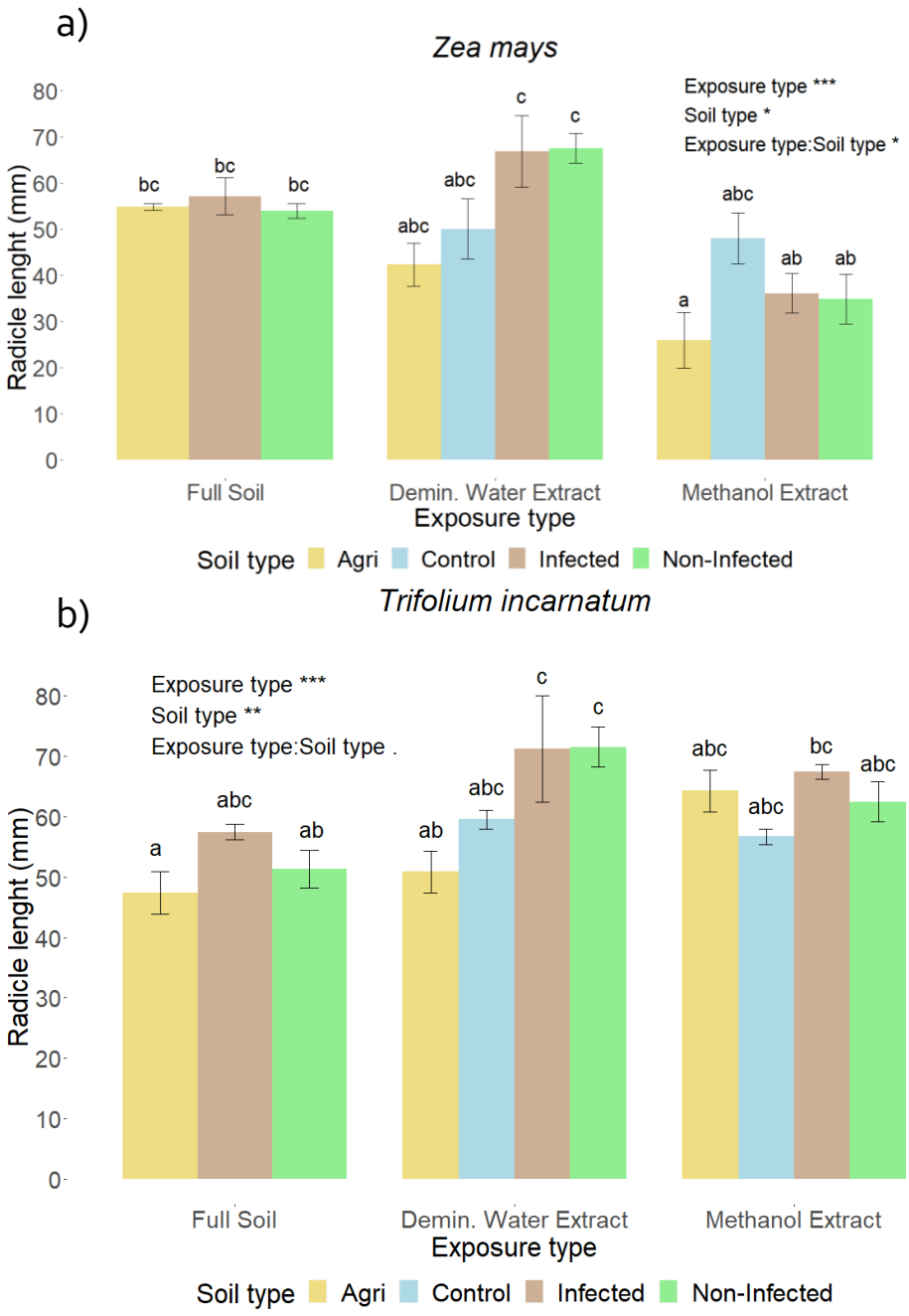


Figure 11: bar plots of the mean radicle length of Zea mays *(a) and* Trifolium incarnatum *(b) by Exposure type and Soil type. "Agri" identifies to soil collected in a field while Infected and Non-Infected refer to soil that was exposed or not to* R. japonica *rhizomes, respectively. Groups not sharing a letter are statistically different. Significance of associated Linear Model is reminded above the graphs. The following significance codes are applied: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

3.1.2 Soil and soil extracts experiment

As shown in Figure 11, impacts of complete infected soils and/or soil extracts on radicle elongation of both test species were less significant than for *Reynoutria japonica* tissue extracts. Soil originating from an agricultural field interestingly presented negative effects across more

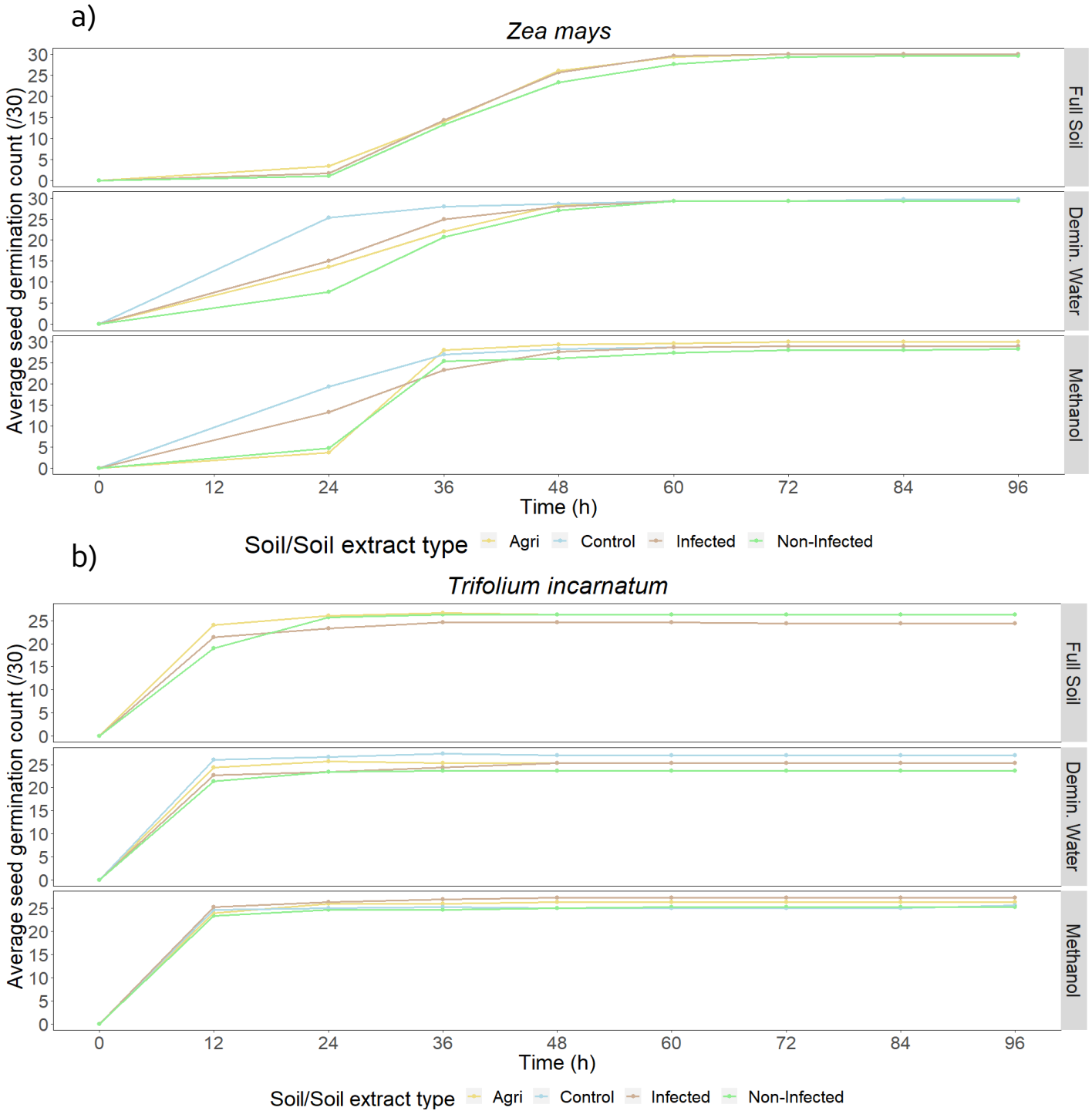


Figure 12: kinetic germination curve of Zea mays *(a) and* Trifolium incarnatum *(b) for each exposure type (solvents or full soil) and soil type. Germinated seeds were counted in each dish every 12 hours for 4 days.*

exposure types than any other soil type in the bioassay and none of the differences among soil types within exposure type groups were significant except for the demineralized water extracts for both test species, in which cases radicles exposed to infected and non-infected soil performed better than the control and those exposed to field soil extract.

Exposure type, or whether the seeds were exposed to complete soil ("full soil"), water extracts or methanol extracts of respective soils, was highly significant in affecting radicle elongation of both test species. This was caused by increased overall effects of methanol extracts on *Z. mays* radicles (Figure 11a) and the contrasting effects of full soils and water soil extracts on radicle length of *T. incarnatum*, which showed tendencies that respectively decreased and increased radicle length (Figure 11b).

Figure 12 shows the kinetics of germination of both test species for the soil and soil extracts bioassay. Consistently with the statistical analysis (Table 3), germination of both *Z. mays* and *T. incarnatum* wasn't affected differently by any of the soil or exposure types except in the case of exposure type on germination of *Zea mays*. We can note the clear effect of full soils in modifying germination kinetics of *Z. mays*, which might explain the weak effect of exposure type on germination rate in the statistical analysis. Furthermore, still for *Zea mays*, water and methanol extract treatments delayed germination even though this effect was most important for non-infected soil treatments. Such a delaying effect wasn't observed in *Trifolium incarnatum* bioassays. In addition, final germination count of *T. incarnatum* was systematically lower in all treatments compared to this of *Z. mays*.

3.2 Greenhouse growth experiments

Substrate treatment was the only significant factor explaining differences in *Trifolium incarnatum* final dry weight whether it grew with or without Japanese knotweed (Table 4). No effect on final *T. incarnatum* dry weight was found for *T. incarnatum* dentity treatment in either growth experiment 1 or 2. None of the substrate treatment groups presented significant differences between the three clover densities (Table 4, Figure 13 & 15). Only results concerning *T. incarnatum* are displayed here, see Annex R1 for data regarding *Reynoutria japonica*.

3.2.1 Growth experiment 1

As mentioned above, substrate treatment was the only significant effect observable in growth experiment one, with Perlite/Vermiculite mix watered with deficient Hoagland solution as the treatment that performed the worse and Perlite/Vermiculite watered with complete Hoagland

Table 4: Statistical data of biometric measurements of Trifolium incarnatum *from both growth experiments. Anova tests were performed on Linear Mixed Models using* T. incarnatum *dry weight as a response variable and the Bloc number as a random factor. When not matching the application conditions, the former was log-transformed as indicated. Values in bold are statistically significant (p-value < 0.05). The following significance codes are applied: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

Growth experiment 1			
Response variable	Explanatory variables	Df	p-value
Log10(*Trifolium incarnatum* (neighbor) individual dry weight(g))	Substrate	2	**1.112e-07*** **
	T. incarnatum density	2	0.3925
	Substrate : *T. incarnatum* density	4	0.6618
Growth experiment 2			
Response variable	Explanatory variables	Df	p-value
Log10(*Trifolium incarnatum* individual dry weight(g))	Substrate	3	**3.541e-08*** **
	T. incarnatum density	2	0.4986
	Substrate : *T. incarnatum* density	6	0.6893

solution as the one that performed best. Natural soil was found to have intermediate final dry weights and this treatment along with the Nutritive solution treatment presented the most dispersion in the data, suggesting a heterogeneity level exacerbated by the resources available for development. None of the substrate groups presented the expected "down-up-down" dry weight per density pattern typical of density-dependent phytotoxicity, with even a clear tendency to show an opposite pattern (e.g. "up-down-up" and Natural soil at density 4 tending

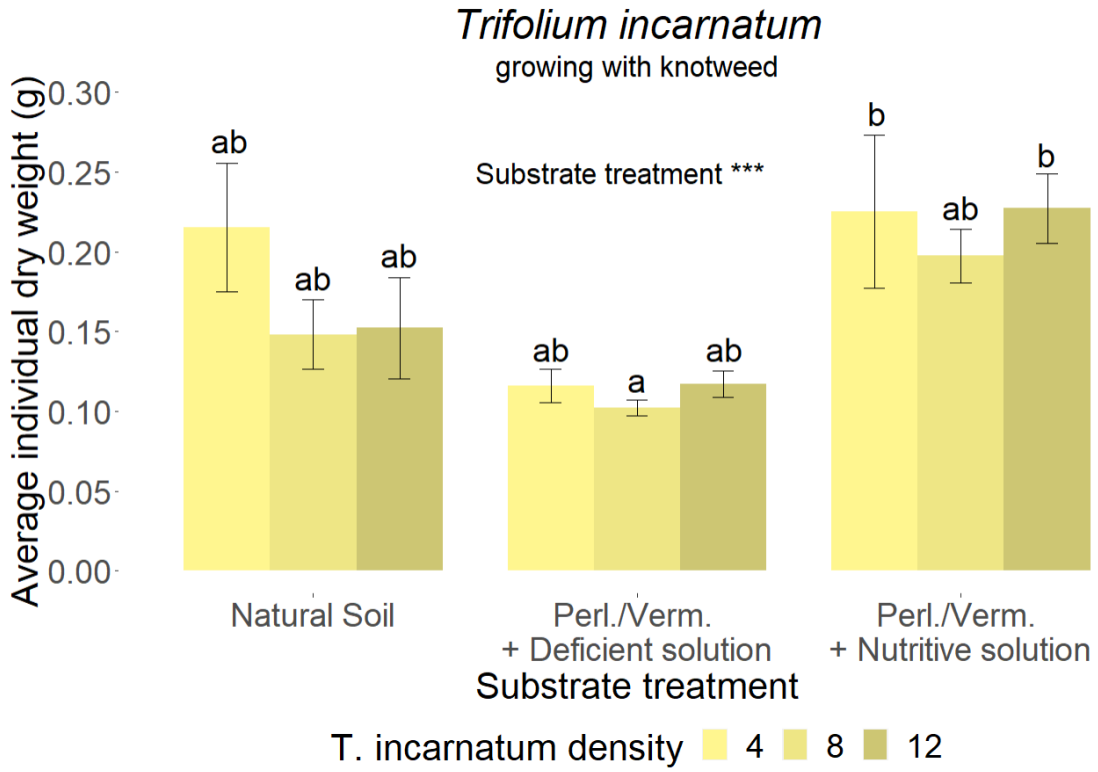


Figure 13: bar plot of Trifolium incarnatum *dry weight by substrate treatment and clover density when growing with Japanese knotweed (Growth experiment 1). Clover and knotweed were left growing for six weeks before harvest. "Natural Soil" refers to soil collected on study site but that wasn't exposed to* R. japonica *rhizomes. Groups not sharing a letter are statistically different. Significance of associated Linear Mixed Model is reminded above the graphs. The following significance codes are applied: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

to have a dry weight higher than the other densities of the group) and no significance among densities whatsoever.

Correlated variables of the experiment are depicted in Figure 14. Starting from the bottom of the figure, fresh and dry total *Reynoutria japonica* weights were positively correlated among themselves and with all other *R. japonica* weight measures of the experiment. Dry aboveground/belowground ratio was correlated with total fresh weight of *R. japonica*. Dry and fresh *R. japonica* aboveground/belowground ratios were positively correlated among themselves. Those same ratios were positively correlated to aboveground *R. japonica* weights and overall negatively with belowground *R. japonica* weights. Latter correlations were only significant in the case of dry belowground weight against fresh aboveground/belowground ratio. All above- and belowground *R. japonica* weights were correlated between themselves.

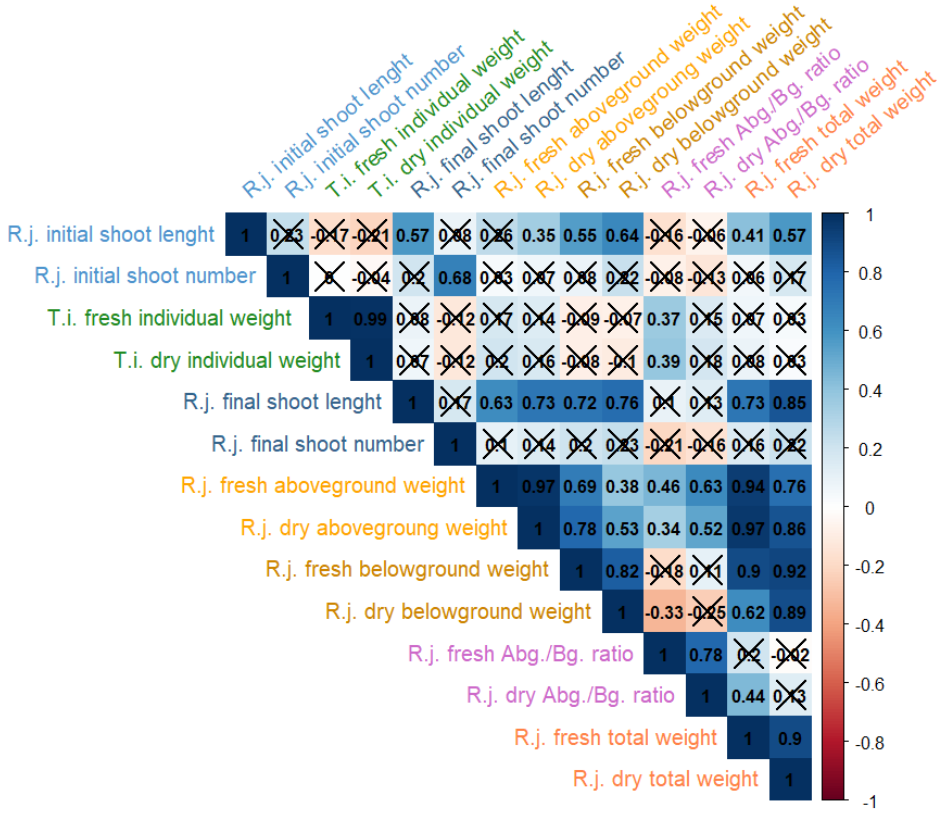


Figure 14: Correlation plot of all continuous variables of Growth experiment 1. Correlation coefficients are given along with matching color which follows the scale color code on the right of the figure. Pearson correlation test was performed for each variable pair. Crosses represent non-significant correlations. R.j. = Reynoutria japonica; *T.i. =* Trifolium incarnatum; *Abg. = Aboveground; Bg. = Belowground.*

R. japonica final shoot number was only significantly correlated to the corresponding initial shoot number and shoot number variables only had this positive correlation as significant. In addition to being positively linked to each other, *R. japonica* initial and final shoot length were significantly correlated with all *R. japonica* weight measurements of the experiment excluding aboveground/belowground ratios and except for *R. japonica* fresh aboveground weight with initial shoot length.

Finally, *Trifolium incarnatum* dry and fresh weights didn't present any significant link to any variables other than *R. japonica* fresh aboveground/belowground ratio, to which they were positively correlated. Interestingly, *T. incarnatum* weights weren't significantly anticorrelated to *R. japonica* initial shoot number and length, reinforcing experimental design's pertinence.

3.2.2 Growth experiment 2

When growing without knotweed, as for growth experiment one, the only impacting factor on *Trifolium incarnatum*'s dry final weight was substrate treatment. In this experiment, as seen in figure 15, the two treatments performing best were Perlite/Vermiculite with full Hoagland solution and Infected soil, the latter consisting of soil taken at study site in which *R. japonica* was found growing. In this experiment again, Perlite/Vermiculite watered with 5-times diluted Hoagland solution presented the lowest dry weights. None of the *T. incarnatum* densities within each substrate treatment groups were significantly differing from one another and no "down-up-down" pattern could be observed. Instead, infected soil and Perlite/Vermiculite nutritive treatments presented a tendency to decrease their dry weight with increasing densities.

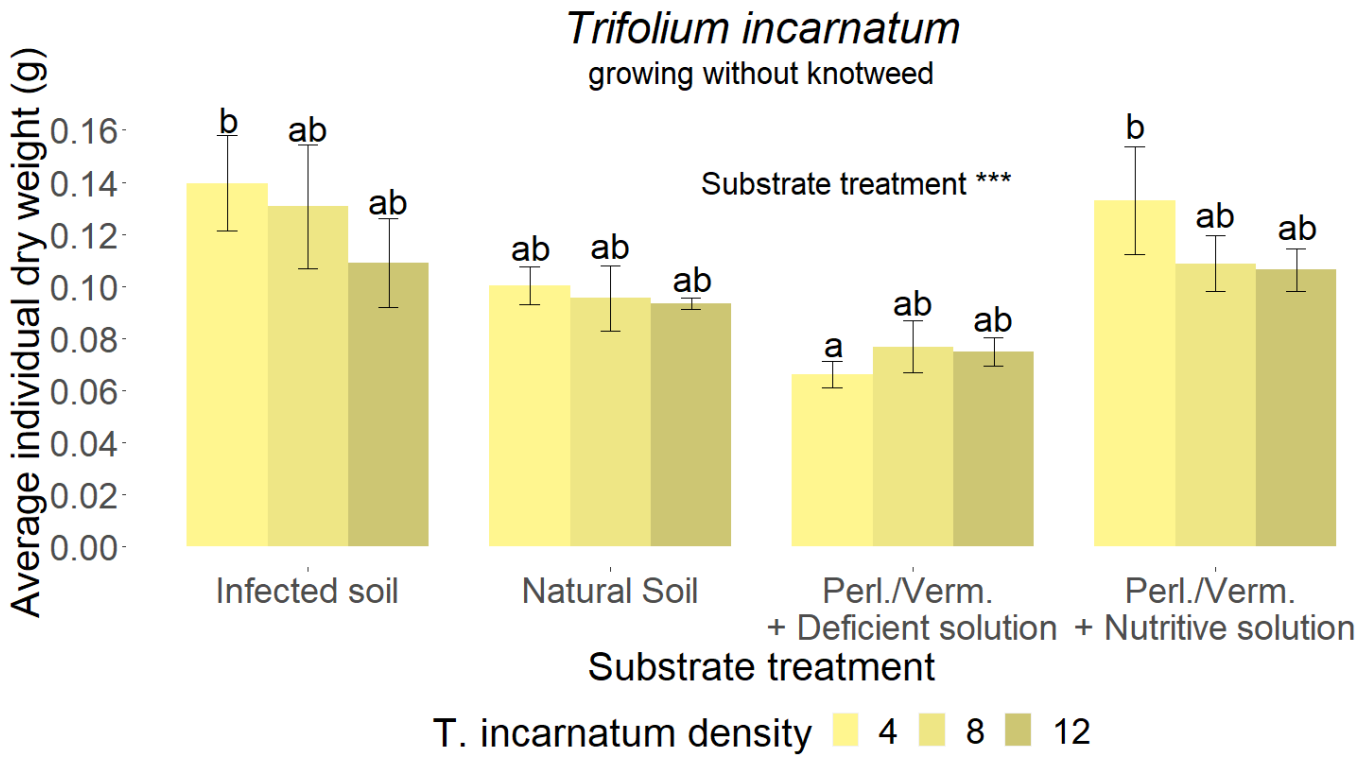


Figure 15: bar plot of Trifolium incarnatum *dry weight by substrate treatment and clover density when growing without knotweed (Growth experiment 2). Clover was left growing for six weeks before harvest. "Natural Soil" and "Infected Soil" refer to soil collected on study site but that wasn't or was exposed to* R. japonica *rhizomes, respectively. Groups not sharing a letter are statistically different. Significance of associated Linear Mixed Model is reminded above the graphs. The following significance codes are applied: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

3.2.3 Mineral content dosage

Mineral content analyses were conducted on *Trifolium incarnatum* tissues of both growth experiments. Statistical analyses of those are found in Table 5. Each experiment data was first analyzed in separate sets of Linear Mixed Models and a third set of LMM was created to investigate the overall effect of knotweed presence on mineral content of crimson clover using the number of experiment (Growth experiment 1 or 2) as a third factor.

In the two first sets of results, it appears clearly that the factor for which groups present significant differences is the substrate treatment. This was true in both growth experiment and across all mineral elements except iron in the first growth experiment (p-value = 0.09101). Details of the mean values for clover tissue mineral content in both experiments are given in Table 6.

Table 5: Statistical data of Trifolium incarnatum *tissue mineral content from both growth experiments. Anova tests were performed on Linear Mixed Models using the corresponding mineral element (Ca,Fe,K,Mg and Zc) as response variables and the replicate number as a random factor. The two first sets of tests focus on each growth experiment, while the third one aims at comparing both experiments. When not matching the application conditions, response variables were log-transformed as indicated. Values in bold are statistically significant (p-value < 0.05). The following significance codes are applied: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

***Trifolium incarnatum* tissue mineral content - Growth experiment 1**			
Response variable	Explanatory variables	Df	p-value
Log_{10}(Calcium (Ca) content ($mg.g^{-1}$))	Substrate	2	**<2e-16*** **
	T. incarnatum density	2	0.2012
	Substrate : *T. incarnatum* density	4	0.8239
Log_{10}(Iron (Fe) content ($mg.g^{-1}$))	Substrate	2	0.1866
	T. incarnatum density	2	0.1274
	Substrate : *T. incarnatum* density	4	0.1776
Potassium (K) content ($mg.g^{-1}$)	Substrate	2	**<2e-16*** **
	T. incarnatum density	2	0.2089
	Substrate : *T. incarnatum* density	4	0.4350
Log_{10}(Magnesium (Mg) content ($mg.g^{-1}$))	Substrate	2	**<2e-16*** **
	T. incarnatum density	2	0.6727
	Substrate : *T. incarnatum* density	4	0.4004
Zinc (Zc) content ($mg.g^{-1}$)	Substrate	2	**0.0003643*** **
	T. incarnatum density	2	0.1483741
	Substrate : *T. incarnatum* density	4	**0.0120167***
***Trifolium incarnatum* tissue mineral content - Growth experiment 2**			
Response variable	Explanatory variables	Df	p-value
Log_{10}(Calcium (Ca) content ($mg.g^{-1}$))	Substrate	3	**<2e-16*** **
	T. incarnatum density	2	0.91550
	Substrate : *T. incarnatum* density	6	**0.04244***
Log_{10}(Iron (Fe) content ($mg.g^{-1}$))	Substrate	3	**0.0001504*** **
	T. incarnatum density	2	0.9929213
	Substrate : *T. incarnatum* density	6	0.9441109

Potassium (K) content ($mg.g^{-1}$)	Substrate	3	**<2e-16*** **
	T. incarnatum density	2	0.6724
	Substrate : *T. incarnatum* density	6	0.5338
Log_{10}(Magnesium (Mg) content ($mg.g^{-1}$))	Substrate	3	**<2e-16*** **
	T. incarnatum density	2	0.9948
	Substrate : *T. incarnatum* density	6	0.4248
Zinc (Zc) content ($mg.g^{-1}$)	Substrate	3	**<2e-16*** **
	T. incarnatum density	2	0.5639
	Substrate : *T. incarnatum* density	6	0.5230
***Trifolium incarnatum* tissue mineral content – Comparison of both growth experiments**			
Response variable	Explanatory variables	Df	p-value
Log10(Calcium (Ca) content ($mg.g^{-1}$))	Substrate	3	**<2e-16*** **
	T. incarnatum density	2	0.29709
	Presence of knotweed	1	**0.01334***
	Substrate : *T. incarnatum* density	6	0.40717
	Substrate : Presence of knotweed	2	0.55537
	T. incarnatum density : Presence of knotweed	2	0.56724
	Substrate : *T. incarnatum* density : Presence of knotweed	4	0.22576
Log10(Iron (Fe) content ($mg.g^{-1}$))	Substrate	3	**1.576e-05*** **
	T. incarnatum density	2	0.5485
	Presence of knotweed	1	**1.378e-05*** **
	Substrate : *T. incarnatum* density	6	0.7263
	Substrate : Presence of knotweed	2	0.2998
	T. incarnatum density : Presence of knotweed	2	0.3264
	Substrate : *T. incarnatum* density : Presence of knotweed	4	0.5647
Potassium (K) content ($mg.g^{-1}$)	Substrate	3	**< 2e-16*** **
	T. incarnatum density	2	0.92846
	Presence of knotweed	1	0.05911.
	Substrate : *T. incarnatum* density	6	0.20423
	Substrate : Presence of knotweed	2	0.12824
	T. incarnatum density : Presence of knotweed	2	0.22895
	Substrate : *T. incarnatum* density : Presence of knotweed	4	0.98358
Log10(Magnesium (Mg) content ($mg.g^{-1}$))	Substrate	3	**< 2e-16*** **
	T. incarnatum density	2	0.87302
	Presence of knotweed	1	**0.01286***
	Substrate : *T. incarnatum* density	6	0.46428
	Substrate : Presence of knotweed	2	**0.01162***
	T. incarnatum density : Presence of knotweed	2	0.80150
	Substrate : *T. incarnatum* density : Presence of knotweed	4	0.38446
Zinc (Zc) content ($mg.g^{-1}$)	Substrate	3	**< 2e-16*** **
	T. incarnatum density	2	0.40186
	Presence of knotweed	1	**0.01368***
	Substrate : *T. incarnatum* density	6	**0.04359***
	Substrate : Presence of knotweed	2	0.10640
	T. incarnatum density : Presence of knotweed	2	0.15633
	Substrate : *T. incarnatum* density : Presence of knotweed	4	0.32574

In addition to such effects, the interaction between substrate treatment and *T. incarnatum* density was shown to be significant in the case of zinc in the first experiment (p-value= 0.03555) and calcium in the second experiment (p-value = 0.0166).

In figure 16 and 17, distribution of substrate treatment groups in both growth experiments along the five mineral element variables can be observed. Substrate treatment groups were unevenly distributed along those five axes, reflecting the statistical significance of substrate treatment. In both experiments, Perlite/Vermiculite substrate groups (complemented with nutritious or deficient solution) were found in locations of the graph corresponding to a higher content in potassium and magnesium and a lower content in calcium and zinc (and iron for growth experiment 2). As for "site soil" groups (Natural soil and Infected soil), they were located in opposite areas to the other groups, which corresponded to high calcium and zinc content and low magnesium and potassium content (and iron for growth experiment 2). All results are consistent with mean values given in Table 6.

Table 6: Mean Trifolium incarnatum *tissue mineral content values for each substrate treatment of growth experiments 1 and 2. Means are given for all five studied mineral elements: Ca, Fe, K, Mg and Zn. All values are given in mg of mineral element per g plant tissue dry weight.*

	Growth experiment 1			*Growth experiment 2*			
	Natural Soil	Perl./Verm. + Nutritive solution	Perl./Verm. + Deficient solution	Natural Soil	Perl./Verm. + Nutritive solution	Perl./Verm. + Deficient solution	Infected Soil
Calcium ($mg.g^{-1}$)	15.5673355	4.90550162	4.44682191	16.95382	5.428177	4.559537	12.28108
Iron ($mg.g^{-1}$)	0.5121035	0.42515017	0.74921597	1.509842	0.762235	1.150312	3.052728
Potassium ($mg.g^{-1}$)	24.418493	67.7062651	56.1273165	31.47668	68.89355	56.24518	35.10538
Magnesium ($mg.g^{-1}$)	3.25364107	7.04772663	8.67080337	3.00737	8.35286	10.30474	2.615887
Zinc ($mg.g^{-1}$)	0.06083696	0.03374374	0.03531885	0.082585	0.040359	0.03801	0.109808

The third set of Anova tests on LMM combined *T. incarnatum* tissue mineral content data available for the two growth experiments and, in addition to substrate and *T. incarnatum* treatments, used the number of experiment as an indicator of knotweed presence (growth experiment 1 = knotweed present, growth experiment 2 = knotweed absence).

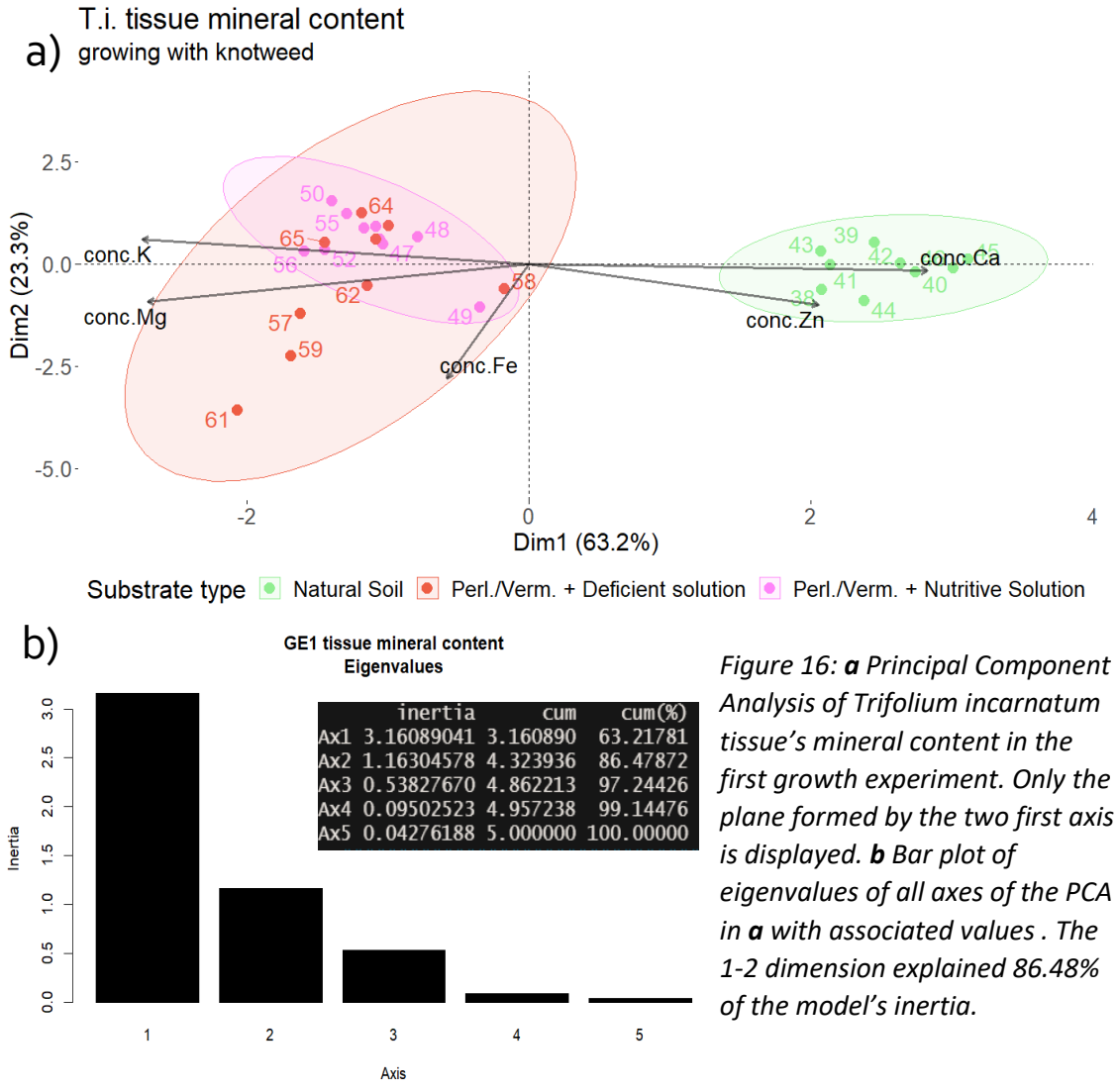
While substrate treatment still had a significant effect in determining mineral content in all element cases, no more significant substrate treatment-*T. incarnatum* density interaction was observed. Presence of knotweed (or the experiment from which measurements came from) significantly shaped results for calcium, iron, magnesium and zinc but not for potassium (p-value = .0.05911). Moreover, interactions between substrate and presence of knotweed as well

as between substrate and *T. incarnatum* density appeared significant in the field case of magnesium and zinc, respectively.

Looking at figure 18 gives us an insight into the meaning of such statistical results. The two groups of substrate treatments as previously described and located on the mineral content gradients can be observed, while distinction between the two groups of knotweed presence (the two distinct experiments) was made especially clear in the "site soils" group with an increased calcium and zinc uptake. The major difference between the two experiments was indeed that plants of the second growth experiment belonging to this group displayed increased iron tissue content, which was imputable to the Infected soil treatment of the second growth experiment alone.

3.2.4 Chlorophyll content dosage

Results regarding chlorophyll a, chlorophyll b and carotenoids content of *Trifolium incarnatum* were somewhat contrasted. In table 7, statistical data for three test sets regarding



	inertia	cum	cum(%)
Ax1	3.16089041	3.160890	63.21781
Ax2	1.16304578	4.323936	86.47872
Ax3	0.53827670	4.862213	97.24426
Ax4	0.09502523	4.957238	99.14476
Ax5	0.04276188	5.000000	100.00000

*Figure 16: **a** Principal Component Analysis of Trifolium incarnatum tissue's mineral content in the first growth experiment. Only the plane formed by the two first axis is displayed. **b** Bar plot of eigenvalues of all axes of the PCA in **a** with associated values . The 1-2 dimension explained 86.48% of the model's inertia.*

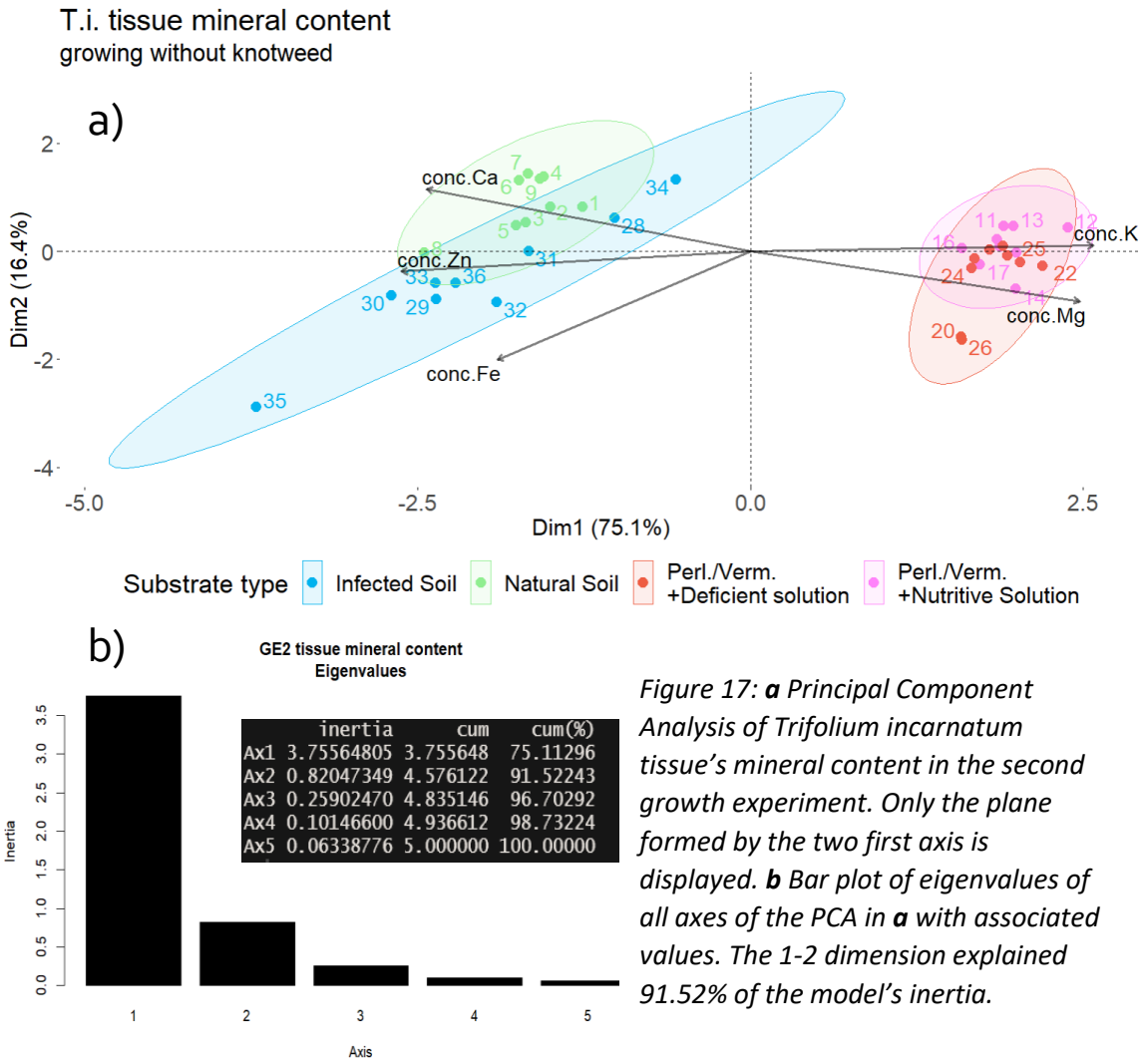


*Figure 17: **a** Principal Component Analysis of Trifolium incarnatum tissue's mineral content in the second growth experiment. Only the plane formed by the two first axis is displayed. **b** Bar plot of eigenvalues of all axes of the PCA in **a** with associated values. The 1-2 dimension explained 91.52% of the model's inertia.*

chlorophyll and carotenoids content can be found. Based on the same rationale as above, the two first sets of Linear Mixed Models concerned each growth experiment separately, and the third one aimed at investigating results combined for both experiments.

No significant effect of neither substrate, *T. incarnatum* density or their interaction was found for tissue chlorophyll and carotenoids content of the first growth experiment. In the second growth experiment, involving crimson clover growing without knotweed, effect of substrate treatment on the three response variables used was found.

Figure 20 gives us an insight into the direction of such effects. In all three molecule content tested, while no differences or patterns in substrate groups is observed for bars associated with growth experiment 1, Natural soil treatment of growth experiment 2 performed best compared to the other treatments of the same experiment.

None of the differences observed between clover density groups were statistically significant. We can however note here the tendency of chlorophyll a, b and carotenoids content to increase

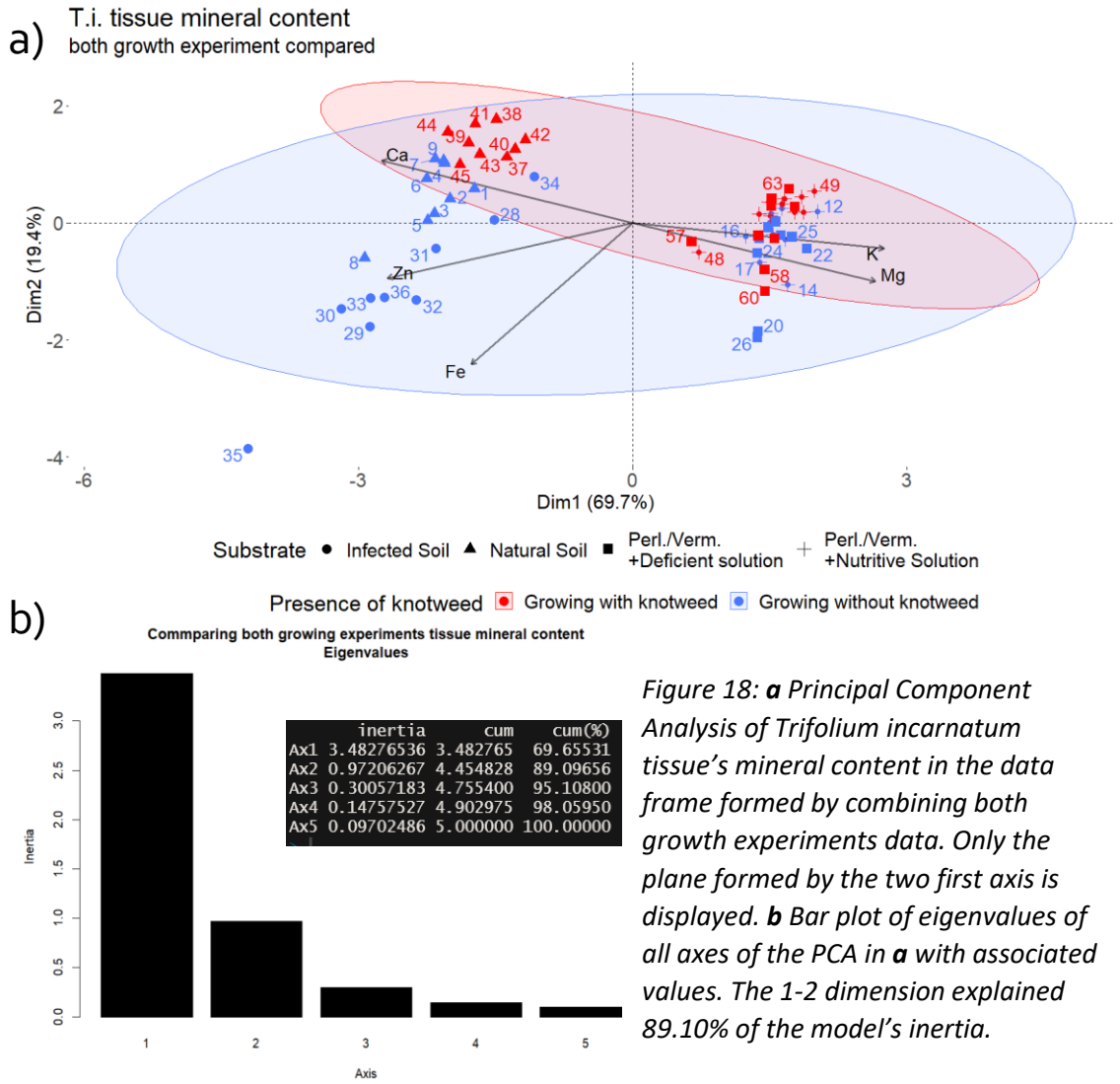


*Figure 18: **a** Principal Component Analysis of Trifolium incarnatum tissue's mineral content in the data frame formed by combining both growth experiments data. Only the plane formed by the two first axis is displayed. **b** Bar plot of eigenvalues of all axes of the PCA in **a** with associated values. The 1-2 dimension explained 89.10% of the model's inertia.*

with density in the Perlite/Vermiculite + diluted Hoagland solution treatment of growth experiment 2, as well as the "down-up-down" shaped pattern of molecule content with clover density in the Infected soil treatment on the same experiment and for all response variables used (Fig. 20).

The third set of LMM performed on combined experiments data revealed systematic effects of substrate, presence of knotweed and interactions of those two factors on each of the three response variables. Chlorophylls and carotenoids content were significantly higher in *T. incarnatum* plants of growth experiment 2. As for the interaction, the most striking observable effect occurred in Natural soil treatments of all three models plotted in figure 20, with contents of growth experiment 2 clearly being higher than those of the first growth experiment.

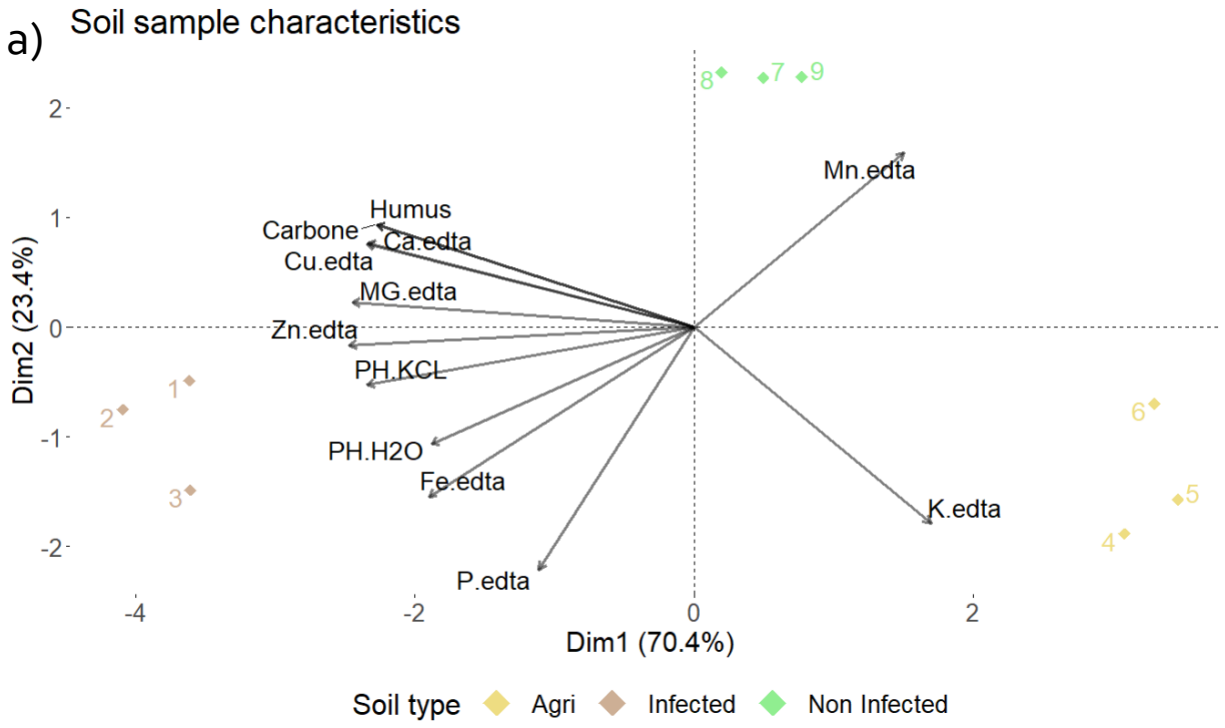
Table 7: Statistical data of Trifolium incarnatum *chlorophyll and carotenoids tissue content from both growth experiments. Anova tests were performed on Linear Mixed Models using the corresponding molecule content (Chlorophyll a, Chlorophyll b and Carotenoids) as response variables and the replicate number as a random factor. The two first sets of tests focus on each growth experiment, while the third one aims at comparing both experiments. When not matching the application conditions, response variables were log-transformed as indicated. Values in bold are statistically significant (p-value < 0.05). The following significance codes are applied: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

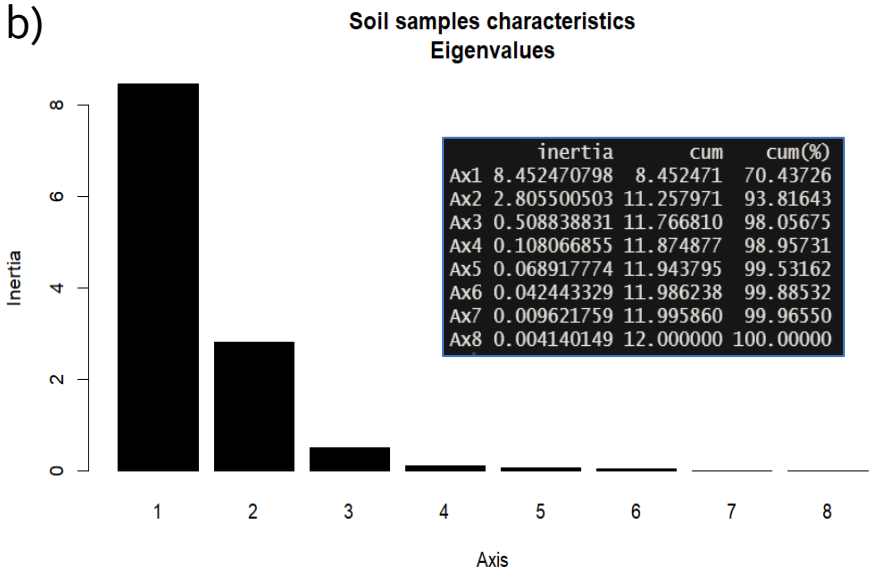
***Trifolium incarnatum* tissue chlorophylls and carotenoids content - Growth experiment 1**			
Response variable	Explanatory variables	Df	p-value
Chlorophyll a content ($mg.L^{-1}$)	Substrate	2	0.3812
	T. incarnatum density	2	0.7933
	Substrate : *T. incarnatum* density	4	0.7453
Chlorophyll b content ($mg.L^{-1}$)	Substrate	2	0.3953
	T. incarnatum density	2	0.8851
	Substrate : *T. incarnatum* density	4	0.7102
Carotenoids content ($mg.L^{-1}$)	Substrate	2	0.3478
	T. incarnatum density	2	0.6966
	Substrate : *T. incarnatum* density	4	0.6829
***Trifolium incarnatum* tissue chlorophylls and carotenoids content - Growth experiment 2**			
Response variable	Explanatory variables	Df	p-value
Chlorophyll a content ($mg.L^{-1}$)	Substrate	3	**0.0002341*** **
	T. incarnatum density	2	0.9595229
	Substrate : *T. incarnatum* density	6	0.2622443
Chlorophyll b content ($mg.L^{-1}$)	Substrate	3	**8.458e-05*** **
	T. incarnatum density	2	0.8511
	Substrate : *T. incarnatum* density	6	0.3227
Carotenoids content ($mg.L^{-1}$)	Substrate	3	**0.000252*** **
	T. incarnatum density	2	0.696427
	Substrate : *T. incarnatum* density	6	0.314756
***Trifolium incarnatum* tissue chlorophylls and carotenoids content – comparing both growth experiments**			
Response variable	Explanatory variables	Df	p-value
Chlorophyll a content ($mg.L^{-1}$)	Substrate	3	**0.002565** **
	T. incarnatum density	2	0.883989
	Presence of knotweed	1	**1.934e-08*** **
	Substrate : *T. incarnatum* density	6	0.315018
	Substrate : Presence of knotweed	2	**0.016198***
	T. incarnatum density : Presence of knotweed	2	0.938938
	Substrate : *T. incarnatum* density : Presence of knotweed	4	0.568164
Chlorophyll b content ($mg.L^{-1}$)	Substrate	3	**0.001419** **
	T. incarnatum density	2	0.937526
	Presence of knotweed	1	**1.77e-08*** **
	Substrate : *T. incarnatum* density	6	0.287109
	Substrate : Presence of knotweed	2	**0.007195** **
	T. incarnatum density : Presence of knotweed	2	0.977616
	Substrate : *T. incarnatum* density : Presence of knotweed	4	0.701343

Carotenoids content ($mg.L^{-1}$)	Substrate	3	**0.007130****
	T. incarnatum density	2	0.555825
	Presence of knotweed	1	**4.491e-08*****
	Substrate : *T. incarnatum* density	6	0.294961
	Substrate : Presence of knotweed	2	**0.005776****
	T. incarnatum density : Presence of knotweed	2	0.926193
	Substrate : *T. incarnatum* density : Presence of knotweed	4	0.674941

3.3 Soil samples analysis

Soil samples from the study sites were characterized following 12 abiotic variables as listed in table 8. A third soil type, field originating soil (Agri), was taken as a control.





	inertia	cum	cum(%)
Ax1	8.452470798	8.452471	70.43726
Ax2	2.805500503	11.257971	93.81643
Ax3	0.508838831	11.766810	98.05675
Ax4	0.108066855	11.874877	98.95731
Ax5	0.068917774	11.943795	99.53162
Ax6	0.042443329	11.986238	99.88532
Ax7	0.009621759	11.995860	99.96550
Ax8	0.004140149	12.000000	100.00000

*Figure 19: **a** Principal Component Analysis of soil sample abiotic characteristics following 12 response variables. Only the plane formed by the two first axis is displayed. **b** Bar plot of eigenvalues of all axes of the PCA in **a** with associated values. The 1-2 dimension explained 93.82% of the model's inertia.*

Values of pH, carbon and humus content, as well as mineral content in potassium, phosphorus, magnesium, calcium, copper, zinc, manganese and iron were all shown to be affected by the soil sample type (p-values ranging between 6.656e-08 and 0.01445).

*Table 8: Statistical table of soil samples abiotic characteristics. Anova tests were performed on simple Linear Models using each 12 abiotic parameters as response variables. Given the low number of observations and after visual and statistical testing, all response variables were considered matching the application conditions. Values in bold are statistically significant (p-value < 0.05). The following significance codes are applied: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

Soil characteristics			
Explanatory variable	*Response variables*	*Df*	*p-value*
Soil type (Agri, Infected, Non-Infected)	PH.H20	2	**0.01445***
	PH.KCL	2	**0.0005739*****
	Carbon	2	**0.0002386*****
	Humus	2	**0.0002504*****
	K	2	**3.393e-05*****
	P	2	**2.634e-06*****
	Mg	2	**2.47e-05*****
	Ca	2	**4.321e-05*****
	Cu	2	**6.656e-08*****
	Zn	2	**2.385e-07*****
	Mn	2	**0.03956***
	Fe	2	**1.636e-07*****

As represented in figure 19, this meant that all three soil types were fundamentally different in such variable's characterization, including infected and non-infected soil with each other. Both Non infected and Infected soil types were characterized by a relatively high humus, carbon, calcium and copper content compared to Agri soil, which contained higher levels of potassium. Finally, Infected soil differed from the Non infected in pH values (Infected soil presenting higher values), and magnesium, zinc, iron and phosphorous content.

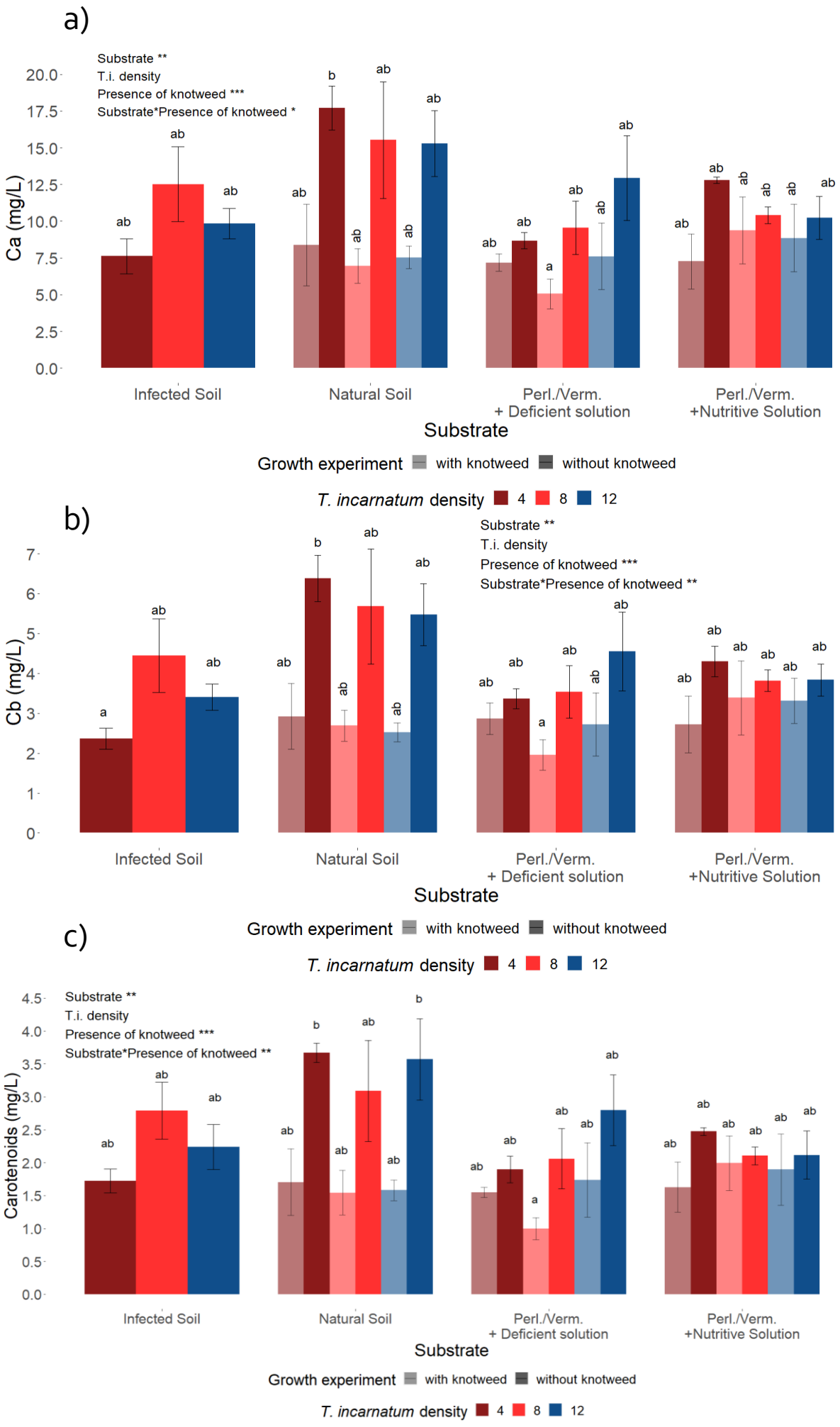


Figure 20: Bar plot of Chlorophyll a (a), Chlorophyll b (b), and Carotenoids (c) content in Trifolium incarnatum *tissue from growth experiments 1 and 2 plotted by substrate and clover density. Each of the three graphs represents data of both experiments. Treatments not sharing a letter are statistically different. Significance of associated Linear Mixed Model is reminded above the graphs. The following significance codes are applied: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

4. Discussion and conclusion

4.1 Soil samples analysis

Analysis of soils used in this study revealed that all three soil types (agri, non-infected, infected) were significantly different from one another (Fig. 19). Statistical differences between soil types were found in all abiotic variables and PCA analysis showed that these differences were well-defined for each soil type, testifying for their stability despite the low number of replicates used (Table 8, Fig. 19). Contradictory to our hypothesis, infected and non-infected soils presented clearly distinct characteristics. Infected soil was slightly more alkaline and richer in Phosphorous, Iron and Zinc than non-infected soil. These differences might be explained by the presence of the Dyle riverbank and its alluvium closer to the infected sampling site than to the non-infected one. Otherwise, heterogeneity of study site soils could be due to soil displacements occurring during likely public landscaping works on the site or because of its habitation history. Japanese knotweed is known to be a niche constructor because of its ability to modify nitrogen cycling in favor of its retention in ecosystems and to increase C and litter content of soil (Dassonville et *al.*, 2011, Bardon et *al.*, 2014). While not providing an explanation for other abiotic parameters, such characteristics of soils invaded by *R. japonica* might account for the slightly higher humus and C content in infected site (Tamura & Tharayil, 2014).

Falling in line with what we hypothesized, both soils from study site had relatively high levels of carbonic content and organic matter in the form of humus when compared with soil from a field (Fig. 19). This is likely due to the differences in land use between the two soil categories, one being extensively used for traditional agriculture and the other being an unexploited wild land in which organic matter accumulated for years.

4.2 Bioassays

There is a rather wide literature covering phytotoxic effects of knotweeds plant material extracts (Šoln et *al.*, 2021, Tucker Serniak, 2016; Vrchotová & Šerá, 2008: Sera, 2012; Moravcová et *al.*, 2011; Novak et *al.*, 2018; Palmeri & Kiviat, 2021; Šoln et *al.*, 2022). However, this set of works is diverse in terms of techniques and test species used, and resulting effects found vary accordingly. To my knowledge, the only study involving soil treatments effects on test species is this of Sera (2012) and consisted of exposing test species to soil mixed with dried knotweed leaves.

4.2.1 *Reynoutria japonica* extracts experiment

No effects of either rhizome and leaves aqueous or methanol extracts were found on germination of both test species except for the interaction between solvent used and plant material on *Trifolium incarnatum* seed germination. This latter effect was due to aqueous rhizome extracts significant inhibition of clover germination (87% of germination) when compared to the control (99% of germination) (data not shown). Although contrasting with our expectations, other authors also found weak or no responses of germination to whole *R. japonica* plant extracts (Novak et *al.*, 2018), aqueous rhizome extracts (Moravcová et *al.*, 2011; Vrchotová & Šerá, 2008) and leaf extracts (Moravcová et *al.*, 2011) exposure (Murrell et *al.*, 2011). However, some authors still report a significant reduction of germination in presence of *R. japonica* aqueous leaves extracts (Vrchotová & Šerá, 2008) or dried leaves mixed with soil (Sera, 2012). While germination kinetic wasn't recorded for this experiment it is very likely that germination was delayed by treatments rather than inhibited (Šoln et *al.*, 2021, Tucker Serniak, 2016).

Contrasting with results for germination and confirming our hypothesis, radicle growth for both *Trifolium incarnatum* and *Zea mays* was significantly affected by all extracts with radicle length decreases up to -77% (methanol rhizome extract on *T. incarnatum* compared to controls) except for aqueous rhizome extracts that didn't reduce maize radicle size. Such impairment of radicle growth is consistent with previous results, which show decrease in radicle length of test species when exposed to aqueous rhizome extracts (Šoln et *al.*, 2021), entire plant aqueous extracts (Novak et *al.*, 2018) or pure phenolic compounds present in *R. japonica* tissues (Tucker Serniak, 2016).

Although one could argue that effects of knotweed tissue extracts on germination and radicle development strongly depends on the test species used, it appeared based on our results and literature that no distinct response patterns could be identified between forbs and monocotyledonous species (Moravcová et *al.*, 2011; Novak et *al.*, 2018). Absence of effect for the rhizome water extract on *Z. mays* radicle length is imputable to the general weaker effect that aqueous extracts exhibited in this experiment. Differences between aqueous and methanol extracts effects are most likely due to the nature of compounds each solvent solubilizes best. *R. japonica* rhizomes and leaves contain phenolic compounds (e.g. resveratrol, emodin, catechin and epicatechin) which are more soluble in organic solvents (methanol, acetone, etc.) than in water although still soluble to some extent in this latter solvent (Šoln et *al.*, 2021; Sera, 2012). These compounds would be responsible for knotweed phytotoxicity (Šoln et *al.*, 2021). This is

supported by the fact that Tucker Serniak (2016) found effects similar to these of the present work using methanol solutions of some of the above-mentioned phenolic compounds.

Such results might attest for the inhibition target of *R. japonica* bioactive compounds to be on early seedling development stages rather than on germination itself. We suggest that given their transportable nature, phytotoxic chemicals from Japanese knotweed need a somewhat developed root system to affect neighboring species (Tucker Serniak, 2016).

4.2.2 Soil and soil extracts experiment

Assessing phytotoxic impacts of soil or soil extracts is crucial in characterizing allelopathy because of how tied this concept is to soil mediated processes (Inderjit & Weiner, 2001; Ens et *al.*, 2009). Despite this, only one study investigated the impact of *Reynoutria xbohemica* soils on test species germination to our knowledge (Parepa et *al*., 2012). Even though this latter study uses bohemian knotweed, its results will be compared with our own because of the relatedness of this species to *R. japonica* and similarities of their respective phenolics profile (Šoln et *al.*, 2021; Mereďa et *al*., 2019; Bailey et *al*., 2009).

Except for germination rate of *Zea mays* exposed to methanol extracts (96.9167%) which was slightly lower than with aqueous extracts (98.1667%) and full soil treatment (99.6667%), none of the exposure and soil types had significant impacts on test species seed germination. Once again, those results contrasted with our hypothesis but are consistent with the knotweed extracts experiment and considering that knotweed's phytotoxic chemicals don't affect germination. Parepa et *al*. (2012) observed positive effects of trained soils compared to control, which differed from the non-significant differences we obtained between infected and non-infected full soil treatments. A clear delay in germination was found for *Z. mays* treated with aqueous and methanol infected soil extracts when comparing to controls, suggesting the presence of phenolic bioactive compounds in those soils (Tucker Serniak, 2016). However, stronger delay was observed for non-infected soil extracts and no such delay were found in *Trifolium incarnatum* seed germination. This would imply that delay in germination of *Z. mays* is caused by other compounds present in soil of the study site that specifically affect maize seeds.

Radicle elongation was effectively affected by the type of exposure, the type of soil and their interaction (*Zea mays* only) but orientation of these effects were not as straightforward as for knotweed tissue extract experiment. Deleterious effects of agricultural soil on radicle length of both species (across all soil treatments) might be explained by presence in low amounts of herbicides in this soil (Wang et *al.*, 2008). Full soil treatment and methanol extracts showed the

most deleterious effects among exposure types on *Trifolium incarnatum* and *Zea mays*, respectively. These could be explained by the small seed size of clover which makes them more susceptible to rot and presence in study site soils of deleterious molecules specifically acting on *Z. mays* radicle length and better extracted with methanol.

Both infected and non-infected soils tended to improve radicle length, which resembles results from Parepa et *al*. (2012) although no significant differences between non-infected and infected treatments were found and similar patterns of the two were even identified. We suggest this to be caused by the rich mineral nutrients content presented by both soils collected on study site.

Overall, we found clear phytotoxic effects of tissue extracts on radicle elongation, but no phytotoxic effect of soil colonized by *R. japonica* could be identified in the bioassays, meaning that we can conclude to phytotoxicity but not allelopathy of the Japanese knotweed on tested species following Ens et *al.* (2009) experimental definitions. This is in contradiction with our hypothesis and with literature reliably concluding to allelopathy of Japanese knotweed (Dommanget et *al.*, 2014) but falls in line with Mincheva et *al.* (2016) findings.

4.3 Growth experiments

There are several studies dealing with potting experiments to assess *Reynoutria* spp. allelopathy (Dommanget et *al.*, 2014; Mincheva et *al.*, 2016; Murrell et *al.*, 2011; Parepa & Bossdorf, 2016; Parepa et *al.*, 2012). However, like for bioassays, those studies employed a variety of methods which lead to difficult comparison of the results. Our experimental design wasn't exactly matching any of the studies. Only two of them tested effects of Japanese knotweed (Dommanget et *al.*, 2014; Mincheva et *al.*, 2016) while the rest used *R. xbohemica* (Murrell et *al.*, 2011; Parepa & Bossdorf, 2016; Parepa et *al.*, 2012). Two used artificial substrates which are known to favor knotweed development (Murrell et *al.*, 2011; Dommanget et *al.*, 2014), two used natural soils (Mincheva et *al.*, 2016; Parepa et *al.*, 2012) and one tested for a gradient of soils (Parepa & Bossdorf, 2016). Three out of the five used Activated Carbon (AC) to assess allelopathy while this was not recommended because of its misleading effects on plant growth (Murrell et *al.*, 2011; Parepa & Bossdorf, 2016; Parepa et *al.*, 2012), and finally only one study could readily tell apart allelopathic effects from resource competition (Dommanget et *al.*, 2014).

4.3.1 *Trifolium incarnatum* biomass measurements

In both growth experiment one and two, the only effect on *Trifolium incarnatum* aboveground biomass was this of the substrate. Infected soil and natural soil in both experiments allowed for

better growth of clover than deficient treatment because of their identified mineral richness, of which the effect was comparable to this of a Hoagland solution. In growth experiment two, we found no statistical difference between clover biomass of infected and non-infected treatments. This falls in line with the overall non-significant effect of invaded or trained soils previously observed (Mincheva et *al.*, 2016; Parepa et *al.*, 2012).

Increased biomass of *T. incarnatum* could have been observed at high or medium densities and in presence of indirect (infected soil) or direct (presence of knotweed) toxin release due to the density-dependent principle of phytotoxicity which was theorized (Weidenhamer, 2006), modelled (San Emeterio et *al.*, 2007), and demonstrated experimentally (Thijs et *al.*, 1994; Canals et *al.*, 2005). Absence of "down-up-down" patterns of densities inside each substrate treatment of experiments one and in infected soil treatment of experiment two, along with the absence of infected soil effect when compared to non-infected soils of this latter experiment accounts for the absence of reflected phytotoxicity.

It appears very unlikely that allelopathy is completely absent in our system because of previous reports providing strong arguments for it (Dommanget et *al.*, 2014; Murrell et *al.*, 2011). A piece of evidence for allelopathy in our results is that *Trifolium incarnatum* dry and fresh weights were positively correlated to *R. japonica* fresh aboveground/belowground ratio (Fig. 14), meaning that clover performed better when knotweed allocated less resources to its rhizomes and roots. However, it is possible for other processes to dominate allelopathy in a setting close to field conditions (Parepa & Bossdorf, 2016; Mincheva et *al.*, 2016; Parepa et *al.*, 2012).

Clover biomass tended to decrease with density, and generally some patterns corresponding to resource competition were identified in both growth experiments (Fig. 13 & 15). This was particularly visible for lower clover densities. We thus suggest that direct competition between *Rj* and clovers and among clovers could be responsible for most of the interactions occurring in our experiments, in such a way that allelopathic patterns would not be visible. This logic is supported by findings of Mincheva et *al.*, (2016).

Soil mediated processes might also have prevented any phytotoxic effect of *R. japonica* material. Those include clay and mineral adsorption of allelochemicals, especially in denser natural soils, and responses of soil biota (mycorrhizal and bacterial) (Parepa & Bossdorf, 2016). As pointed out by Stefanowicz et *al.* (2016), knotweed effects on soil microbiota can vary depending on the study site. Previous analysis of invaded soils or extract-exposed non-invaded

soils found high saprophytic fungi abundance due to their C-rich nature and low bacterial abundance (Tamura & Tharayil, 2014; Mincheva et *al*., 2014; Stefanowicz et *al*., 2022), low fungal, microbial, and mycorrhizal fungi abundance because of antibiotic properties of knotweed bioactive compounds and their disruption of N cycling (Kato-Noguchi, 2021; Stefanowicz et *al*., 2022; Stefanowicz et *al*., 2016) or yet no response of either microbial and fungal biomass (Abgrall et *al*., 2018). In addition, soil biota can both increase resistance to invasion through detoxification of putative allelochemicals (Parepa et *al*., 2012) or, influenced by the invader plant species, shift towards community composition facilitating invasion (Parepa et *al*., 2013). While exact soil biota processes at play in our experiment remain very unclear, it appears that bacterial (nodules) and mycorrhizal symbionts of *Trifolium incarnatum* neighbors remained unaffected or even favored and this suggests a resistance mechanism of natural soil biota communities to *Reynoutria japonica* phytotoxicity (Zhang et *al*., 2019).

Overall, clover biomass was higher in our experiment when knotweed was present than when it was absent (data not shown). This was in direct contradiction with negative effects of knotweed presence or leachate exposure previously described (Dommanget et *al.*, 2014; Murrell et *al.*, 2011; Novak et *al.*, 2018; Mincheva et *al.*, 2016) but not with the findings of Viard-Crétat et *al.* (2012).

Increased densities of neighbor plants also meant increased intraspecific interactions, and as pointed out by Viard-Crétat et *al.* (2012), such an increase could modify sensitivity of neighbor plants to allelopathic effects. Viard-Crétat et *al*. (2012) study shows that a case of interspecific competition between two grass species improves resistance to allelopathic effects of one of the two species. Authors argue that such a pattern is due to the counteracting effect of *Agrostis capillaris* on nitrogen immobilization effects of *Festuca paniculata* leachates. This example of facilitation under allelopathic conditions adds a new dimension to the study of allelopathy using common growth experiments. Although once could argue that such facilitation is more likely to occur in interspecific interactions, if kinship facilitation occurs among crimson clovers under allelopathic conditions, it is only possible to account for allelopathy by comparing plants growing with or without a putative allelochemical stress. Indeed, toxin dilution effects in pots with high density of clover exposed to *R. japonica* presence could then be confounded with positive effects of facilitation on clover growth. Following Viard-Crétat et *al*. (2012), clear differences in growth patterns between plants undergoing allelopathic stress or not should nevertheless be observed.

When looking at the growth difference between the two experiments, it is found that clovers grew more biomass when knotweed was present than without (data not shown). This could be due to facilitation processes as pointed out by Viard-Crétat et *al*. (2012) or to heterogenous abiotic growth conditions between the two experiments, the first one starting from the 1st of September to the 15th of October 2021 and the second from the 13th of October to the 26th of November 2021. The first hypothesis would argue for allelopathy in our study system as facilitation would occur only under allelopathic stress.

Ultimately, it is possible that other factors that were not assessed in our experimental design played a role in the absence of phytotoxicity by confounding its effects or preventing their detection. Such factors would include competition for light between clover and knotweeds, with previous studies indicating that it might play a role in knotweed success (Moravcová et *al*., 2011; Siemens & Blossey, 2008). Such competition is likely in our experimental design given the important final shoot length of *R. japonica* that often led to it covering clovers in its own pot and/or overlapping some of the neighboring pots. In addition, we could have missed some effects by not assessing clover belowground biomass which is likely to be sensible to allelopathic effects as this was demonstrated (Dommanget et *al.*, 2014). Furthermore, belowground biomass trends in opposite directions depending on the process involved: decreased belowground biomass when under phytotoxic stress (Dommanget et *al.*, 2014) and increased belowground biomass when in resource competition with other species (San Emeterio et *al.*, 2007). Given the phenolic content of leaves of Japanese knotweed, the controlled fashion by which this pant displaces molecules in its senescent leaves, and the effect that its litter has on soil C and microbial characteristics, it is also suggested that a complementary way through which *R. japonica* does allelopathy is via its leaf litter on a larger time scale than this considered in this work (Sera, 2012, Dassonville et *al.*, 2011; Tamura & Tharayil, 2014).

These findings contrast with our hypothesis in that we could not find additional evidence for *R. japonica* allelopathy through our experimental design, even under nutritive deficiency (no "down-up-down" patterns and no differences between infected and non-infected treatments). However, our initial idea that phytotoxic effects of Japanese knotweed are weak, and that allelopathy is surely not the only process accounting for its ecological dominance was verified.

4.3.2 *Trifolium incarnatum* tissue mineral content

The main effects on plant mineral content were those of the substrate. In both experiments, there was a clear distinction between mineral nutrient uptake of plants growing in substrate watered with nutritious solutions or in soils collected on study site. This was due in part to the

direct nutrient content of the study site soils, of which high calcium and zinc contents and low potassium content translated in the clover tissue content. As therefore expected, values of zinc and calcium were two to three times higher than those of solution treated plants, and potassium content values were approximately two times lower than in those same plants (Fig. 19, Table 6). Iron content was also two to three times higher in plants growing in infected soil compared to other treatments, as it was reported especially abundant in this soil.

However, magnesium tissue content of clovers growing in study site (infected and non-infected) soils were two to three times lower than those of solution treated plants despite identified abundance of this mineral in both study site soils (Fig. 19, Table 6). We suggest such marked differences arise from the difference in nutrient availability between Hoagland solutions and site soils, with calcium, iron, potassium, and zinc considered as being highly available in soils and magnesium poorly available.

Interestingly iron tissue contents of all comparable treatments between growth experiments one and two had different values, with those of experiment two two to three times higher than those of first experiment. This wasn't the case for any other mineral extracted and is likely due to different soil collection times between the two experiments and perhaps to variations in iron content of solutions mistakenly introduced during preparation. All effects tied to the presence of knotweed factor found when comparing both growth experiments for each mineral tissue content are most likely caused by this unexpected increase in iron content between both growth experiments (Fig. 18).

Tissue content of clovers treated with solutions show that plants supposed to be nutrient deficient due to application of a 5 times diluted Hoagland solution did not present different values from those treated with full Hoagland solutions. This might account for the fact that no optimum density pattern due to phytotoxicity could be observed for the deficient solution treatment and contrasts with our hypothesis (Fig. 13 & 15). Absence of mineral tissue content differences between nutritive and deficient solutions is likely to be due to a growth proportional to nutrient availability: deficient treated plants grew less and had less nutrients available than nutritive treated ones which grew more and had more nutrients available, resulting in comparable nutrient tissue concentrations.

Apart from potential allelopathic stress and nutrient deficiency, plants in our study design were not subject to stresses that can cause modification of nutrient uptake (Gomes et *al*., 2011). Allelopathy doesn't have a clear effect on nutrient uptake, as contradictory results exist in

literature (Hussain & Reigosa, 2015; Sánchez-Moreiras et *al*., 2008) and that such effects are very likely to be species-specific. Either it favorizes (Hussain & Reigosa, 2015) or impairs (Sánchez-Moreiras et *al*., 2008) nutrient uptake should have been clearer when looking at deficient treated plant because of the nutrient stress inducible nature of allelopathy (Inderjit & Weiner, 2001; Inderjit & Duke, 2003). However, mineral tissue content between the two solution treatments didn't differ and factors involving *Trifolium incarnatum* densities or their interactions were mainly insignificant, suggesting an absence of allelopathic influence on tissue mineral content overall. This is contrasting with what we hypothesized.

4.3.3 *Trifolium incarnatum* tissue chlorophyll and carotenoid content

Chlorophyll and carotenoid tissue contents were significantly affected by the substrate in growth experiment two only. This was caused by a great increase in photosynthetic pigments in tissues of clovers growing in non-infected soil (natural soil) compared to all other treatments. Yet magnesium and potassium tissue contents of this treatment, which could have an influence on chlorophyll content (Sitko et *al*., 2019), were actually lower than those recorded for solution treatments and did not differ from the infected soil treatment (Fig. 20, Table 6). We propose that this striking effect is due to the different characteristics of soil used as non-infected treatment in growth experiment two, as mentioned above. This observation is also responsible for the effect of the interaction between substrate and presence of knotweed in the common model. No statistical difference was observed between deficient and nutritive solutions, which reflects the tissue mineral content results and contradicts our initial hypothesis.

When comparing both growth experiments, we found higher overall chlorophyll and carotenoid content in clover plants that didn't grow along with knotweed. Plants used in this study were not exposed to other stresses than potential allelopathic stress and nutrient deficiency and chlorophyll content is usually used as a good stress indicator (Gomes et *al*., 2011; Hussain & Reigosa, 2015). It is known that allelochemicals can affect the performance of photosynthesis main processes, which is considered as a major feature of allelopathic effects given the importance of these processes for plants (Hussain & Reigosa, 2011; Huang & Bie, 2010). Once again, however, literature provides contradicting results when it comes to the impact of allelochemicals on chlorophyll a and b content. Hussain & Reigosa (2015) therefore found that application of Artemisinin on adult *Arabidopsis thaliana* plants was causing a decrease in chlorophyll a and b contents, while Huang & Bie (2010) applied cinnamic acid (CA) on cowpea leaves and recorded an absence of effect on the chlorophyll content. Adding to this, a recent study using *Reynoutria japonica* rhizome extracts on radish seedlings also found no effect on

the chlorophyll a, b and carotenoid contents and suggested that this might be due to the shoot not yet relying on the seedling's root system to develop (Šoln et *al.*, 2021).

Therefore, despite the significant effect of knotweed presence, given that we observed no effects of density or subsequent patterns of phytotoxicity, and based on the recent work of Šoln et *al.* (2021), we conclude to non-significant effects of treatments on clover photosynthetic pigments and that Japanese knotweed allelopathic potential couldn't be observed via their measurement. We however acknowledge that our results are inconclusive to some extent and that this latter conclusion is in contradiction with our hypothesis regarding physiological measurements.

4.4 Experimental design improvement

4.3.1 Growth experiments

While neighbor density treatments are deemed sufficient as they were designed to yield sufficient results, changes to other treatments and conditions of greenhouse experiments' design could have led to substantial improvement of results quality.

First, experimental duration could have been increased as putative phytotoxic effects of *Reynoutria japonica* may not have had the time to impact *Trifolium incarnatum* plants. In addition, such effects may have been more visible once clover reached later development stages (Parepa et *al*., 2012) and authors investigating phytotoxic effects of knotweeds in greenhouse experiments designed experiments lasting significantly longer than six weeks (Parepa & Bossdorf, 2016; Mincheva et *al.*, 2016; Dommanget et *al.*, 2014, Murrell et *al.*, 2011).

Another possible change of the design concerns one of its fundamental components: the quantity/size of *R. japonica* rhizomes present in each pot of the growth experiment one. As envisioned by Weidenhamer (2006), rhizome presence is supposed to serve as a putative toxin source acting upon neighbor plants growth through phytotoxicity. The absence of observed phytotoxic patterns in growth experiment one could thus be due to an insufficient toxin quantity released in soil by Japanese knotweed rhizomes. A way to account for this would be to increase rhizome number and/or size in each pot while maintaining such changes constant across all pots of the experiment.

Using both neighbor plant species from the native and invaded range of *Reynoutria japonica* in a similar design would also have helped gaining substantial insights on the applicability of the

Novel Weapons Hypothesis in the case of *Reynoutria japonica* (Hierro et *al.*, 2005). In fact, as highlighted by Hierro et *al.* (2005), comparison between putative allelopathic plant effects on co-occurring species from native and invaded range constitute a robust test of this hypothesis. This is because, if comparison between native and invaded neighbors is plausible (it is preferred that they are as taxonomically related as possible), experiments designed this way allow for a very straightforward interpretation: either invaded and native neighbors both react, react differentially, or both don't react.

Other investigation techniques and designs could also be envisioned. To better understand the physiological responses of neighboring plants, it could be valuable to investigate suspected allelochemicals presence in those plants' tissues using fluorescence spectrophotometry (Tucker Serniak, 2016). Tucker Serniak (2016) effectively found that *R. japonica* bioactive compounds could be taken up by in radish tissues. Activated Carbon (AC) used in allelopathy studies introduces a confounding effect on plant growth, which may be due to involuntary nutrient addition or modification of soil microbial communities, and should thus be carefully considered for new designs (Lau et *al*, 2008; Viard-Crétat et *al.*, 2012, Parepa & Bossdorf, 2016). The donor pot-target pot design used by Dommanget et *al.* (2014) and Viard-Crétat et *al*. (2012) efficiently proves allelopathic potential and was used by the former to demonstrate the allelopathic potential of *Reynoutria japonica*. Without querying the validity of their results in highlighting phytotoxic effects of live plants leachates that were not tied to resource competition, we can however question the ecological relevance of those studies. As incentivized by Inderjit & Callaway (2003), field experiments making used of the great abundance in *R. japonica* potential study sites could also be conducted.

5. Conclusion

While this work didn't pretend fully explaining Japanese knotweed ecological success, which was suggested to be due to numerous different hypotheses (Moravcová et *al.*, 2011; Heděnec et *al.*, 2014; Catford et *al*., 2009), it aimed at replacing the allelopathic potential of this plant in its ecological context. To do so, we used innovative methods based on the theorical and empirical frames of both phytotoxicity and allelopathy concepts and on previous methodological shortcomings identified in similar studies (Weidenhamer, 2006; Ens et *al*., 2009, Thijs et *al*., 1994; Parepa & Bossdorf, 2016; Inderjit & Callaway, 2003; Lau et *al*., 2008).

Bioassays using knotweed tissue and invaded soil extracts highlighted the insensitivity of test species seed germination to bioactive compounds of *Reynoutria japonica*. Radicle length of

target species was however negatively affected by those compounds, and it was suggested that what allows them to excerpt their effect is to be taken up by a functional root system (Tucker Serniak, 2016). We found phytotoxic effects of *R. japonica* tissue extracts but not of invaded soil extracts, which provided evidence for phytotoxicity but not allelopathy of Japanese knotweed following Ens et *al*. (2009) definitions. This was in contradiction with the allelopathic potential of *R. japonica* we had hypothesized.

Greenhouse experiments involving increasing crimson clover densities and natural substrates only identified positive effects of soils from study site on clover biomass due to their rich mineral content. No phytotoxicity related patterns could be observed in either clover aboveground biomass, tissue mineral contents, and tissue chlorophyll and carotenoid contents, which contradicted all our experimental hypotheses relative to those parameters.

It is very likely that *R. japonica* does allelopathy to some extent, since strong arguments in favor of this hypothesis exist (Dommanget et *al.*, 2014; Murrell et *al*., 2011; Parepa & Bossdorf, 2016). Absence of allelopathy related patterns in growth experiments and bioassays may be due to other processes dominating allelopathy when considering an ecologically relevant setting (e.g. resource competition and soil microbiota mediated processes). It is also probable that some parameters providing more insights were not assessed and that facilitation among clovers influenced the observed patterns (Viard-Crétat et *al*., 2012).

As for the question of the role of allelopathy in *Reynoutria japonica* ecological dominance, no studies, including the present one, provided results significant and reliable enough to answer it with certainty yet. Studies making use of conditions close to field ones couldn't find strong evidence for knotweed allelopathy (this work, Parepa et *al*., 2012, Mincheva et *al*., 2016) while other studies provided arguments in its favor but using questionable methods in terms of ecological relevance (Dommanget et *al.*, 2014; Murrell et *al*., 2011; Parepa & Bossdorf, 2016). The experimental design of the present study, while being subject to improvements, didn't find substantial evidence for an allelopathic component of Japanese knotweed ecological behavior. Application of the Novel Weapons Hypothesis in the case of *R. japonica* isn't obvious when looking at our results. Our initial hypothesis stating that allelopathic effects of Japanese knotweed are integrated in a set of distinct processes was verified.

We would also like to highlight here the lack of methodologically relevant studies focusing on knotweed allelopathy. This topic is not enough covered by studies using relevant experimental design, that is, avoiding the use of Activated Carbon, using natural soils over artificial ones,

using invaded soils along with plant tissue extracts in bioassays, or yet properly disentangling allelopathy from resource competition via donor-target pots or target-neighbor density designs (Weidenhamer, 2006; Ens et *al*., 2009, Thijs et *al*., 1994; Parepa & Bossdorf, 2016; Inderjit & Callaway, 2003; Lau et *al*., 2008). In fact, none of the studies considered applied all of the above experimental criteria simultaneously. We incentivize further research to implement such experimental characteristics and the general standardization of knotweed allelopathy investigation.

Kato-Noguchi review (2021) and numerous authors suggest that Japanese knotweed and knotweeds in general do allelopathy and that it partially explains the success of those species in their invaded range. However, proving allelopathy experimentally in an ecological meaningful way remains complicated and studies pointing out to experimental shortcomings of knotweed allelopathy investigation or arguing for the weak ecological relevance of knotweed allelopathic effects remain (Kato-Noguchi, 2021; Mincheva et *al.*, 2016; Parepa & Bossdorf, 2016).

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Annexes

Annex M1

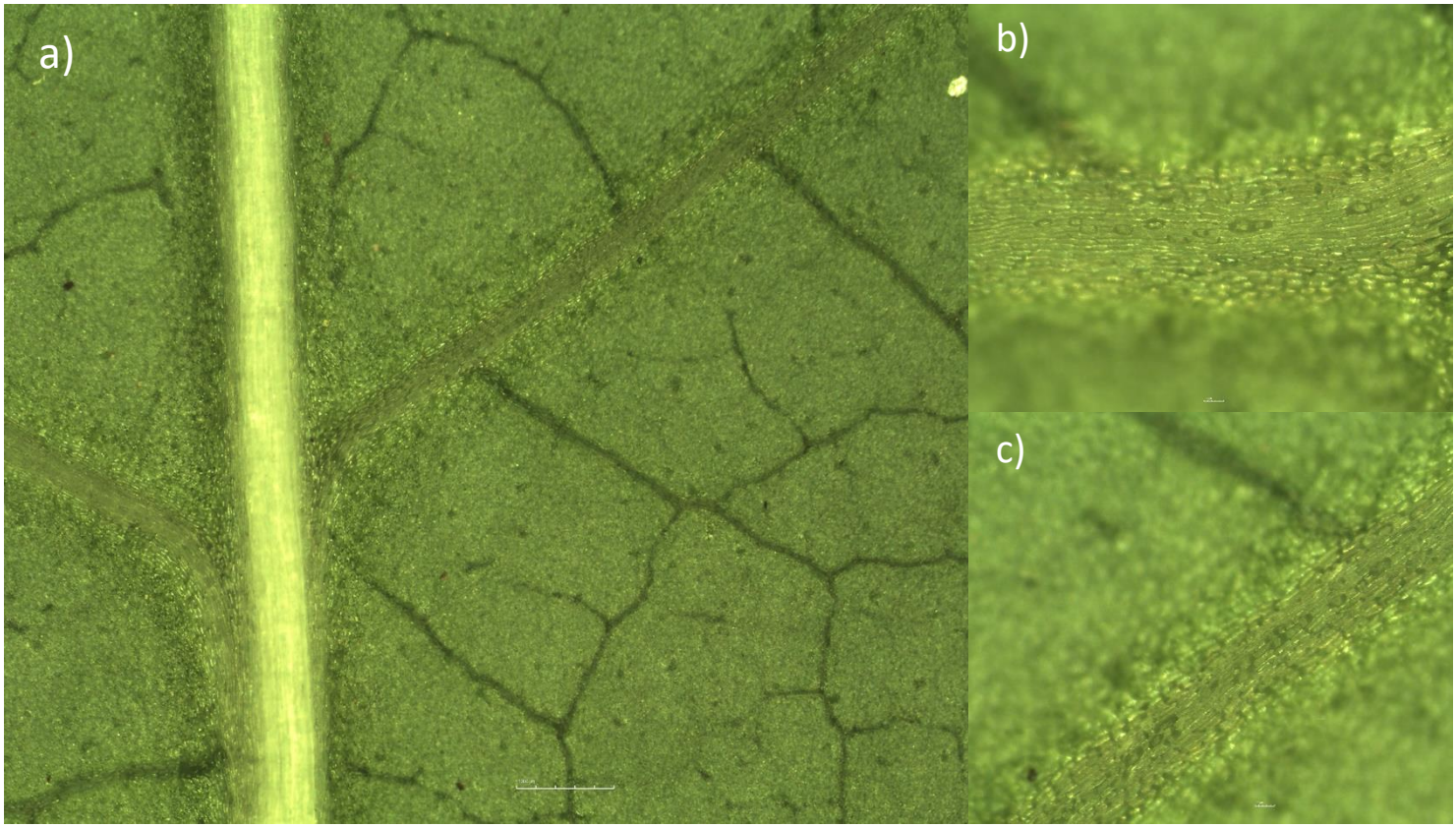


*Figure M1.1: Optical microscope photographs at 40x (**a**) and 100x (**b** & **c**) magnification of a mature leaf's abaxial surface. The leaves were harvested from* Reynoutria japonica *plants grown from rhizomes collected on the study site. Image **a** shows the leaf nerves and the absence of multicellular trichomes on them, scale bar = 1000µm = 1mm ; **b** and **c** show a close-up on the nerves, revealing the unicellular bump structure of the hairs, scale bar = 100µm.*

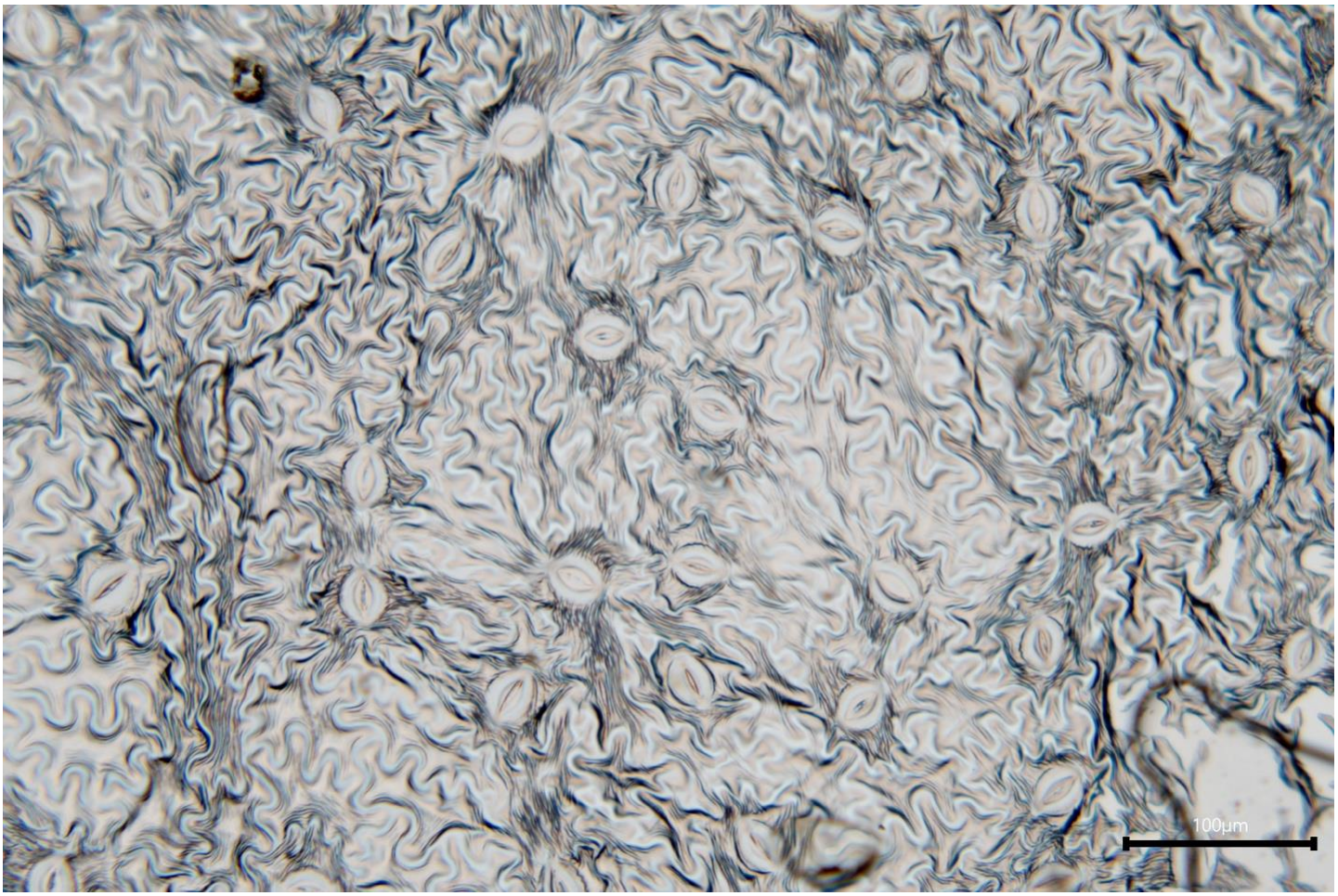
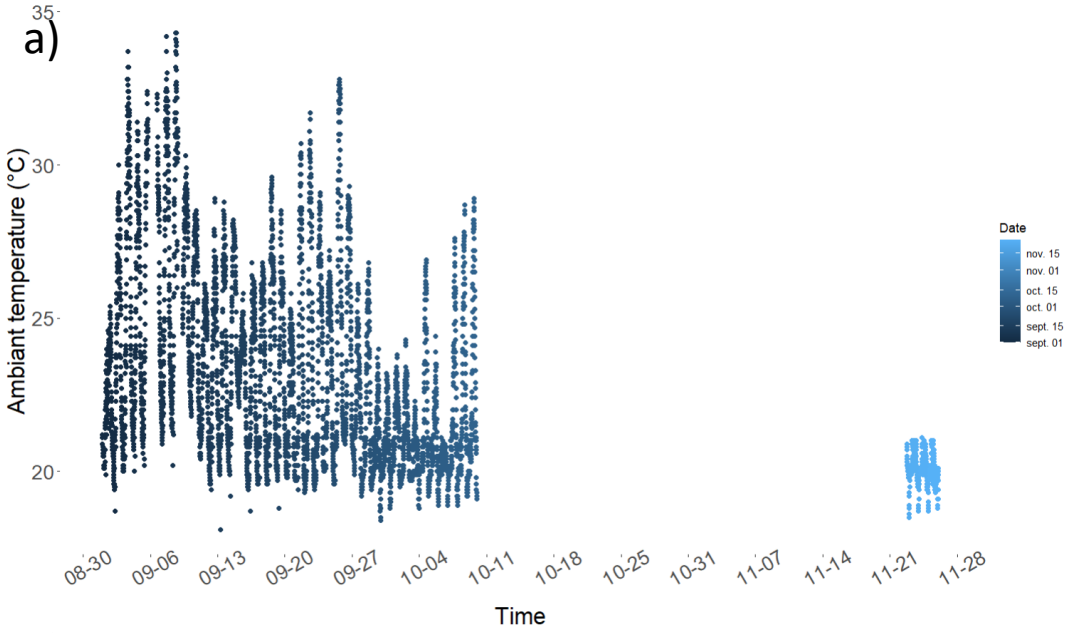
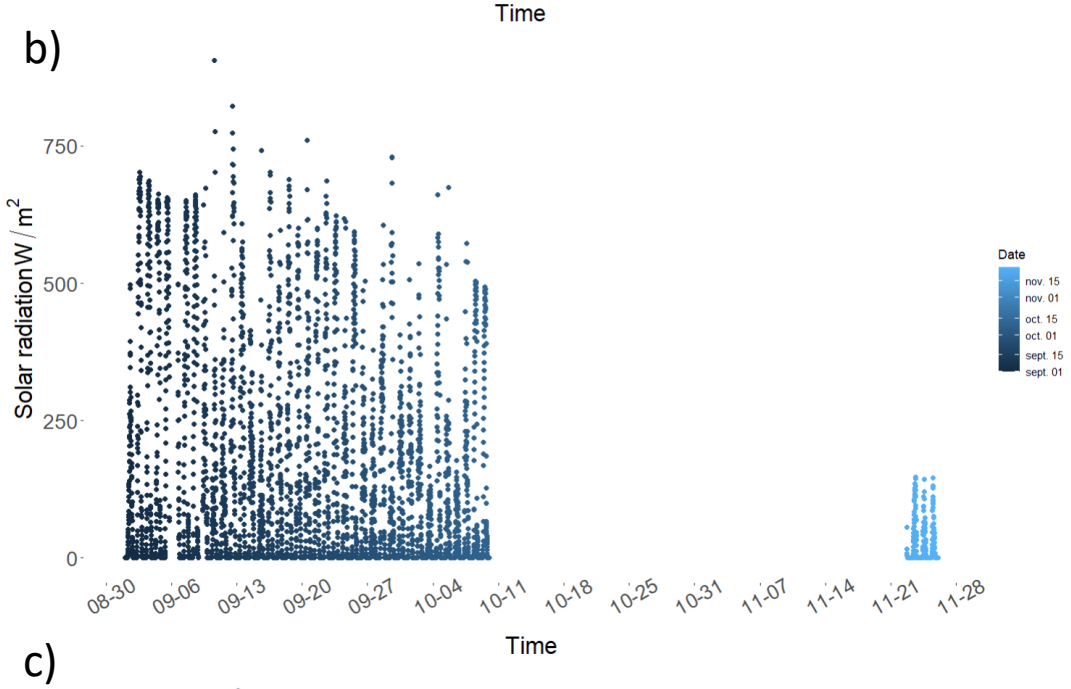


Figure M1.2: Optical microscope photographs at 100x magnification of epidermis-imprinted nail varnish sampled from a mature leaf's abaxial surface. The leaves used for sampling are from Reynoutria japonica *plants grown from rhizomes collected on the study site. Image shows epidermal cells along with stomata of the abaxial surface as they were imprinted on the nail varnish layers. Striation level of the epidermal cells is clearly observable, scale bar = 100µm.*

Annex M2





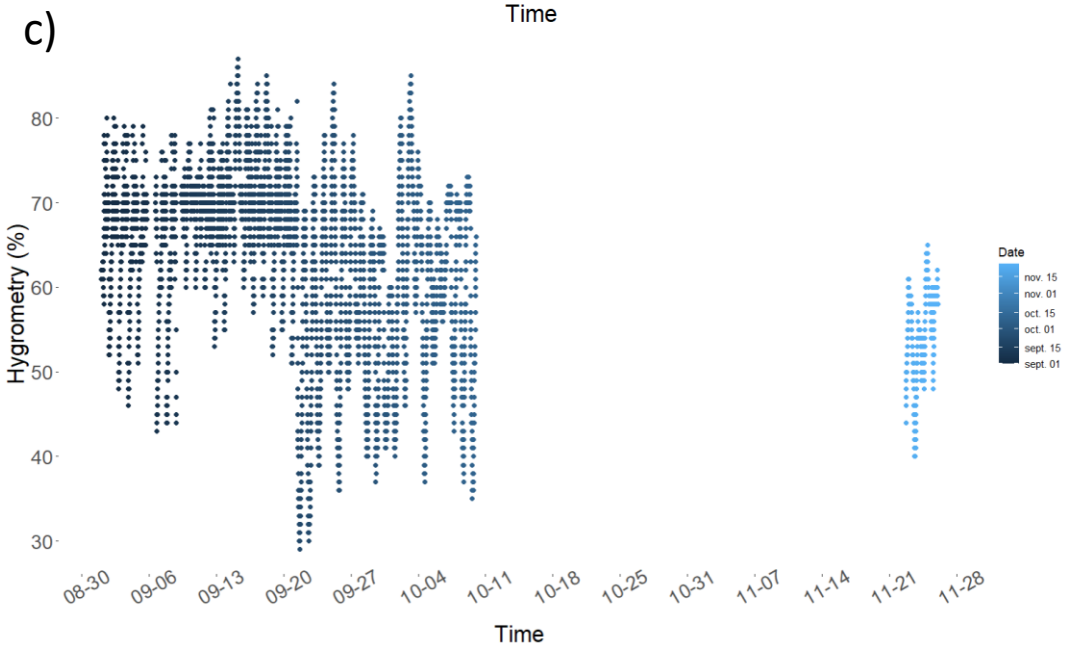


*Figure M2: Dot plots of ambient temperature (**a**), solar radiation (**b**) and hygrometry (**c**) in the greenhouse over the time span of both growth experiments (from 1st of September to 26th of November 2021). Due to a technical problem, data is only available for September and early October, and at the end of November. Each three abiotic variables were recorded every ten minutes for the entire period.*

Annex M3

Table M3: Geographic coordinates list of all knotweed-colonized sites recorded during field outings. The 61 sites were all visited between early February and mid-June 2021. Approximative locations and exact dates on which each site was recorded are given in headers above concerned sites. All sites except those colored in red were reported on iNaturalist (https://www.inaturalist.org/).

Site N°	*Latitude*	*Longitude*
<10/02/2021 – *Mont Saint-Guibert*		
1	50.636056	4.623278
2	50.632999	4.622971
10/02/2021 – *Ottignies*		
3	50.670157	4.570977
4	50.670851	4.57158
5	50.666276	4.570868
6	50.665134	4.570139
7	50.662739	4.568176
8	50.660645	4.566591
9	50.658908	4.567613
20/02/2021 – *from Ottignies to Basse-Wavre*		
10	50.671193	4.572072
11	50.673377	4.574821
12	50.674156	4.574826
13	50.674881	4.574865
14	50.676594	4.575648
15	50.677405	4.575487
16	50.678171	4.576769
17	50.679274	4.575918
18	50.679746	4.575128
19	50.683637	4.576165
20	50.682328	4.576733
21	50.681876	4.576253
22	50.681538	4.576619
23	50.693013	4.576726
24	50.694281	4.577661
25	50.692175	4.57684
26	50.692712	4.578697
27	50.693396	4.579247
28	50.695946	4.585649
29	50.696645	4.584836
30	50.696429	4.585591
31	50.716249	4.613316
32	50.717344	4.614949
33	50.719658	4.620769
34	50.720339	4.621094
35	50.721287	4.621794
21/02/2021 – *Vieusart, shore of le Train*		

36	50.677202	4.648431
37	50.667521	4.653809
38	50.680801	4.722721
39	50.68388	4.695843
6/03/2021 – *South, Bois des rêves, Ottignies' railroad, Thyle, Beaurieu*		
40	50.657216	4.578868
41	50.660939	4.574445
42	50.661647	4.574277
43	50.658518	4.578046
44	50.660511	4.569075
45	50.654443	4.568869
46	50.652958	4.568997
47	50.655653	4.565998
48	50.651884	4.569021
49	50.647344	4.56517
50	50.645552	4.598426
51	50.646965	4.565698
20/04/2021 – *Ottignies-Louvain-La-Neuve*		
52	50.658394	4.57412
53	50.657821	4.587411
54	50.660398	4.575168
18/06/2021 – *Louvain-La-Neuve behind Bruyères*		
55	50.659268	4.607583
56 + 57	50.662059	4.604102
58	50.66093	4.568193
59	50.660072	4.603962
60	50.661556	4.60447
61	50.661343	4.604062

Annex R1

Table R1: Statistical data of biometric measurements of Reynoutria japonica *from growth experiment 1. Anova tests were performed on Linear Mixed Models using* R. japonica *dry weight and dry aboveground/belowground weight ratio as response variables and the Bloc number as a random factor. When not matching the application conditions, response variables were log-transformed as indicated. Values in bold are statistically significant (p-value < 0.05).*

Growth experiment 1			
Response variable	Explanatory variables	Df	p-value
Log10(*Reynoutria japonica* total dry weight(g))	Substrate	2	**0.0001244*** **
	T. incarnatum density	3	0.2245776
	Substrate : *T. incarnatum* density	6	0.7075408
Reynoutria japonica dry aboveground/belowground ratio	Substrate	2	**1.763e-13*** **
	T. incarnatum density	3	0.5864
	Substrate : *T. incarnatum* density	6	0.6558

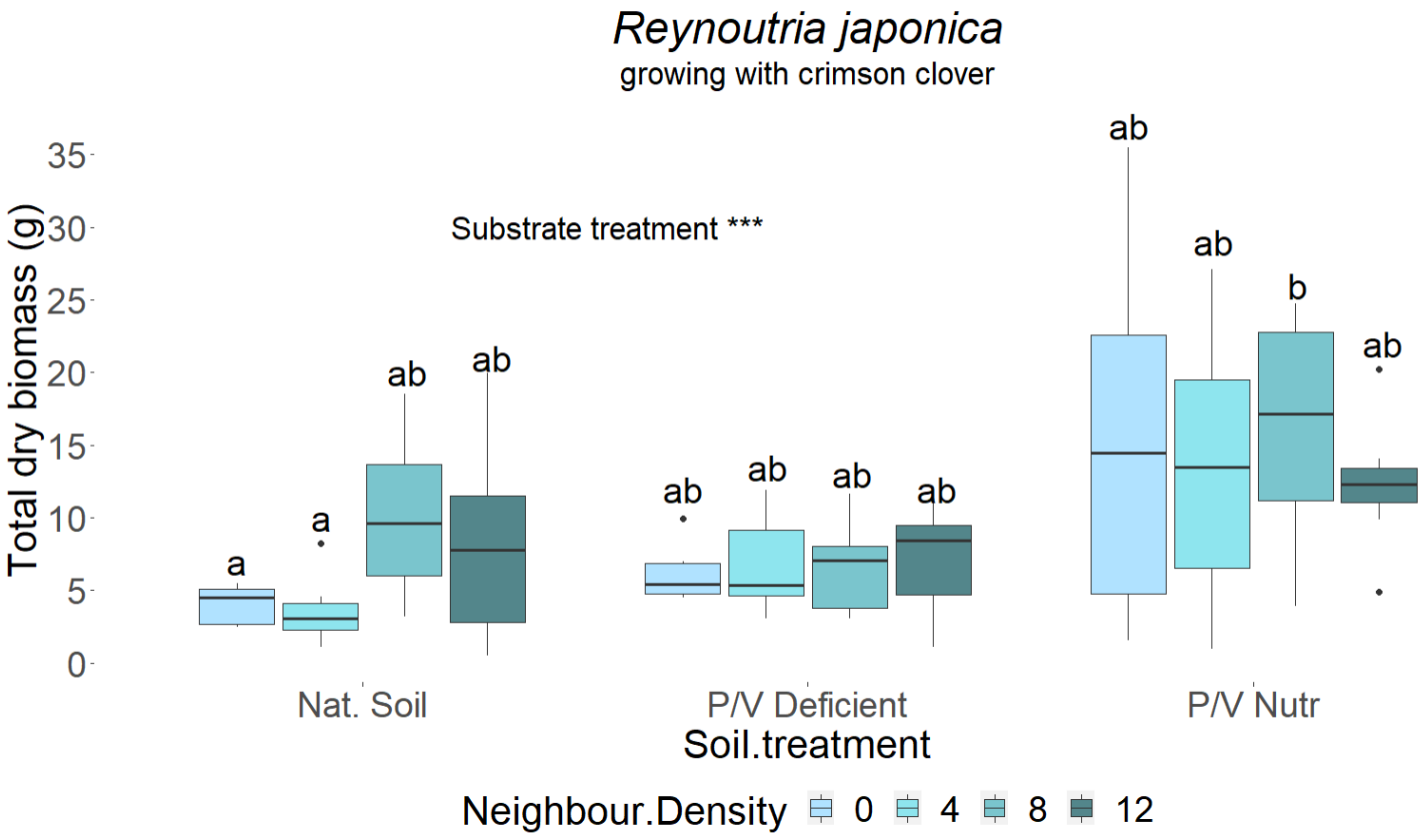


Figure RZ1: box plot of Reynoutria japonica *dry weight by substrate treatment and clover density (Growth experiment 1). Knotweed was left growing for six weeks before harvest. "Natural Soil" refers to soil collected on study site but that wasn't exposed to* R. japonica *rhizomes. Groups not sharing a letter are statistically different. Significance of associated Linear Mixed Model is reminded above the graphs. The following significance codes are applied: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

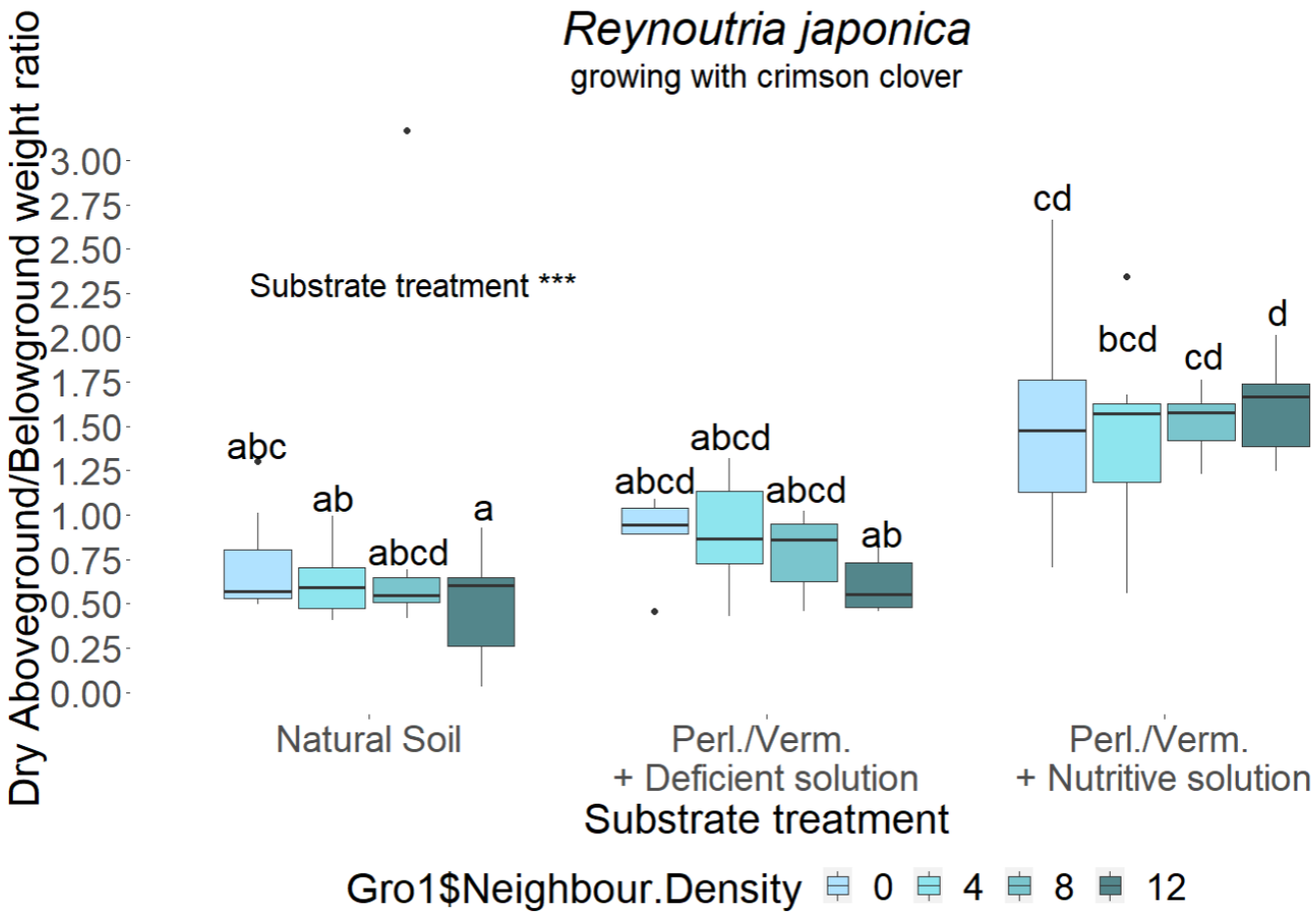


Figure RZ2: box plot of Reynoutria japonica *dry aboveground/belowground weight ratio by substrate treatment and clover density (Growth experiment 1). Knotweed was left growing for six weeks before harvest. "Natural Soil" refers to soil collected on study site but that wasn't exposed to* R. japonica *rhizomes. Groups not sharing a letter are statistically different. Significance of associated Linear Mixed Model is reminded above the graphs. The following significance codes are applied: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*