



From proof of concept to optimization - the use of a predatory mite species to control the population of the Western Corn Rootworm (*Diabrotica virgifera virgifera*) (LeConte, 1868)

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THÈSE DE DOCTORAT

De la preuve de concept à l'optimisation : L'utilisation
d'une espèce d'acarien prédateur pour contrôler la
population de la Chrysomèle des racines du Maïs
(*Diabrotica virgifera virgifera*) (LeConte, 1868)

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écologie

d'Université Côte d'Azur

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From proof of concept to optimization: the use of a predatory mite species to control the population of the Western Corn Rootworm (*Diabrotica virgifera virgifera*) (LeConte, 1868)

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RÉSUMÉ

Le maïs est la première céréale cultivée dans le monde (162 millions d'hectares). Il joue un rôle capital dans l'alimentation humaine et animale et sa culture occupe environ 9% de la surface agricole utile en Europe. Depuis son arrivée en Europe en 1992, la chrysomèle des racines du maïs appartenant à l'ordre des Coléoptères menace cette culture d'intérêt. Le stade larvaire de cet organisme est particulièrement nuisible pour les plantes car il s'attaque aux parties souterraines de cette céréale causant des pertes de rendement pouvant atteindre 80 % du potentiel de la culture dans les cas les plus critiques. Les pesticides chimiques, l'utilisation d'OGM Bt et la rotation des cultures alternant le soja et le maïs sont les principales méthodes de lutte pour contrôler les populations de ce ravageur actuellement. Cependant, des études ont démontré que certaines populations étaient résistantes à ces insecticides et étaient capables (i) de persister dans le sol plus longtemps et (ii) d'étendre leur gamme d'hôtes en s'attaquant aux racines du soja. Dans ce contexte, il est nécessaire d'explorer de nouvelles méthodes de protection des cultures qui permettront une lutte plus intégrée de ce ravageur. Une étude a récemment démontré le rôle potentiel des acariens prédateurs du sol en tant qu'agents de lutte biologique. Ces organismes sont au sommet des chaînes trophiques dans les systèmes agricoles et ont d'ores et déjà montré de fortes capacités de contrôle de populations de ravageurs souterrains. L'objectif principal de ma thèse est de développer une stratégie de lutte biologique viable d'un point de vue agronomique et économique pour lutter contre la chrysomèle des racines du maïs. Dans un premier temps, j'ai évalué la capacité de prédation des trois acariens du sol (*Stratiolaelaps scimitus*, *Gaeolaelaps aculeifer* et *Macrocheles robustulus*) sur les premiers stades de développement de la chrysomèle en laboratoire. Ces résultats ont été concluants et un des trois candidats a particulièrement attiré notre attention : *G. aculeifer*. Cet acarien prédateur a montré une capacité de prédation plus importante sur le premier stade larvaire du ravageur. Dans un second temps, j'ai étudié l'effet de la présence et de la densité (100, 500 et 1000 acariens par plants de Maïs infestés par le ravageur) de cette espèce d'acarien prédateur sur la population de chrysomèle en condition semi-contrôlée. Cette expérience a permis de montrer que les densités d'acariens testées permettent toutes de protéger efficacement le plant de Maïs. Enfin, et dans le but de faciliter l'utilisation de ces acariens prédateurs en tant qu'agent de biocontrôle, j'ai introduit une faible densité d'acariens prédateurs en présence de nourriture alternative au moment du semis de Maïs (méthode 'Predator-in-first') dans un champs naturellement infesté par les chrysomèles. Les paramètres physiologiques des plants de Maïs et populationnel de la chrysomèle ont tous deux montré que cette méthode permet de revenir à des valeurs comparables à celles des pesticides utilisés classiquement contre ce ravageur. Ces expériences ont permis de confirmer le potentiel des acariens prédateurs et de mettre en valeur le potentiel de l'approche dite de "Predator-in-first". Ces travaux devront être confirmés par une campagne plus large d'essais en conditions réelles mais ils apportent d'ores et déjà des informations primordiales dans la compréhension du potentiel de lutte biologique de ces organismes du sol peu étudiés jusqu'ici.

Mots clés: Acariens prédateurs, *Gaeolaelaps aculeifer*, biocontrol, predator-in-first,

ABSTRACT

Maize is the most important cereal crop in the world (162 million hectares). It plays a major role in human and animal nutrition and its cultivation occupies about 9% of the useful agricultural area in Europe. Since its arrival in Europe in 1992, the corn rootworm, belonging to the order Coleoptera, has threatened this important crop. The larval stage of this organism is particularly harmful to plants because it attacks the underground parts of this cereal causing yield losses that can reach 80% of the crop's potential in the most critical cases. Chemical pesticides, the use of Bt GMOs and crop rotation alternating soybeans and corn are the main control methods for this pest currently. However, studies have shown that some populations are resistant to these insecticides and are able to (i) persist in the soil longer and (ii) expand their host range by attacking soybean roots. In this context, there is a need to explore new crop protection methods that will allow a more integrated control of this pest. A study recently demonstrated the potential role of soil predatory mites as biological control agents. These organisms are at the top of trophic chains in agricultural systems and have already shown strong abilities to control populations of subterranean pests. The main objective of my thesis is to develop a biological control strategy agronomically and economically viable to control corn rootworm. First, I evaluated the predation capacity of three soil mites (*Stratiolaelaps scimitus*, *Gaeolaelaps aculeifer* and *Macrocheles robustulus*) on the early developmental stages of western corn rootworm in the laboratory. These results were conclusive and one of the three candidates particularly attracted our attention: *G. aculeifer*. This predatory mite showed a higher predation capacity on the first larval stage of the pest. In a second step, I studied the effect of the presence and the density (100, 500 and 1000 mites per corn plant infested by the pest) of these predatory mites on the western corn rootworm population under semi-controlled conditions. This experiment showed that the mite densities tested were all effective in protecting the maize plant. Finally, and in order to optimize the effectiveness of these predatory mites as a biocontrol agent, I studied the population dynamics of these predators when a low density of predatory mites has been introduced at the time of seeding (Predator-in-first method) and in the presence of alternative food, in a field naturally infested by corn rootworm. These experiments allowed me to identify an application method that would facilitate the introduction of the biological control solution and reduce its cost. My work will provide essential information in understanding the biological control potential of these previously understudied soil organisms.

Key words: Predatory mites, *Gaeolaelaps aculeifer*, biocontrol, predator-in-first

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GENERAL INTRODUCTION

1. A global crop and a global change for a global threat ?

Maize is the most important cereal produced with 1137 millions mt annually, the second in terms of surface covered with 197 million of hectares (Erenstein et al., 2021) and among the three most important crops in the world considering human nutritional value (Tanumihardjo et al., 2020) with rice and wheat. The growing need for human and animal nutrition in competition with its use for biofuel made this crop a strategic issue worldwide, which is planned to outmatch any other crop by 2030 (Tanumihardjo et al., 2020). Economic interests (Hodgson et al., 2015) and agronomic ease to grow this crop made it used in Europe, Asia, America, and Africa (Faostat, 2018). This global presence leads to global trade with a total value of maize exportation in 2017 evaluated at 3,23 billions dollars (Maize, OEC). However, these international trades are confirmed to be an increasing factor of invasive pest emergence (Dawson et al., 2017; Perrings et al., 2010) which can threaten this widely spread crop. Another global change, the worldwide temperature increases (Sorokin and Mondello, 2018), could force more farmers to grow this heat-resistant crop if suitable humidity is present (Abate et al., 2015). Although global warming can promote plant growth in special cases, it mainly harms the environment by increasing length and frequency of drought episodes which increases distribution of crop pests (Barford, 2013; Bebber et al., 2013; Quarles, 2007) in addition to facilitating invasion (Vilà and Hulme, 2017). Monoculture, the most commonly used agronomic practice in maize production (Fenzi and Couix, 2021), is another source of pests and disease outbreaks (Lopes et al., 2016; Rao et al., 2012; Trenbath, 1993) that promotes resistance to traditional pest management (Roush and Tabashnik, 2012). Global distribution, global trade, global change and monoculture are four important factors enhancing the risk of invasion and pest nuisance on maize cultivation. Indeed, insect damages and management are the main costs in maize production worldwide (Oerke et al., 2012). Corn earworm (*Helicoverpa zea*) (Boddie), European corn borer (*Ostrinia nubilalis*) (Hübner, 1796), Fall armyworm (*Spodoptera frugiperda*) (J.E. Smith, 1797) and Western Corn Rootworm (WCR) (*Diabrotica virgifera virgifera*) (Lecomte, 1868) are identified as the main pests of

maize worldwide, and impact its production on more than a single continent (Diffenbaugh et al., 2008; Ramasamy et al., 2021). Among these 4 major pests, WCR is the least studied with 1078 articles using the latin name in Web of Science, whereas the three other pests *Ostrinia nubilalis*, *Helicoverpa zea*, *Spodoptera frugiperda* appears in 1945, 1992, 4966 articles respectively. In addition to this observation, Chave et al., (2014) highlight that despite soil pests being responsible for a loss of 10% of total crop yield (Oerke et al., 2012), they attracted less attention compared to the aerial pest (Hiddink et al., 2010). We assume that WCR life cycle being mostly in the soil, it is more difficult to observe compared to the three other pests, damaging only the aerial parts of the plant. That difficulty being responsible for the least attention given to WCR. Despite this difference, WCR was considered in 1986 the most severe maize pest in North America and known as “a one billion dollar beetle” (Krysan et al., 1986). Moreover, in 1992, WCR was discovered for the first time in Europe near Belgrade, Serbia (Bača, 1994). This discovery deeply alarmed maize producers and the scientific community. The most important structure and experts of maize and its pests gather together to assess WCR spread through Europe (Bažok et al., 2021). The International Working Group on Ostrinia and Other Maize Pest (IWGO), European and Mediterranean Plant Protection Organization (EPPO), and Food and Agriculture Organization (FAO) all considered this invasive pest as a major threat to maize production in Europe. Since 1995, they have organized an annual meeting to share any new information that can help to control its populations. This cluster helped to identify the origin of WCR european population in North America (Ciosi et al., 2008; Miller, 2005), how the species spread in Europe (Edwards et al., 1999; Kiss et al., 2005), and much knowledge on its ecology, damages, and control methods (Bažok et al., 2021). In conclusion of this work, the Commission of the European Union issued emergency measures to control the spread of WCR (2003/766/CE, 2003) by recommending pest eradication anywhere it was detected. This strategy was lightened/eased in 2006 by switching to containment measures / prescriptions (2006/564/CE, 2006). While these measures have helped limit the impacts of WCR in Europe, which have remained far less than the 700 million euros of cost per year that was predicted by Wesseler and Fall, (2010), they did not allow to stop the spread of WCR. The pest is now reported from 21 European countries and has been removed from the quarantine list (EPPO). This successful spread in Europe despite the resources invested to control the invasion is worrying as we mentioned earlier the

global interest of maize and its growing importance for nutrition and biofuel production. It is thus necessary to gather information on WCR biology and how it impacts maize production.

1.1. WCR biology and its impact on maize

The adult stage can feed on corn silks and might cause economic losses in case of high abundance (Elliott et al., 1990). However, the main damages come from the larvae that feed on maize roots, which increases the risk of lodging and attack from pathogens (Palmer and Kommedahl, 1969), reduces nutrients and water uptake (Kahler et al., 1985) leading to a reduction in maize size and yield loss (Allee and Davis, 1996; Branson et al., 1980; Roth et al., 1995; Urías-López et al., 2000). Eggs are laid at the end of the Summer in soil rifts at 5-15 cm depth and overwinter at this stage (Pierce and Gray, 2006) (Figure 1). Larvae emergence is triggered by a temperature superior to 10 degrees (Schaafsma et al., 1991). Larvae need to find maize roots to establish within the first 24 hours after emergence, 95% of first instar larvae fail to establish and die at this stage (Hibbard et al., 2010, 2004; Pilz, 2008; Strnad and Bergman, 1987a). Soil physical properties can impact the path to reach the roots since the bulk density and pore size can slow larvae movement (Ellsbury et al., 1994; Macdonald and Ellis, 1990; Spencer et al., 2009). To find their way to the maize root system, first instar larvae are able to recognize plants volatile compounds such as CO₂ (Bernklau et al., 2004; Strnad et al., 1986; Strnad and Dunn, 1990). Once the larvae reach the roots, plant secretion activates feeding activity. Monogalactosyldiacylglycerol (MGDG) and its byproducts in addition to sugar such as glucose, fructose, and sucrose plus linoleic or oleic acid and a complex of Fe were identified as feeding triggering factors (Bernklau and Bjostad, 2008; Schumann et al., 2014). Once they accumulate enough energy on the nearest root, they move toward younger roots (Robert et al., 2012; Strnad and Bergman, 1987b) with higher nutritional value especially in Fe(III)(DIMBOA)₃ essential to complete development (Hibbard et al., 2010). Considering larvae movement, they will spread away from maize roots if too many individuals feed on them which reinforces the proof that each individual needs to ensure to have sufficient food source to reach the adult stage (Spencer et al., 2009). The pupae stage happens in the soil when larvae consume sufficient food but also depends, like all the other stages, on temperature in soil higher than 11°C (Bergman

and Turpin, 1986; Fisher, 1987). Once adults emerge from pupae, they leave the soil and go toward the top of the maize plant to feed on leaves and silks (Elliott et al., 1990). WCR is a univoltine species and females can fly up to 2 km (Yu et al., 2019) and lay up to 1200 eggs with 400 eggs on average. Eggs overwinter at this stage during a required 18-weeks cold period (Jackson, 1986). The estimated period of each stage is provided in Figure 1. WCR was considered monophagous on maize but few studies showed their capacity to reach second instar, third instar, and sometimes even adult stage on rabgrass, barnyardgrass, sand lovegrass, western wheatgrass and Texas panicum (Clark and Hibbard, 2004; Oyediran et al., 2004; Wilson and Hibbard, 2004). This has become of particular importance since monophagous species are sensitive to the lack of their host plant. This biological characteristic leads to the historically first efficient pest management methods: the use of crop rotation to deny larvae stage feeding for a year. This particular pest management method in addition to many others are described in the following part.

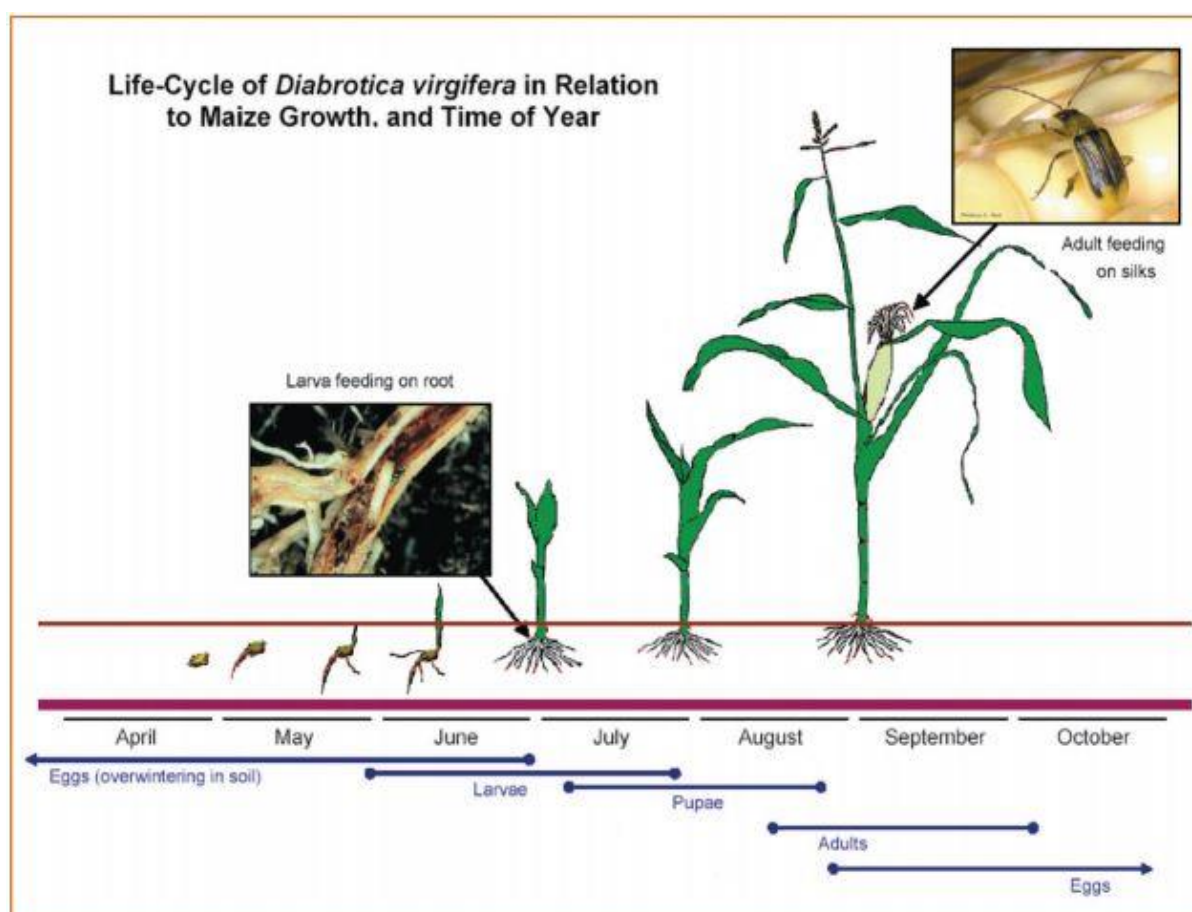


Figure 1 : *Diabrotica virgifera virgifera* life cycle. From (Edwards et al., 2006).

1.2. WCR management methods

To prevent WCR damages, many management methods have been explored through the decades but none of them help to sustainably control the pest. It was crop rotation that was first used followed by pesticide to avoid the need to diversify crop use but both showed their limits. Genetically Modified Organisms, Pest/host interference and Biocontrol has also been explored. We detail these different management methods below.

1.2.1. Crop rotation

Crop rotation is the practice of growing a sequence of plant species on the same land (Yates, 1954). This method was the first method used to control WCR damages on maize in the early 20th century (Levine et al., 2002). Crop rotation presents many advantages to sustainable crop management. At the field scale, it can help to maximize crop yield, decrease pest pressure, diversify nutrients availability, increase soil organic matter, improve soil structure which reduces soil degradation (Selim, 2019). When climatic conditions allow it (Abate et al., 2015), crop rotation is particularly useful to starve monophagous species and interrupt their life cycle. Crop rotation was found efficient against WCR damages in Europe (Bažok et al., 2021) leading to a lack of major economic impact in the maize countries producers. However, for this method to be efficient, regional coordination of rotations in the entire production area is needed. Hodgson et al., (2015) showed that maize prices will rise when production is lowered by pest pressure, which increases local economical pressure to plant more maize. WCR beetles are good dispersers able to fly 2 km away from their natal site into neighbouring fields (Isard et al., 2004; Spencer et al., 2005), which may act as a population reservoir while the crop rotation occurs. Flight capacity is not the only parameter helping WCR to bypass culture rotation. A strain was found in the early 90's to be able to feed on soybean, one of the species used for crop rotation, due to a digestive enzyme overexpression in this particular population. This strain has now spread to the entire Cornbelt in the USA (Curzi et al., 2012; Gray et al., 2009; Levine et al., 2002). Another species of the same genus, *Diabrotica barberi* (Smith and Lawrence, 1967), has the capacity to spend a year in an extended diapause to wait until an appropriate food source has grown again. Despite these localized adaptations, WCR biology regionally overcomes crop rotation.

1.2.2. Pesticides

Pesticides were deployed against WCR to avoid the need to rotate crops, but they are now recognized as a non-solution. Many studies have repeatedly demonstrated the dangers of pesticides to the environment and human health (Carson, 2002; Levitan, 2000; Pimentel et al., 1992) and the increasing resistance of organisms targeted by these chemical products (Powles, 2018; Roush and Tabashnik, 2012). In the case of WCR, Meinke et al., (2021) drew a good overview of the pesticides used. This work highlighted that agronomic practices in the Corn Belt in the US, such as large irrigated monocultures, continuous planting, and strong aerial pesticide application, lead to multiple resistance and cross-resistance to cyclodiene, organophosphate, carbamate, and pyrethroid. In Europe, the legislation promotes a sustainable use of pesticides, to prevent environmental and human health issues (Directive 2009/128/EC). These biological and legislative adaptations induced the need to reduce the use of pesticides to the benefit of more integrated pest management strategies.

1.2.3. Genetically Modified Organisms

A technology based on Genetically Modified Organisms (GMOs) is used in the US. Transgenic corn produces insecticidal toxins derived from the bacterium *Bacillus thuringiensis* (Bt) (Shrestha and Gassmann, 2019). Genetically modified maize was widely used in the Corn Belt (Paddock et al., 2021) and different variants of Bt toxin were incorporated into the maize genome through the years. However, WCR has developed resistances to all strains available for commercial use, thus decreasing the efficiency of these methods. In Europe, GMO maize seeds are banned by legislation (Directive 2001/18/EC). While GMOs producing Bt toxin, pesticides, and crop rotation have been the most widely used methods to control WCR populations, many other methods have been explored, of which Paddock et al., (2021) provided a good overview.

Another technology of genetically modified organisms has been tested to control WCR. RNA interference (RNAi) methods rely on the production by the plant of small fragments of RNA that will be ingested by insects and block the expression of specifically-targeted genes (DvSnf7 dsRNA), which can result in the death of individuals (Katoch et al., 2013). However, WCR managed to develop resistance and

cross-resistance to this technology rapidly in laboratory experiments (Khajuria et al., 2018). Furthermore, many ethical concerns can be raised around this technology, as very specific RNAi has to be designed to avoid non-intentional effects on the environment (Jackson et al., 2003).

In addition to GMOs, maize strain selection was widely used to enhance maize yield, resistance to drought and herbivory (Fontes-Puebla and Bernal, 2020). Selecting WCR resistant strains has also widely been explored. Unfortunately, they were not able to reach yields comparable to actual commercialized strains. This leads to an absence of this strain's availability for the farmer (Paddock et al., 2021) due to economic loss.

1.2.4. Pest / host interference

Interference in pest / host relation has been widely studied but barely applied. Throughout the decades of research on WCR biology and ecology, much information was collected on this organism and its relation to its host plant. One major potential lever for limiting WCR populations that has been identified is related to the highly sensitive stage between egg hatching and establishment within maize roots. About 95 % of first instar larvae die before reaching maize roots (Hibbard et al., 2010, 2004; Strnad and Bergman, 1987a) due to their need to feed within the first 24 hours of their life (Pilz, 2008). Many lines of research focused on physical or chemical soil properties, aiming at extending the time between hatching and the first feed on maize roots to increase first instar larvae mortality even more. Soil compaction can limit larvae movement and slow down their access to the maize root system (Ellsbury et al., 1994; Macdonald and Ellis, 1990; Strnad and Bergman, 1987a) but little is known about WCR larvae capacity to burrow and dig through the soil to reach the maize roots. Maize crops are extremely dense with a range from 30 000 to 90 000 plants/ha (Sangoi, 2001), which allows soil pests to find a plant within a mean range from 19 to 33 cm around them (Maize density/total surface with sphere surface applied), whereas WCR larvae have shown to be able to travel on these distance (Macdonald and Ellis, 1990). Soil compaction can reduce accessibility of maize roots to WCR larvae but this method is limited since it can not prevent larvae emerging on the furrow to damage the root system.

Chemical compounds that disturb larvae movement toward maize root have been tested but failed to reduce larvae establishment. Studies showed that WCR larvae are attracted to the CO₂ produced by maize roots (Bernklau et al., 2004; Strnad et al., 1986; Strnad and Dunn, 1990). A release of CO₂ in the soil could then be used to blur the chemical track pointing towards the maize root system. Moreover, CO₂ could be used to attract WCR first instar larvae toward traps or compounds able to kill them following the attract-and-kill strategy. This method has been tested and encapsulated CO₂ was released in the soil with or without pesticide to assess whether disturbing larvae movements provided any level of protection to the plant. In both cases, this method showed no significant results on larvae establishment on the roots (Schumann et al., 2014).

1.2.5. Biocontrol

Plants modification, agronomic practices, and pesticides solutions were widely explored through the last decades but they were all limited by their lack of impact, their cost, or by WCR rapid adaptation. An alternative to these solutions exists: The Biocontrol. Biocontrol which relies on crop protection services using natural mechanisms such as living organisms or substances derived from them (in this work, minerals sources are not considered as biological control solutions according to Biological Control Journal (2021) definition) is planned to increase from 3 billion dollars in 2018 to 7.5 billion dollars in 2025 in the world (Global Market Insights, 2019). This alternative to traditional pest management practice is recommended by Europe ("Directive 2009/128/EC of the European Parliament and of the Council of 21 October 2009 establishing a framework for Community action to achieve the sustainable use of pesticides") and this arises at the national level. In France, the Ecophyto plan's objective is to reduce 50% the use of pesticides from 2015 to 2025 ("Le plan Écophyto, qu'est-ce que c'est ?," 2015). Beyond political and state willingness, International Biocontrol Manufacturers Association (IBMA) surveyed 540 french farmers on the biocontrol subject. It showed that 93% of farmers want to use more biocontrol solutions in the future if products improve in their affordability, availability (more diverse), and convenience (IBMA, 2019). In field crops, the need is even more important. The IBMA, (2019) survey showed that only 34% of farmers use biocontrol products compared to 57%, 65%, 84% for viticulture, arboriculture, and vegetable cultivation, respectively.

Field crops are characterized by extensive surface and lower profits margin which induced the two main farmers drags related to biocontrol products used : the complexity to apply the products in extended area and its price. Biocontrol is the broad term to consider using living organisms to control pests but there are few different strategies to deploy it. Conservation biological control aims to protect crops by improving natural enemies' habitat (Barbosa, 1998). Importation of biological control, also called classical biological control, purpose is to introduce exotic organisms (natural enemies of the pest in its native area) that will control the invasive pest population (Heimpel and Cock, 2018). Augmentative biological control aims to release appropriate numbers of additional biological control agents to ensure pest control (Sivinski, 2013). Within the maize field to control WCR, conservation biological control seems infeasible due to the lack of natural enemies sufficient impact in the maize field environment (Toepfer et al., 2009b), especially in Europe (Toepfer and Kuhlmann, 2004). However, despite natural enemies' limited impact on the maize field, many parasitoids, predators, entomopathogenic nematodes, viruses, bacteria and entomopathogenic fungi were identified as potential biological control agents in the laboratory (Toepfer et al., 2009b). None of them were used at large scale yet but few studies focus on applicable biocontrol solutions exploration to find solutions against WCR.

The earliest biocontrol strategies developed against WCR targeted the adult stage: in 1993, three species of Centistes (Hymenoptera: Ichneumonoidea) were shown to parasite adult corn rootworm in the laboratory (Shaw, 1995). Then, despite additional work aiming at evaluating the rearing potential of these parasitoids (Schroder and Athanas, 2002), no further developments were made towards a viable control solution. Another parasitoid, *Celatoria compressa* Wulp (Diptera: Tachinidae) showed its potential as a biocontrol agent (Kuhlmann et al., 2006) but was considered too generalist and possibly harmful for the environment. Toxin from the bacteria *Chromobacterium subtsugae* (Martin et al., 2007) showed very high efficiency on WCR adults in the laboratory (80-100% kill) but no production or formulation was developed further. More recently, several researches aimed at controlling WCR underground stages, with a strong focus on entomopathogenic organisms. Entomopathogenic fungi reduced WCR adults emergence by 31% (Pilz et al., 2009) and were proved not to be harmful to the environment (Mayerhofer et al., 2019) but this solution still requires large scale production, formulation, and suitable application to be efficient, which are not

available at present. Multiple species of entomopathogenic nematodes were also tested against WCR (Toepfer et al., 2005). They were used alone, with engineered bacterial symbionts (Machado et al., 2020), combined with pesticides (Nishimatsu and Jackson, 1998), Bt GMO (Petzold-Maxwell et al., 2012), with fungi and bacteria (Jaffuel et al., 2019), or with fungi and chemical insecticides (Rauch et al., 2017). Among all these studies, *Heterorhabditis bacteriophora* showed the most promising results (Nishimatsu and Jackson, 1998; Toepfer et al., 2009a) with conclusive field trials (Toepfer et al., 2010). However, this study highlighted the dependence of nematodes on rainfall, which can alter the regularity of biocontrol results or require additional interventions to control irrigation in the field. Another promising, yet underinvestigated strategy, is the use of predatory mites against the WCR larval stages.

THESIS AIM: CAN SOIL-DWELLING PREDATORY MITES BE AN EFFICIENT BIOCONTROL SOLUTION AGAINST WESTERN CORN ROOTWORM ?

Predatory mites showed their capacity to control pests such as phytophagous mites that cause damages to multiple types of crops throughout the world (Rao et al., 2018) when other predators failed to control the pest with efficiency (Fathipour and Maleknia, 2016). Their capacity to be mass-reared (Hoy, 2011), their voracity (Escudero and Ferragut, 2005), and their capacity to survive on multiple food sources (Nguyen et al., 2014) enhance their potential as biological control agents.

Predatory mites are Arthropoda belonging to the class Arachnida and the subclass Acari. Their life cycle is composed of 5 distinct stages: Egg, larva, protonymph, deutonymph and adult (Figure 2). After hatching, predatory mite larvae are mobile but do not feed. They live on their reserve before moulting in protonymph. The protonymph feeds mainly on small prey and accumulates energy resources for a few days before moulting into a deutonymph and then an adult. The sex determination of predatory mites is a little known phenomenon. But their reproduction is arrhenotokous parthenogenesis (unfertilized female gives male and fertilized female gives both sexes) and is internal (Usher and Davis, 1983). Adults feed on organisms ranging from 200 micrometers to several millimeters and have a lifespan of a few weeks depending on the species.

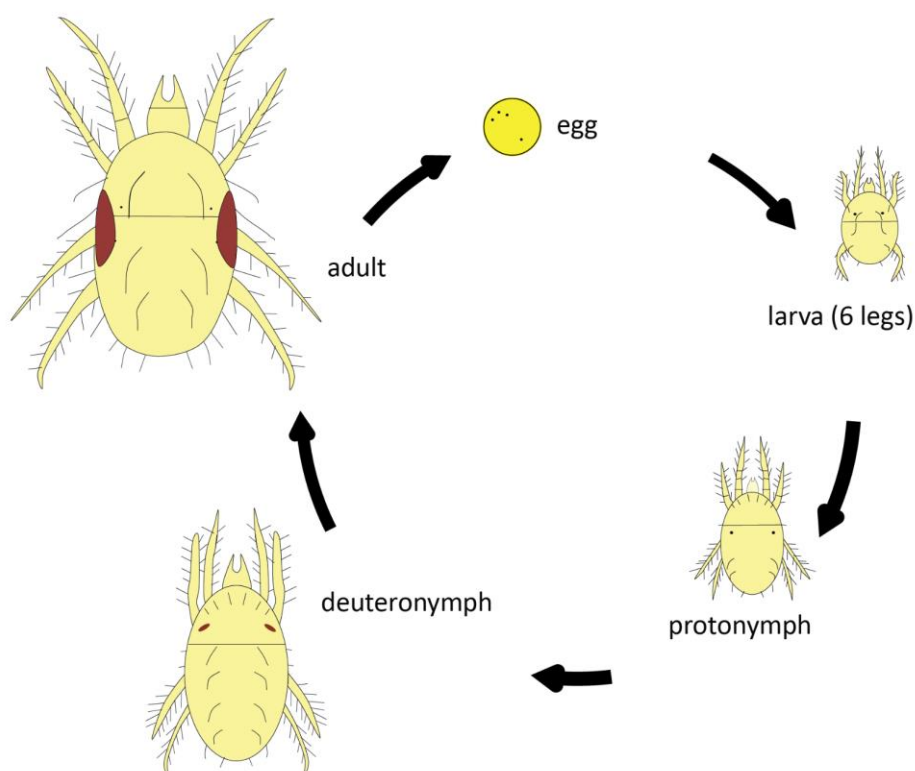


Figure 2: Generalised mite life cycle (source: ©2021 Sonya Broughton). Deuteronymph = Deutonymph.

Soil-dwelling predatory mites, in particular, are at the top of the trophic web in the soil agronomic system (Postma-Blaauw et al., 2010). Most species are mobile predators feeding on collembola, nematodes, insect larvae, insects eggs and other microarthropods (Baatrup et al., 2006; Koehler, 1997; Walter and Proctor, 2013). Their effectiveness as biological control agents in regulating soil pest populations has contributed to their use both in the field and in the greenhouse (Gerson and Weintraub, 2012; Lesna et al., 2000). Two species, *Gaeolaelaps aculeifer* Canestrini (Arthropoda: Mesostigmata) and *Stratiolaelaps scimitus* Womersley (Arthropoda: Mesostigmata), are commercially available to control *Frankliniella occidentalis*, a western flower pest and mushroom flies (Premachandra et al., 2003) and to reduce the density of shore fly larvae and acarid mites regardless of the substrate of introduction (Jess and Schweizer, 2009). As Fathipour and Maleknia (2016) suggest, this soil-dwelling predatory mites show good potential to control arthropods in the soil. However, it is necessary to assess if soil-dwelling predatory mites can recognise WCR early stage

as a prey, if it can control the WCR population in the soil and if this control is still present in field conditions.

Can soil-dwelling predatory mites be an efficient biological control solution against Western Corn Rootworm ? This question is addressed in the following 3 chapters of this manuscript.

Chapter 1 is a **proof of concept of soil-dwelling predatory mites capacity to attack WCR early stages**. Before investing a lot of time and money in a long experimental set up we choose to test the reaction of multiple species of predatory mites in presence of WCR early stages in very controlled conditions and in a short experiment duration.

Chapter 2 relies on **testing one species of soil-dwelling predatory mites in presence of WCR in a soil / plant / pest / biocontrol agent system**. First applied in the greenhouse, then in a maize field, we tested different introduction timings and observed their impact on WCR population and maize physiology in a single environmental condition.

Chapitre 3 is about **assessing the effects of environmental conditions on the predatory mites population and their impact on biological control efficiency**. This work is a part of a bigger project called Mites Against Diabrotica (MAD) funded by Semae (ex-GNIS) which charge Bioline AgroScience and a consortium composed of INRAE, Arvalis and Axereal Serbia to develop a biological control solution against WCR. The project was divided in the following subtasks (i) formulate alternative food to enhance or attract predator populations, (ii) develop molecular tools to assess predatory mites efficiency and impact, and (iii) identify new species of naturally present soil-dwelling predatory mites. Most of the work in the project scope will remain confidential but we will specify if the work done by the consortium supplies information that helps to guide the thesis in respect to its confidentiality.

CHAPTER 1: DEMONSTRATION OF SOIL-DWELLING PREDATORY MITES' ABILITY TO ATTACK WCR EARLY STAGES

Introduction

The evaluation of the efficiency of a new biological product is often a progressive process, starting from small-scale tests under controlled conditions in the lab to confirm its mode of action, to full-fledged field trials in the latest stages of development. Because large-scale testing is typically costly, only products that have demonstrated their potential efficiency in the lab will be subjected to further investigations. In this entire manuscript, we show how we explored step by step at increasing scales the ability of soil-dwelling predatory mites to control WCR early stages. In this first chapter, we evaluate the potential of soil-dwelling predatory mites to recognize the early stages of WCR as prey and to kill them when they are put into direct contact. This is a necessary condition for efficiency, before considering further trials. Prischmann et al., (2011) first explored this idea by confronting 6 species of soil-dwelling predatory mites to different stages of WCR larvae in an artificial environment over 10 days. They showed that the predatory mites were able to attack and kill the first instar larvae within the test duration. However, our goal is to control WCR early stages before they reach maize roots and start to damage the crop, which happens within 24 hours (Pilz et al., 2009). We kept the very simple experimental set up presented in Prischman et al., (2011) work and shortened the observation period to 10 min. This short period allowed us to observe the entire prey / predator interaction in the small arena and ensured visually that predatory mites encounter the potential prey in addition to ensuring that the artificial environment will not harm the prey and facilitate attack or larvae death. The choice of predatory mites species used for the experiment was based on French legislation. We chose to focus on the species currently allowed to be released in France, considering that if the results of this research were positive we would be able to avoid supplementary registration and provide a biological control solution at mid-term for the farmer. Three soil-dwelling predatory mites species are currently allowed for introduction in France and commercialized: *G. aculeifer* and *S. scimitus*, also tested

by Prischman et al., (2011), and *Macrocheles robustulus* (Berlese). Results are presented in Article 1.

Article 1 (Short communication) : Predation capacity of soil-dwelling predatory mites on two major maize pests.

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Abstract

The western corn rootworm *Diabrotica virgifera virgifera* (WCR), and the wireworm *Agriotes sordidus* (WW), whose eggs and first instar larvae develop in the first few centimeters of soil, are major crops pests. As soil-dwelling predatory mites are known to be efficient biocontrol agents against many pests, we investigated the predation capacity of *Stratiolaelaps scimitus*, *Gaeolaelaps aculeifer* and *Macrocheles robustulus* on immature stages of WCR and WW in a laboratory setting. While eggs of WCR and WW were never consumed, all three predator species attacked both WCR and WW first instar larvae. These results need to be confirmed in natural conditions, our work identifies the early larval stage instead of the egg stage as the most vulnerable stage for control against WCR and WW with soil-dwelling predatory mites.

Keywords

Click beetle; Corn pest; *Laelapidae*; *Macrochelidae*; Biological control.

Introduction

Western corn rootworm (WCR) *Diabrotica virgifera virgifera* LeConte, 1868 (Coleoptera: Chrysomelidae) and Wireworm (WW) *Agriotes sordidus* Illiger, 1807 (Coleoptera: Elateridae) are two underground crop pests present in Europe and North America. WCR used to be a specific corn (*Zea mays* L.) pest but one population recently became a pest for soybean (Curzi et al. 2012), and its economic impact in Europe is estimated at 700 million euros per year in case of no control (Wesseler and Fall 2010). WW causes economic damages on many vegetable and arable crops (Burgio et al. 2012) and is considered as one of the most harmful *Agriotes* species from an agronomic point of view (Furlan 2004).

Some pest management tools exist to control WCR populations such as pesticides, GMOs producing the *Bacillus thuringiensis* toxin, or crop rotation. However, at least one WCR population evolved and became resistant to each of these pest control methods (Gassmann et al. 2011; Pereira et al. 2017). Regarding WW, pesticide is the main method used to control pest population but the commission implementing regulation 2013/485/EU is restricting the use of neonicotinoids.

In this context, Prischmann et al. (2011) opened a new perspective for the biological control of WCR by showing that soil-dwelling predatory mites species may feed on WCR eggs and first instar larvae. Soil-dwelling predatory mites that inhabit the first centimeters of the soil showed a good potential to control pests such as nematodes, thrips or flies (Carrillo et al. 2015). Three species are already commercialized for their interest in biological control: *Gaeolaelaps aculeifer* (Canestrini), *Stratiolaelaps scimitus* (Womersley) and *Macrocheles robustulus* (Berlese). The purpose of this study is to document the predation success of these three species of generalist soil-dwelling predators on WCR and WW immature stages in a low cost/low time experimental set up, before investing in a larger scale trial.

Materials and methods

Biological material

Koppert Biological systems provided *Macrocheles robustulus*, commercialized under the name Macro-mite®. They were maintained on vermiculite for 2 months. *Gaeolaelaps aculeifer* was provided by EWH Bioproduction, Denmark. The population

was maintained during 8 months on a substrate made of 1/3 third blond sphagnum peat and 2/3 of fine vermiculite. *Stratiolaelaps scimitus* individuals are commercialized by Bioline AgroSciences, under the name Hypoline©. This population has been maintained on blond sphagnum peat for 2 years.

The three species used in the experiment were kept in climatic chambers at $25^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ and $70\% \pm 10 \text{ RH}\%$ and in darkness. A 100 mm x 94 mm bugdorm-5002 with 30µm nylon screen port sold by Bugdorm© were used as a rearing unit. 1 cm of plaster of Paris was flown in the Bugdorm. The colonies were also maintained permanently moist by addition of water three times a week. A mix of *Aleuroglyphus ovatus* stages was used as food and provided three times a week.

WCR diapausing eggs were provided by the Centre of Agriculture and Bioscience International (CABI), Hungary. They were stored at $7^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ below their temperature of development (Vidal et al. 2005). Before starting the experiment, we sieved the eggs from their substrate and selected only turgescient eggs for predation assays. We placed WCR eggs on plaster of Paris in a climatic chamber at $25^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ and $70\% \pm 10 \text{ RH}\%$. We added water twice a week on the substrate to lift the diapause and trigger eggs development. We checked daily for hatched eggs and collected first instar larvae for predation assays. Arvalis provided WW eggs and first instar larvae in Petri dishes filled with a sample of soil from their collection site. Once an egg or a first instar larva was isolated, we inserted it in the predation device with a predatory mite.

Predation tests

Adult mites (unsexed) were isolated and starved during 7 days in 2 mL Eppendorf tube containing 1 mL of dried plaster of Paris before the predation tests (El Adouzi. et al. 2017). The top of the tube was pierced and covered with a 106 µm wide nylon fabric to allow ventilation. 100µL of water was added every 3 days during the starvation period to maintain relative humidity necessary for the survival of the mites. The tubes were stored in a climatic chamber at $25^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ with $70\% \pm 10\% \text{ RH}$.

For each test, one egg or one larva of WCR or WW was introduced in a tube containing a predatory mite and the predation success was observed during 10 minutes with a stereo-microscope. We used an indirect source of light, controlled at

100 lux (measured with the Digital Illuminance meter TES 1335), to minimize natural behavior disruption of these lucifugous species. We considered predation to occur when mites impaled the prey with their chelicerae. We chose to observe predation on a short duration because some of the preys could suffer from the abrasive texture of the plaster of Paris if left to dry. The experiment was replicated twenty times for each prey/predator combination.

Statistical analysis

The statistical analyses were done on R. 4.0.3. Predation success when encountering a larva of WCR or WW was compared among the predator species using a General Linear Model (GLM) following a binomial distribution. As almost no predation on eggs was observed, except for *G. aculeifer* on WCR for which the confidence interval was not different from zero, predation from all species on eggs was considered inexistent and these data were not statistically analyzed.

Results and Discussion

The aim of this study was to investigate the predation potential of soil-dwelling predatory mites on WCR and WW. The predation success varied from 30 to 65% and was similar among the predator species either on WCR larvae ($\chi^2_{22} : 3.8$, $P = 0.150$) or WW larvae ($\chi^2_{22} : 2.2$, $P = 0.329$) (Figure 1). These results confirm the conclusion from Prischmann et al. (2011) on this development stage for *G. aculeifer*, and open larger perspectives for the biological control of both WCR and WW with other soil-dwelling predatory mites.

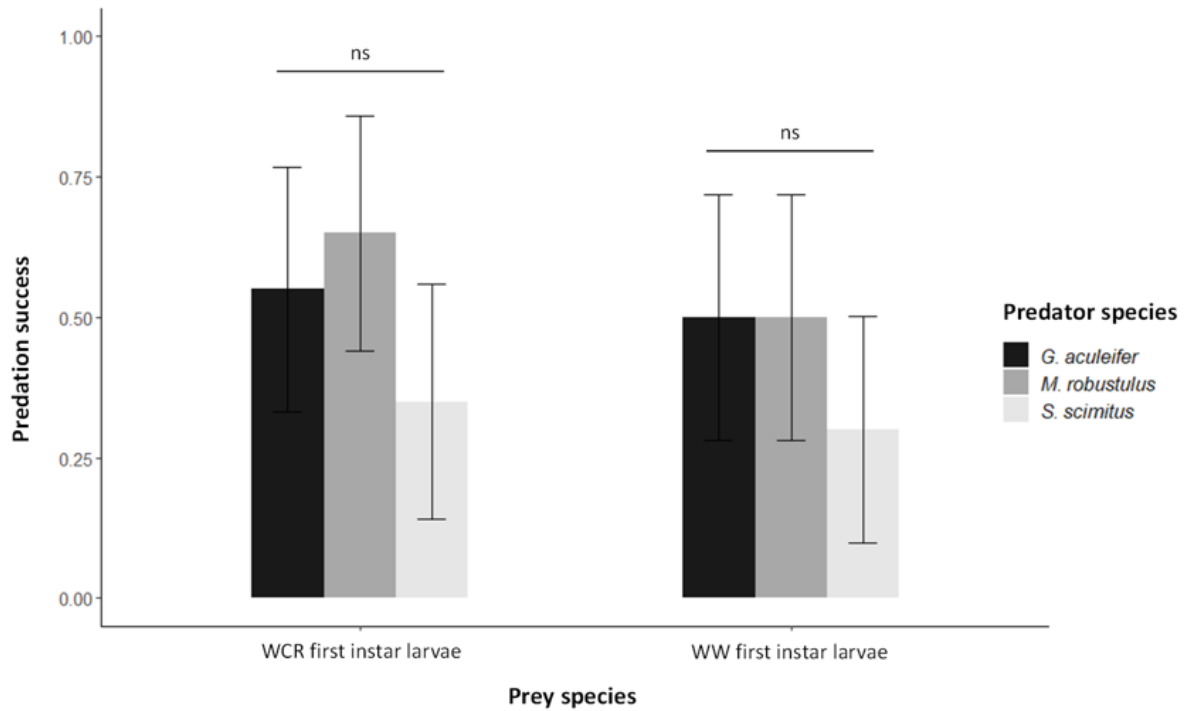


Figure 1. Predation success of *G. aculeifer*, *S. scimitus* and *M. robustulus* on WCR and WW first instar larvae during the 10-minutes predation assays. $n=20$. NS = no significant difference among predator species (p -value < 0.05). The error bars represent the 95% confidence interval for the predation success.

Regarding egg predation, WW eggs were never attacked by any of the three species, while in contrast with Prischmann et al. (2011) WCR eggs were attacked by *G. aculeifer* but only in 10% (+/- 13%) of the assays. The duration of experimentation (7 to 14 days for Prischmann et al. (2011)) might explain this difference in our results. In any case, our results emphasize the need to target a specific developmental stage to reduce damage most efficiently on plants. Indeed, eggs are not consumed by the predatory mites while later stages of WCR and WW are able to protect themselves against predators thanks to their hemolymph properties or the presence of a sclerotized cuticle (Furlan et al. 2004; Lundgren et al. 2009). First instar larvae might thus be the most sensitive stage for efficient control of WCR and WW by predatory mites.

Despite our simple experimental design, we established a proof of concept that predatory mites might consume larval stages of major maize pests, which open perspectives for the development of a biocontrol strategy. It is worth noting however

that our protocol did not reflect realistic environmental conditions. First, soil-dwelling predatory mites are very sensitive to low humidity (El Adouzi et al. 2017), yet as a total of 240 predatory mites were kept alive and active for 7 days using our protocol, this suggests that these conditions were adapted to all three species survival. Second, Abrams (2000) enhances the effect of many parameters such as population density which could prevent predation success. This stresses the necessity to set up larger scale experiments to consider whether predatory mites will effectively be able to find and control the first instar larvae of these pests in the soil.

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Discussion

Both Prischmann et al., (2011) and the work presented above showed that soil-dwelling predatory mites can effectively prey upon WCR early stages but not their eggs. This observation is of particular importance since WCR life cycle is univoltine and that larvae emergence happens at only one period of the year (Vidal et al., 2005). There was no significant difference between *M. robustulus* and *G. aculeifer* predation rate. However, being able to observe the entire duration of the test provided qualitative information that helped us select the best candidate for further research. We noticed that *G. aculeifer* tended to head towards its prey as soon as the prey was inserted in the arena, whereas *M. robustulus* used a sit and wait strategy by staying immobile, waiting for the prey to approach to consume them. This observation was also coherent with some observation made in the rearing unit, *G. aculeifer* was mobile around the food source whereas *M. robustulus* population was observed staying immobile on it. Based on this observation, we think that the small arena set up in our experiment increased *M. robustulus* predation success compared to more realistic conditions. Indeed, the biological control agent we select will need to find and eat WCR larvae in the soil before they reach maize roots, so the predator needs to be mobile to actively prospect the soil. This assumption is supported by the comparison of foraging strategies in the same class: Arachnida. A meta-analysis showed that the sit and wait strategy was less efficient compared to an active hunting strategy (Liu et al., 2015; Michalko et al., 2019) to control pests. Based on this observation and the favorable legislation we mentioned earlier, *G. aculeifer* seemed a suitable candidate for further investigation.

The potential of a biological control agent is often reduced at its ability to kill a pest. However, before reaching the field biological control agents have to be mass-reared, shipped and introduced. Once in the same environment as the pest, they also have to be active at the same time and location as the harmful organism. This lack of biological control products fulfilling these conditions lead to a lack of biological control solutions, claims by farmers (IBMA, 2019). In order to confirm the potential of *G. aculeifer*, we investigated whether these constraints might limit its efficiency in the field. First, the net reproduction rate, which defines the quantity of offspring per female, reaches 17.2 at 24 +/- 1°C and 100 %HR for *G. aculeifer* fed with *Tyrophagus*

putrescentiae (Barker, 1969), which confirms the possibility to produce large quantities of individuals in less than 3 weeks (Lobbess and Schotten, 1980). Second, despite a good population growth, high rates of cannibalism might harm the population either in rearing units or during shipment. However, multiple studies assessed this parameter for *G. aculeifer* and showed that cannibalism occurs only after weeks of starvation (Berndt et al., 2003; Usher and Davis, 1983). Third, Jensen et al., (2019) showed that *G. aculeifer* can resist 24h to -2°C and few days at 10°C. This resistance to cold temperature facilitates shipping in slowing down population development (Amin et al., 2014) during the transportation and still be active and able to control the pest when temperature rises. They also showed that this predatory mite species stay active at 9°C and achieve life cycle at 11°C whereas WCR eggs hatch when soil temperature is above 11°C (Levine et al., 1992), meaning that *G. aculeifer* will be active at WCR first hatches to ensure an efficient plant protection. Finally, *G. aculeifer* lives in the first centimeters of soil (Moreira and de Moraes, 2015), where WCR eggs hatch and where larvae feed on maize roots (Vidal et al., 2005), which increases the probability for the predatory mites to control WCR early stages.

We summarized here the multiple reasons why we chose *G. aculeifer* for the future research to control WCR. However, theoretical information and proof of concept in the lab are not sufficient to determine whether this biological control agent can effectively control the targeted prey. Many parameters can still interfere in the predator capacity to control the pest and efficiency has to be evaluated in more realistic conditions. This is the main objective of chapter 2.

CHAPTER 2 : TESTING A SPECIES OF SOIL-DWELLING PREDATORY MITES IN PRESENCE OF WCR IN A SOIL/PLANT/PEST/BIOCONTROL AGENT SYSTEM.

Introduction

The success of the predation test of *G. aculeifer* against WCR in the lab by Prischmann et al., (2011) led to two field trials on the Eastern South Dakota Soil using the soil-dwelling predatory mite: *G. aculeifer* (Prischmann-Voldseth and Dashiell, 2013). In these trials, the authors introduced both mites and WCR at sowing to assess the biological control agent efficiency to control WCR population and prevent damages on the plant. Multiple densities of mites (300, 600 mites per plant) were tested to determine the threshold density for maize protection. However, despite promising results in the lab, this work showed no significant impact of *G. aculeifer* presence on maize roots damage or WCR adult emergence and the authors concluded that the use of these organisms was not viable for WCR pest management. However, two details discussed in this work retained my attention. First, the authors highlighted that *G. aculeifer* was never recovered after their synchronized introduction with WCR eggs. They hypothesized that either mites spread away from the sample area or that they died from a lack of food. However, one might object that in this case, the actual capacity of *G. aculeifer* to control WCR was not tested here, but rather their capacity to remain alive around their introduction site until the emergence of WCR larvae, which occurred 15 days after release (Fisher, 1987). Moreover, the trial took place in Eastern South Dakota Soil and Water Research Farm near Brookings within a single set of agronomic and climatic conditions whereas the authors suggest a role of local fauna in the field trial results (Prischmann-Voldseth and Dashiell, 2013). These elements highlighted that the presence of predatory mites around the roots at the exact timing of WCR larvae emergence might be a key characteristic in their capacity to control WCR. To investigate this issue, we set up two trials with slight but significant variation from Prischmann-Voldseth and Dashiell, (2013). In the first trial in a greenhouse, we introduced various mite densities (100, 500 and 1000 mites per corn plant infested by the pest) at the estimated time of larvae emergence to highlight the impact of predatory

mites on first instar larvae and determine the optimal mite density enabling the control of WCR population under the harmfulness threshold. In the second trial in a maize field, we introduced the predatory mites at sowing (Predatory-in-first method), but combined with a known alternative food source : *Aleuroglyphus ovatus* (Barbosa and de Moraes, 2016; Rueda-Ramirez et al., 2018) to maintain and multiply the population on the furrow to ensure optimal presence of predatory mites when WCR eggs hatch. This work is presented in article 2.

Article 2 : A Promising Predator-In-First Strategy to Control Western Corn Rootworm Population in Maize Field

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Abstract:

Western Corn Rootworm is a pest of maize that mostly damages roots. Many alternative strategies have been explored to control this species, with little or non-lasting success, and it remains a threat to maize production worldwide. *Gaeolaelaps aculeifer*, a soil-dwelling predatory mite that inhabits the first centimeters of the soil, showed high predatory potential against WCR larvae in the laboratory. In this study, we explored the efficiency of *G. aculeifer* against WCR in more realistic contexts. First, we infested maize plants isolated in pots in a greenhouse with WCR, and tested the impact of different densities of mites on plant protection. Using standard indicators of WCR population presence and impact, we confirmed that *G. aculeifer* has the potential to control WCR at densities starting from 100 mites/plant. Then, considering that the release of a large amount of biocontrol agents at WCR emergence might be too costly and constraining for large-scale implementation, we tested the efficiency of a Predator-in-First strategy in a maize field infested by WCR. The goal was to introduce fewer *G. aculeifer* combined with *Aleuroglyphus ovatus* eggs as an alternative food source in order to let the mite population grow in the field and reach sufficient density at the critical stage for protection. This strategy gave comparable results to pesticide on all indicators examined in our field trial, highlighting the potential to manage this pest sustainably.

Keywords: predatory mites; *Diabrotica virgifera virgifera*; biological control; *Gaeolaelaps aculeifer*; underground pests; predator-in-first

Introduction

With 1068 million tons produced in 2017 [1], Maize (*Zea Mays* L., 1753) is the first cereal produced in the world. The Western Corn Rootworm (WCR) (*Diabrotica virgifera virgifera*) LeConte, 1858 is a ground beetle originating from Central America [2,3] whose larvae inhabit the first centimeters of soil and consume Maize roots [4], which decreases water and nutrients uptake [5] and increases the risk of lodging and attack from plant pathogens [6]. Pupae complete their development in the soil while adults feed on the aerial part of the plant such as silks. Although most economic losses are related to root damages by larvae, high densities of adult individuals can also affect plant reproductive organs, leading to a reduction in grain production and yield loss [7]. WCR is now established all over North America [8] and has been spreading rapidly in Europe since its first observation in Serbia in 1992. The lack of coevolution with native species prevents natural regulation of WCR by local predators, parasitoids or pathogens [9,10], thus resulting in huge economic losses. In 1986, the total cost resulting from yield decrease and pest management in North America was estimated over 1 billion USD [11].

Due to these important threats, major efforts were devoted to developing control strategies against WCR. These efforts were particularly challenged by the species' important capacity to adapt to traditional pest management methods. Some populations became increasingly resistant to pesticides [12] and Genetically modified organisms (GMOs) [13]. Although the situation in Europe appears currently under control [14] due to culture rotation use promoted by European legislation [15], resistance to crop rotations has been observed in the USA [16], and the demand for alternative control methods remains high. With this objective, many biological control solutions have been investigated, such as parasitoids [17–19], toxin from bacteria [20], entomopathogenic fungi [21,22] or nematodes [23–30] but none of these solutions proved suitable for large-scale use in maize fields.

Another promising, yet under-investigated strategy is the use of soil-dwelling predators of WCR larval stages. In particular, predatory mites are at the top of the soil trophic web [31]. Most species are mobile predators feeding on collembola, nematodes, insect larvae, insects eggs and other microarthropods [32,33]. Their effectiveness as biological control agents in regulating soil pest populations have contributed to their use both in the field and in the greenhouse [34,35]. A first test of their potential against WCR was done by Prischman et al., [36], confirmed recently by

Pasquier et al., [37], who tested the predation success of 6 mite species from 4 different genera on WCR in the laboratory. Among the different species tested, *Gaeolaelaps aculeifer* Canestrini (Arthropoda: Mesostigmata) showed the best results. *Gaeolaelaps aculeifer* is a soil predatory mite used as a biological control agent in greenhouse for controlling populations of nematodes, mealybugs, soil flies, and thrips pupae [32,38–40] in 17 European countries [41]. This cosmopolitan organism naturally present in the soil [42] can move and burrow up to several tens of centimeters to find its prey. [43] highlight its capacity to overwinter and its resistance to low temperature. A laboratory experiment demonstrated the voracity of the species since in 1 out of 2 times, it is able to detect and consume prey of varying sizes (200 µm to 5 mm) in less than 10 min [37]. This work also showed that *G. aculeifer* does not feed on WCR eggs, meaning the biocontrol strategy needs to target the neonate stage.

In the present study, we first tested the potential of *G. aculeifer* to control WCR in greenhouse. We released different density of *G. aculeifer* around single maize plants infested with WCR at the estimated time of larvae emergence [44] to establish (a) whether predatory mites are able to find and eat WCR larvae directly in the soil (b) to estimate *G. aculeifer* density to control WCR populations at larvae emergence. However, to be applied efficiently in the field, this method of introduction needs to overcome several limitations: (i) introducing high density of predatory mites is particularly expensive; (ii) introducing biocontrol agent at larvae emergence requires supplementary intervention for the farmer at 4–5 leaves stage; (iii) the vulnerable developmental stage, i.e., the WCR larvae emergence [37] is difficult to predict, may vary between environments, and may be missed when releasing predatory mites [45]. Based on all these constraints, we explored a second time another strategy in a field trial. The Predators In First (PIF) strategy, first suggested by McMurtry et al., [46], aims to let the predators establish before the targetable pest or stage is present. This method relies on generalist predators' capacity to feed, survive and reproduce on an alternative food source provided by the environment [46–49]. This method showed its efficiency on predatory mites *Amblyseius swirskii* (Acari: Phytoseiidae) Athias Henriot, 1962 previously introduced and fed with pollen to control thrips [50,51]. In this second experiment, we investigated the efficiency of a PIF in one maize field naturally infested by WCR at the egg stage, which is not targetable by predatory mites [37]. We introduced a smaller number of predatory mites (compared to the inundative strategy in the greenhouse experiment) early in the season (at sowing), combined with

alternative food in order for the mite population to reach a sufficient density when WCR larvae, i.e., both the sensitive and the harmful stage emerge. Indeed, WCR overwinters as eggs and emerge only when temperature increases at spring (Fisher, Hampton, NH, USA, 1987) and maize plants have grown enough to provide food for the larvae [8]. Because the delay between introduction (sowing) and larvae emergence is hard to predict [52], it was necessary to sustain the *G. aculeifer* population with an alternative food source during this period. We chose to use a known prey for *G. aculeifer*, *Aleuroglyphus ovatus* (Troupeau 1879) [53,54], which is also used in our rearing unit at Bioline AgroSciences. To establish a proof of concept of this PIF strategy in realistic conditions before wider experimental plan, we led the trial in one maize field and compared it with pesticide treatment and negative control.

Material and Methods

Greenhouse Trial

Plant Material

The maize variety used in the greenhouse experiment is “Figaro”, commercialized by the seed company “Semences de France”. The seeds used were produced in 2019 and were not treated by the manufacturer. They were introduced at 5 cm depth into the soil and covered with soil.

Arthropods Production and Preparation

Diabrotica virgifera virgifera.

Diabrotica virgifera virgifera eggs were provided by CABI (Centre for Agricultural Bioscience International) laboratory in Hungary, then stored for 22 days below their developmental threshold temperature, at 7 ± 0.5 °C. Eggs were separated from soil using metal sieves of 355 and 600 µm mesh. Eggs contained in the 355 µm mesh sieves were collected and inspected using an Olympus SZ 61 binocular microscope to separate viable eggs from damaged ones. Color and turgidity were used as criteria to distinguish viable, light-coloured eggs from presumably damaged, dark-coloured eggs. Viable eggs were collected with a polyamide fibers brush, 1.3 mm (Ref: Pebeo Lotus Deco Round No.10-0) and used in the greenhouse trial.

Gaeolaelaps aculeifer.

In the greenhouse experiment, mites came from EWH Bioproduction, Tappernøje

Denmark. The population was maintained during 8 months on a substrate made of 1/3 blond sphagnum peat and 2/3 of fine (0.5 to 2.8 mm) vermiculite and fed a mixture of all *A. ovatus* stages [37]. Predatory mites were stored in climatic chambers at 25 ± 0.5 °C and $70 \pm 10\%$ RH with a constant obscurity. A mixture of all stages of *A. ovatus* was used as food, and extra water was provided three times a week in a 100 × 94 × 60 mm³ bugdorm-5002 with 30 µm nylon screen port sold by Bugdorm© Megaview science corporation, Taiwan. Before application, *G. aculeifer* population sizes in the bugdorms were assessed. For doing so, a sample of the sphagnum / vermiculite was weighed and then sieved using a 500 µm metal sieve to remove substrate and a 250 µm metal sieve to retrieve mites. *G. aculeifer* individuals were then poured into 50 mL of distilled water then homogenized. Three samples of 2 mL of this mix were placed on a counting chamber under a SZ61 Olympus binocular microscope with a drop of surface-active agent to break the water surface tension and counted (37 ± 1.73 mites/2 mL). *Gaeolaelaps aculeifer* density in the rearing medium was then calculated, and Falcon tubes containing the required weight of substrate for each maize pot were prepared. At last, each Falcon tube containing the mix of growth substrate and *G. aculeifer* individuals was released close to the maize plant on the soil surface of each maize pot at the estimated time of WCR larvae emergence [44].

Experimental Setup

The experiment was performed in greenhouses at Bioline Agrosiences research site located in Valbonne, France, in 2019. Greenhouses ventilation and cooling parameters were set not to exceed 30 °C (Range 15 °C–30 °C). The cooling system is made of a ventilation spreading water inside the greenhouse reducing temperature as well as increasing humidity (~60%). The experiment to test the effect of *G. aculeifer* density to control the WCR population was performed in a compartment (48 m²) with 6 rows composed of ten maize plants (60 plants in total). Each maize plant grew up isolated in a black plastic 50L pot with a top-diameter of 46 cm and a bottom-diameter of 39 cm. Each plant was watered with 1 L of water each day except on Fridays where 2 L were poured in the pots and no extra water was added until Monday. Three 25 mm diameter holes were drilled at 4.5 cm of the bottom of each pot, to make sure no excess of water would result from plant watering. 50 L of topsoil were used as growth substrate for each maize plant. This topsoil was composed of 72% dry matter and organic matter represented 5.9% of the dry matter content. Water holding capacity was estimated at

150% by the supplier, conductivity was 1.36 mS / cm and pH was equal to 7.5. The 60 plants were equally distributed between the 5 treatments: 12 Maize plant only (Maize), 12 Maize infested by *D. virgifera virgifera* (maize + WCR), and 3 treatments involving maize infested by *D. virgifera virgifera* and inoculated with *G. aculeifer* (maize + WCR + mites) with various mite densities: (i) 12 plants with 100 *G. aculeifer* (WCR + 100 mites), (ii) 12 plants with *D. virgifera virgifera* + 500 *G. aculeifer* (WCR + 500 mites) and, (iii) 12 plants with *D. virgifera virgifera* + 1000 *G. aculeifer* (WCR + 1000 mites). Pots placement within the greenhouse and the distribution of treatments over pots were both randomized to prevent any edge effects.

For treatments with WCR infestation: Twenty-one eggs of *D. virgifera virgifera* sub-divided in three separated Eppendorf tubes were introduced at three distinct spots around each plant, at a depth of 10 cm beneath the soil surface [8] at sowing. For treatment with mites: A mix of *G. aculeifer* stages were then introduced at the estimated time of WCR larvae emergence (300-degree days base 11 after WCR eggs introduction, [44]) as previous work suggested that eggs are not identified as prey by *G. aculeifer* [37].

Maize development was assessed by monitoring the nitrogen levels of maize. Nitrogen deficiency is a commonly used indicator to assess WCR physiology impact on plants [55]. Nitrogen level was measured using a N-tester Nitrogen testing device commercialized by Yara [56]. This device optically measures the leaves chlorophyll content, which is correlated with plant nitrogen level. Thirty measurements are necessary to obtain one value for chlorophyll content per plant. These measurements were taken on the youngest fully developed leaf of each plant for each group when plants were at the 7–8 ligulate leaves stage. In order to assess the impact of the presence of various mite densities on *D. virgifera virgifera* development, WCR adult emergence was monitored. WCR adults were encased around each plant using tights as insect-proof nets. Traps were glued on the inside of each pot before adults emerged [44], and checked daily for emergence during 60 days. However, inspection of all parts of the pot was also necessary since adults could be found on the soil surface, on the plant or on the net. WCR adults were removed from pots and traps after each monitoring and placed in 70° alcohol to prevent spread in the trial area. Finally, to assess the potential damages induced by WCR larval stages on the root system of maize plants, each maize plant was pulled and washed out once adult emergence was over. The root system damage was ranked, using the widely used 0–3 IOWA ranking

scale [57]. The lower level, i.e., the grade “0”, was used when no visible damage was present; the grade “1” was used when damage marks were visible. The grade “1.5” was used when damage marks were visible with less than three roots severed and the higher grade i.e., the grade “3”, was used when damage marks were visible with three or more than three roots severed.

Statistical Analyses

All statistical analyses were carried out using R software (R Development Core Team, version 4.0.3). The chlorophyll index or the residuals of the following models involving chlorophyll index as the dependent variable followed a normal distribution, confirmed by a Shapiro–Wilk test and visual interpretation of quantile–quantile plots, and were therefore analyzed using Linear Models (LM). By contrast, the number of WRC emerged or the residuals of the following models involving the number of WRC emerged as the dependent variable did follow a Poisson distribution (commonly used to represent count data distribution) and were therefore analyzed using Generalized Linear Models (GLM). The root system damage estimated using the 1-3 IOWA ranking scale was analyzed using the Mood’s median test enabling the comparison of ordinal dependent variables (‘RVAide-Memoire’ package).

To test the hypothesis that the presence of predatory mite (i) reduces the population of WRC, (ii) limits their impact on plant nutritional quality (N level), and (iii) reduces the root system damages in the greenhouse trial, the impact of the treatments (maize vs. maize + WRC vs. maize + WCR + mites) on the number of emerged *D. virgifera virgifera* adults, the chlorophyll index per plant and the 1–3 IOWA ranking scale were evaluated, respectively (treatments using varying mite densities were pooled in this analysis). As an impact of the mite presence has been detected and in order to identify which density of predatory mite is necessary to control WRC populations with a limited impact on plant quality under the same conditions, the impact of the mite density (100, 500 or 1000 individuals per plant) in the maize + WCR + mites treatment on the number of WRC emerged, chlorophyll index per plant and the 1-3 IOWA ranking scale has been tested.

Multi-comparison tests were used to compute pairwise comparisons of the number of emerged *D. virgifera virgifera* adults and the chlorophyll index among the treatments (maize vs. maize + WRC vs. maize + WCR + mites) using the ‘multcomp’

package (Tukey method). Multi-comparison tests of the 1-3 IOWA ranking scale among the treatments were performed using the Bonferroni adjustment method.

Field Trial

Plant Material

For the field trial, the maize variety used was DKC5830, commercialised by “DEK-ALB” (Annexe 1). The seeds used were produced in 2020. Seeds were sowed the 17th April 2020 using a pneumatic plot drilling machine regardless of the treatments.

Arthropods Production and Preparation

Diabrotica virgifera virgifera were naturally present in the field of experimentation.

Sterilized *A. ovatus* eggs were used as an alternative food source for predatory mites during the field trial. These sterilized *A. ovatus* eggs are produced by Bioline AgroSciences and commercialized as Predafix© Bioline AgroSciences, Clacton-on-Sea, England. *A. ovatus* eggs were formulated as aggregated particles, whose formulation is currently protected by a consortium agreement. These particles were produced in March 2020 and stored for 33 days at 4 °C before sending. After arrival at the research site, they were kept 44 days at 2.2 °C. Calibrated 0.25 g doses of formulated sterilized *A. ovatus* eggs (~126400 eggs) were introduced in the entire maize plot immediately after sowing at a depth of 5 cm. A micro granulator was used to ensure homogeneous distribution of the product in the furrow.

Gaeolaelaps aculeifer. In the field trials, mites were produced by Biological Services, Australia (product and commercialized as Killer mites©). Predatory mites were stored for 71 days before introduction in climatic chambers at 25 ± 0.5 °C and $70 \pm 10\%$ RH with a constant obscurity on a substrate made of 1/3 blond sphagnum peat and 2/3 of fine (0.5 to 2.8 mm) vermiculite. A mixture of all *A. ovatus* stages was used as food, and extra water was provided three times a week in a 325 × 150 × 135 mm³ Bugdorm© Megaview science corporation, Taiwan with 30 µm nylon screen port sold by Saulas. Mites counting followed the same process as in the greenhouse experiment (see greenhouse trial) except the mites and substrates were prepared in 500 mL saltshakers. Due to the lack of knowledge on the potential effect of a mechanical introduction on mites' viability, mites were spread by hand on micro-plot of 1 m².

Experimental Setup

The experiment was performed in an irrigated maize field located in Castagnole Piemonte, in Piedmont Region (Northwestern Italy) in 2020 (coordinates: 44.90603611 N; 7.57085277 E). Soil composition and environmental conditions are provided in Annex 1. This field was chosen because WCR was already present at high density and source of yield loss. No additional WCR individuals were introduced. The trial area was set up at least 50 m of field edge to prevent any edge effect. An area of 35 m length (48 rows) per 10 m width was isolated from the surrounding field treatment by an additional 10 m width with no treatment zone as a buffer zone. Among the 48 rows, 12 plots of 4 consecutive rows were designed and treated. Within each plot, only the two central rows were sampled to prevent edge effects. Plots were assigned randomly to one of the treatments giving this sequence: (i) 100 mites in each microplot over an area of 1 m² + 0.25 g / plant of alternative food composed of *A. ovatus* eggs particles ("Biological control treatment"), (ii) no pest control treatment ("control treatment") and (iii) FORCE Ultra pesticide application on the furrow during the sowing at 12.2 kg/ha ("pesticide treatment"). This sequence of biological control/control/pesticide was kept for the 9 remaining plots. In each "biological control" plot, mites were spread on 4 micro-plot of 1 m² (c.f production of *G. aculeifer*), WCR population assessment and damage on maize were conducted only on this micro-plot whereas assessment for control and pesticide treatment were conducted randomly over the entire surface of each plot.

Maize development was assessed by monitoring the nitrogen levels of maize at two different plant development stages: when the plant had 7–8 leaves (12th June) and during the flowering period (16th July), for 4 plants randomly selected in each plot. Nitrogen levels were measured using a SPAD testing device commercialized SDEC. This device optically measures the chlorophyll content of leaves which is correlated with plant nitrogen level [58]. The thirty required measurements were taken on the youngest fully developed leaf of each plant for each group when plants were at the 7–8 ligulate leaves and at the flowering stage. In order to assess the impact of the presence of predatory mites (associated with alternative food source) on *D. virgifera virgifera* population dynamics, the number of emerging WCR adults was assessed. In each plot, 4 emergence cages were set up before first emergence. One plant is trapped inside a metallic cylinder of a diameter 25 cm and 60 cm high which half of it is inserted in the soil. A net is attached to the cylinder and maintained taut with a stick to prevent the net from impacting maize growth. This device prevents WCR adults from escaping.

Individual removal from the net is done through a zipper present all along the net length. WCR adult collection started once first emergence was recorded on yellow chromotropic traps placed in the field, then after 0,8,16,22,29,37 and 45 days WCR adults were counted and removed from the cage. Finally, the assessment of the potential damages induced by WCR larval stages on the root system of maize plants was realized as described above. Four maize plants per plot were pulled on 16th July and washed out once adult emergence was over. The root system damage was ranked, using the 0-3 IOWA ranking scale [57].

Statistical Analysis

All statistical analyses were carried out using R software (R Development Core Team, version 4.0.3). The chlorophyll index or the residuals of the following models involving chlorophyll index as the dependent variable followed a normal distribution, confirmed by a Shapiro–Wilk test and visual interpretation of quantile–quantile plots, and were therefore analyzed using Linear Mixed Models (LMM). By contrast, the number of WRC emerged or the residuals of the following models involving the number of WRC emerged as the dependent variable followed a Poisson distribution (commonly used to represent count data distribution) and were therefore analyzed using Generalized Linear Mixed Models (GLMM). The root system damage estimated using the 1-3 IOWA ranking scale was analyzed using the Mood's median test enabling the comparison of ordinal dependent variables ('RVAideMemoire' package).

In the models analyzing the number of emerged *D. virgifera virgifera* adults per plant, the chlorophyll index in both the 7–8 leaves stage and flowering stage and the 1-3 IOWA ranking index, pest control treatment (no treatment vs. introduction of both mites and food vs. pesticide application) was modelled as a fixed effect. In the LMM, each plant represented a technical repetition (30 and 20 measures have been taken for each 7–8 leaves stage and flowering plant, respectively) and was modelled as a random effect to consider the dependence of replicates from the same plant. In the GLMM, the emergence cage was modelled as a random effect to consider the dependence of replicates from the same cage.

Multi-comparison tests were performed to compare the number of emerged *D. virgifera virgifera* adults per plant and the chlorophyll index among the pest control treatments (no treatment vs. introduction of both mites and food vs. pesticide application) using the 'multcomp' package (Tukey method). Multi-comparison tests of

the 1-3 IOWA ranking scale among the treatments were performed using the Bonferroni adjustment method.

Results

Greenhouse Trials

The chlorophyll index varied depending on the treatments (maize vs. maize + WRC vs. maize + WCR + mites) but not the mite density in the maize + WCR + mites treatment (Table 1, Figure 1). Using pairwise comparison, the control mean chlorophyll index (590 ± 7.04) was 19% higher than the maize + WCR treatment (484 ± 16.9 , $p < 0.001$) (Figure 1). The maize + WCR + mites treatment had a similar Chlorophyll Index (586 ± 7.04) as the control treatment ($p = 0.926$), and significantly higher than the WCR-only treatment ($p < 0.001$).

Table 1. Results of Linear (F value) and Generalized Linear Model (χ^2 deviance) on the number of adult *D. virgifera virgifera* emerged per plant, the nitrogen content of plant, and the root damage index (IOWA) in both greenhouse and field. Factors included in the models are described as explanatory variables. Significant effects are indicated in bold text. Df corresponds to the degree of freedom.

	Variables to Explain	Number of Adult Emerged			Chlorophyll Index						Root Damage Index		
		Deviance	Df	<i>P</i> Value	Young Plant			Flowering Plant			Deviance	Df	<i>P</i> Value
Greenhouse Experiment	Maize vs. Maize + WCR vs. Maize + WCR + Mites	151.0	2	<0.001	34.6	2.56	<0.001				23	2	<0.001
	Maize + WCR + 100 Mites vs. Maize + WCR + 500 Mites vs. Maize + WCR + 1000 Mites	1.3	2	0.532	0.13	2.33	0.877				2.9	2	0.235
Field Experiment	Control vs. Biological control vs. pesticide	67.1	2	<0.001	23.9	2	<0.001	1.5	2	0.223	56.4	2	<0.001

Root damages represented by the IOWA index varied significantly according to the treatments but not the mite density in the maize + WCR + mites treatment (Table 1, Figure 1). The mean IOWA Index for maize + WCR treatment was close to the highest possible value (2.55 ± 0.24) and significantly higher than both the maize (no damage) and the maize + WCR + mites treatment (0.57 ± 0.12 ; $p < 0.001$).

The number of emerging adults also varied depending on the treatments but not the mite density in the treatment using *G. aculeifer* (Table 1, Figure 1). The number

of adults was close or equal to zero in control pots and in maize + WCR + mites (0.56 ± 0.12) treatment while on average 6.1 ± 0.48 adults per plant were captured in maize + WCR treatment ($p < 0.001$).

Field Trials

The SPAD reading showed slight, but significant differences among the three treatments for the 7–8 ligulate leaves stage (control: 36.6 ± 0.38 , pesticide: 38.81 ± 0.49) (Table 1, Figure 2) with an advantage for the biological control treatment (41.04 ± 0.48 ; all $p \leq 0.001$). These differences did not persist at the flowering stage (control: 48.43 ± 1.03 ; pesticide: 50.63 ± 0.93 ; biological control: 50.08 ± 0.85 ; Table 1).

Root damages represented by the IOWA index also showed a significant and similar impact of both pesticide (0.73 ± 0.10) and biological control (0.25 ± 0.11) treatments when compared to the control (2.45 ± 0.13 ; all $p < 0.001$) (Table 1, Figure 2). Despite very little variation and the lowest mean response, results from the biological control treatment were not significantly different from those obtained under the pesticide treatment ($p = 0.46$).

WCR adult emergence, represented by the mean number of WCR per plant, varied significantly with the treatments (Table 1, Figure 2) and was higher in the control treatment (1.52 ± 0.09) than either in the pesticide (0.65 ± 0.06 ; $p < 0.001$) or the biological control treatment (0.54 ± 0.05 ; $p < 0.001$). No significant differences among biological control and pesticide treatment were found ($p = 0.50$).

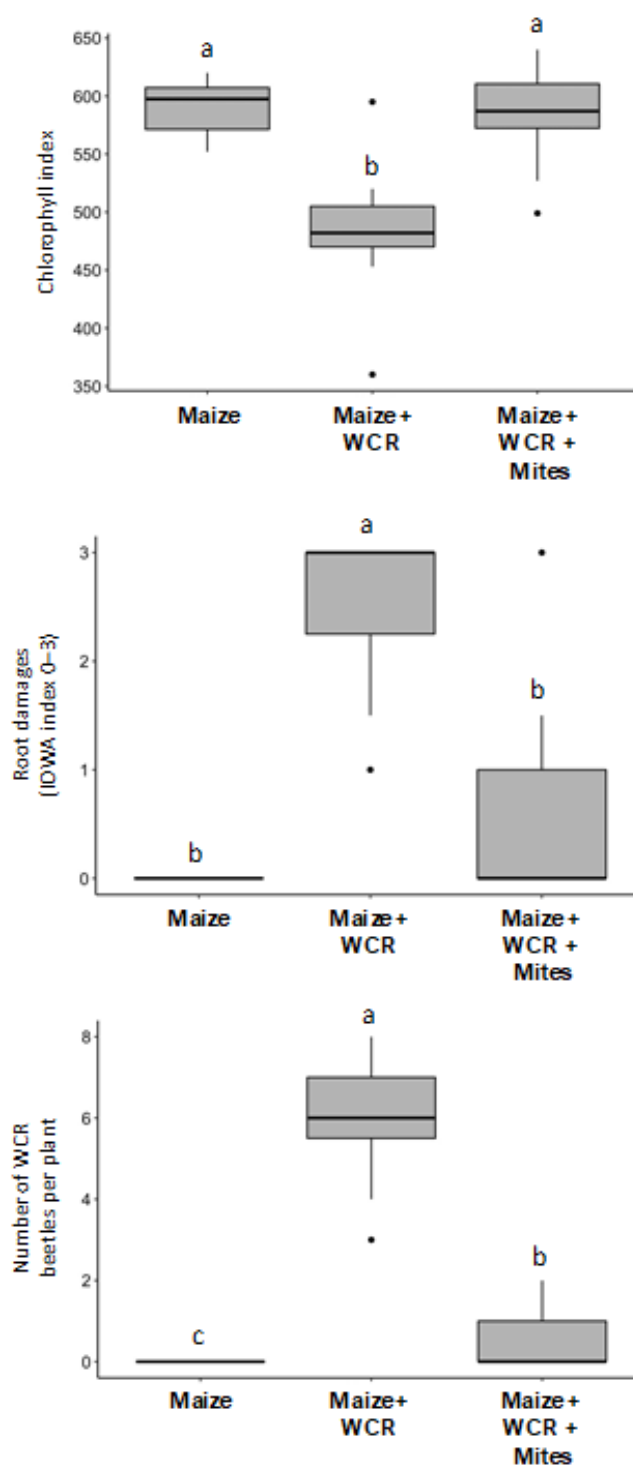


Figure 1. Greenhouse trial. Impact of the maize treatments (maize vs. maize + WCR vs. maize + WCR + mites) on the chlorophyll index (N-tester; top panel), the root-damage index (IOWA index: 0-3; middle panel) and the total number of adults emerging. Boxplot topped by the same letters did not differ significantly.

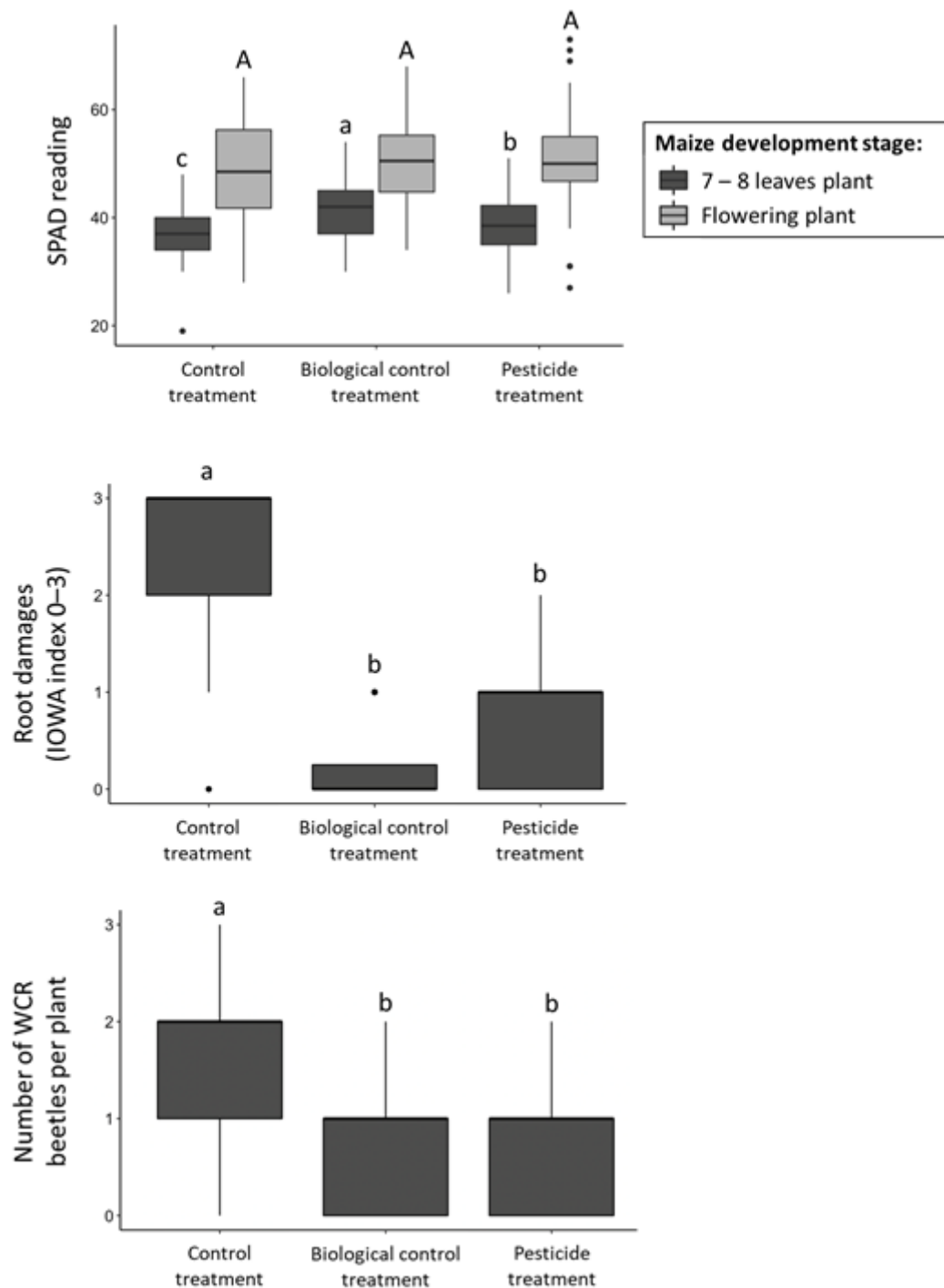


Figure 2. Field trial. Impact of the treatment type (control, biological control through the use of predatory mites and pesticide treatments) on the maize chlorophyll index (SPAD-reading; top panel), the root-damage index (IOWA index: 0-3; middle panel) and total number of WCR adults emerging per plant (low panel). Boxplot topped by the same letters did not differ significantly.

Discussion

Our results provide the first empirical demonstration of the ability of soil-dwelling predatory mites to prevent WCR damages on maize plants. Furthermore, we show preliminary evidence that a Predator-in-First strategy may provide a level of control equivalent to pesticide treatment, while reducing the costs related to the production and introduction of predatory mites.

To evaluate WCR impact on plants and approximate potential yield loss, we used indicators of plant health, WCR population and WCR damages. We first estimated larvae density and development by observing root damage using a 0–3 scale called IOWA index, a proven reliable indicator of WCR damage on roots. However, observable damage does not necessarily induce physiological deficit for the plant due to plant resilience to pest attack [59]. We thus collected the chlorophyll Index in order to identify the global physiological impact of WCR on the plant once larvae already started to attack roots (7–8 leaf stages). Then, we assessed adult emergence to verify whether the biocontrol treatment affects the capacity of WCR to achieve its life cycle. We showed that adult emergence, roots damage and chlorophyll index were all correlated indicators of the WCR population impact on maize plants. These parameters can thus be used confidently to assess the capacity of treatments to control WCR population and limit their impact on maize plants.

We considered that treatments in both trials were conducted on surfaces insufficient to have a representative yield loss but Haegele et al., [60] highlighted the strong relationship between chlorophyll and grain yield for maize. Urías-López et al., [55] showed that decreases of the chlorophyll index in maize attacked by WCR led to a reduction in plant height. Many studies showed that WCR physiological impact on the plant can lead to significant yield loss [59, 61–63]. In both experiments (greenhouse and field trials), chlorophyll index in young plants decreased in the presence of WCR larvae and remained high when pest populations were absent or controlled (by pesticide treatments or using predatory mites). These results confirmed that decreasing WCR population density would improve plant physiology and yield. Previous work [64] confirmed this statement by showing 4 adults per plant induced damage on maize whereas 1 adult per plant did not decrease yield. The WCR density for biological control and pesticide treatment was below 1 in our study even if this threshold has to be used carefully since plant response to WCR attack also depends of plant health [62]. Conversely, the presence of pests does not seem to modulate the

chlorophyll index when the plants have reached the flowering stage. However, according to Urías-López et al. [55] plant height or grain yield could already have been impacted by chlorophyll index decrease at younger stages.

All indicators showed that the inundative strategy allowed to control WCR population and preserve plant health. The greenhouse experiment aimed at determining the optimal mite density that should be introduced to achieve efficient control of WCR larvae in a soil. The three mite densities tested (100, 500, or 1000 mites per plant) showed similar and positive results since both WCR population density and root-damage index decreased significantly, while physiological plant indicator (chlorophyll index) improved with mites presence. Further studies were planned to assess effect of *G. aculeifer* smaller density on WCR population, but they were not feasible within the timeline of the project due to global conditions (2020 covid-19 pandemic). Minimum *G. aculeifer* density threshold to control WCR population at larval stage remains to be determined.

Gaeolaelaps aculeifer timing release is a key element for the successful control of WCR population. A previous study conducted by [65] also tested *G. aculeifer* inundative strategy against WCR. They used density comparable to what we introduced in the greenhouse trial (150 mites/plant or 300 mites per plant) and surprisingly showed no effect on WCR population. The main difference between their trial and ours is the timing of introduction of the predatory mites. In their study, they introduced mites and WCR eggs at the same time whereas we introduced mites secondarily at the estimated time of larvae emergence. Soil-dwelling predatory mites and especially *G. aculeifer* showed very high capacity to disperse to find food and sufficient humidity (personal observation). We assume that in the Prischmann-Voldseth and Dashiell experiment, *G. aculeifer* might have spread away from the plot while the eggs were maturing, so that they were not present around the plant when the eggs hatched. Alternatively, if the amount of natural resources in the soil was insufficient to sustain the mite population, the number of mites surviving until WCR larvae emergence might have been greatly reduced. This highlights the need to sustain reliably the population of predatory mites close to the plants over a prolonged period until WCR larvae emerge.

100 mites per plant still represent a large number of individuals at field scale but the alternative Predator-in-first strategy we tested in the second experiment allows reducing the density introduced while maintaining efficiency. In the greenhouse trial,

100 mites per plant were introduced. Since a maize field contains up to 90000 plants per hectare over extended surfaces [1], this quantity is not expected to be viable in agronomic conditions. To reduce the density, 100 mites/m² with alternative food were introduced in the field trial, amounting for approximately 11 mites per plant. The density was then expected to reach higher levels at the time of WCR larvae emergence and keep them along the furrow. We found that this predator-in-first strategy coupled with the introduction of alternative food was efficient to control WCR populations, with a performance equivalent to the pesticide treatment. The introduction of a low number of individuals at sowing with alternative food has many agronomic advantages. It could decrease the purchase cost of predatory mites, the production of these organisms being particularly expensive under current technology. The predator-in-first strategy is expected to reduce the work load of farmers by allowing them to introduce the individuals at the same time as planting. It also helps ensuring that the predatory mites are present in the soil at the vulnerable stage of WCR development, as the precise timing of WCR larvae emergence in natural conditions can be difficult to predict [45]. Predator-in-first strategy was first developed on phytoseiid predatory mites to control thrips [46]. This method's main concept is to introduce predatory population before the peak of pest emergence, so that the predator population is established and amplified when the sensitive stage for predation occurs. The main limit to this strategy is that it requires predators to be able to feed on alternative food sources, but in our case *G. aculeifer* showed its capacity to feed on many different food sources such as pollen [66], dust mites *Tyrophagus putrescentiae* [67] or eggs from *Aleuroglyphus ovatus* [54]. The last was used in our experiment with a small amount of predatory mites introduced at sowing in order to initiate the population. Our results showed that predatory mites with alternative food were highly efficient to protect the plant against WCR under field conditions. Indeed, all the indicators used in the experiment lowered pest pressure on roots, decreased adult emergence and increased chlorophyll index at 7–8 leaves compared to the area without treatment. Moreover, biological control showed an efficiency comparable to pesticide treatment, enhancing its potential as an alternative to the common solution in this pest management. More than a promising result for WCR control, it showed that biological control relying on population dynamics and Predator-in-first strategy can help farmers in their work. Indeed, Predator-in-First strategy decreases the necessary amount of biocontrol agents to introduce and thus reduces cost, it does not require knowledge of larvae emergence timing to spread

solution, and the farmer may introduce the biological agent simultaneously with another mechanical intervention.

Natural predation is unlikely to have caused WCR population control in our field trial. In the present study, we added *A. ovatus* eggs in the soil to enhance *G. aculeifer* population. The introduction of an alternative food source could by itself attract other predators such as other predatory mites species or Staphylinidae also present at the top of the food chain in crops [31]. However, [68,69] highlighted the negative impact of continuous crops and pesticide on predatory organisms. Both these factors being frequently present in maize crops, we assume that natural predatory density is degraded enough to prevent it from controlling WCR population in most agronomical contexts. This assumption is confirmed by the lack of natural control by endemic predators both in North America and in Europe and the necessity to apply culture rotation to lower WCR population density [14] despite years of WCR presence in the soil. Our own samples from 3 maize fields in 3 maize production areas in France and Italy also showed extremely low natural densities of soil predators (T. Andrieux, personal communication, less than 200 predatory mites for 650 samplings of approximatively 150 g of wet dirt).

The potential long-term presence of predatory mites in soil has not been considered yet. *G. aculeifer* presence in the soil and its capacity to overwinter suggest that temperature conditions are not the issue for the population to maintain from one spring season to another [43]. The second essential parameter, high amount of humidity in soil, is not considered as a limiting factor either because a low density of individuals is still present in the soil meaning they found appropriate conditions to survive all year long. We assume that food availability is the most likely limiting factor for sustaining predatory mites populations at sufficient levels to control WCR because of low biomass in continuous maize fields [68,69] (e.g., 1.94% organic matter in the Castagnole Piemonte field see Supplementary material). We assume the PIF strategy we explored in this work can help to transition from a chemical pesticide habit which significantly impacts biodiversity and biomass to a more integrated management by providing supplementary biomass in this damaged environment.

Adopting relevant farming practices could enhance predatory mites efficiency to control WCR. Kautz et al., (2006) highlight the positive impact of different types of manure introduction on predatory mites populations whereas Cortet et al., (2002) showed a negative impact when tillage is applied. Hamers and Krogh, (1997)

concluded on the negative effect of pesticide on *G. aculeifer* specifically. In the scope of reducing the impact of agronomic practices on the environment, these conclusions suggest that adopting European legislation recommendation (directive 2009/128/EC) could further improve the performance of the biocontrol solution we investigate in this study.

As a generalist predator, *G. aculeifer* could be used against another major maize pest. Wireworms are another coleopteran species able to feed on maize roots and many other crops of economic interest [70]. In previous work in laboratory conditions ([37], *G. aculeifer* showed its capacity to attack *Agriotes sordidus* first instar larvae but these promising results will require further investigation in field conditions. *Agriotes sordidus* neonate stages are asynchronized with WCR neonate emergence [8,71] which would complicate a single, inundative introduction strategy. In contrast, a strategy based on the early introduction of soil-dwelling predatory mites combined with alternative food as we proposed in the present work could maintain the population through both emergences to control both population pests.

This work is definitely promising but requires further investigation to confirm its potential. As we mentioned earlier, the population dynamics of predatory mites, and biological control agents in general, highly depend on environmental conditions such as food availability, temperature and humidity [72,73] which in turn depend on agronomical practice such as pesticide use, irrigation, manure or tillage [74,75]. Predators-in-first strategy lets the biocontrol agent confront these conditions for a long period and we only tested this method in one scenario of predatory mites dose, WCR density, food source quantity, temperature, humidity, soil composition whereas each of these parameters can impact positively or negatively mites efficiency to control WCR population. This work will have to be particularly addressed to consider an applicable biological control solution against WCR in all its areas of nuisance [76].

Supplementary Materials:

The following are available online at <https://www.mdpi.com/article/10.3390/agronomy11101984/s1>, Field Trial Report: Efficacy and crop selectivity of *Aeuroglyphus ovatus* egg's strategy against *Diabrotica* spp. (DIABVI) on *Zea mays*.

Author Contributions:

A.P., T.A., M.F., A.M., B.K., C.C. and E.V. conceived and designed the experiments;

A.P., T.A., A.M., B.K. and C.C. performed the experiments; A.P., L.S.M., M.F. and E.V. analysed the data; and A.P., L.S.M., M.F. and E.V. wrote the paper. All authors have read and agreed to the published version of the manuscript.

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Discussion

The precedent article shows how one greenhouse and one field trial gave evidence that soil-dwelling predatory mites might be an efficient biological control agent against WCR provided if the mites are present at larvae emergence. Considering the absence of difference among the three mites density impact to control the pest in the greenhouse trial, we were willing to lower the density tested in a second year experiment. Our objective was to confirm that a dose of 100 mites was enough to protect one maize plant against WCR, and test lower densities (50 and 75 mites per plant). Despite using the exact same material and methods as in the greenhouse trial, no emergence of WCR was observed and no damages were measured on maize roots. We assumed the WCR eggs provided by CABI were damaged during transportation, which was highly impacted by covid-19 pandemy. We chose not to present these results.

In the field trial, we concluded that the combination of alternative food and predatory mites can be efficient to control WCR population. However, the relative influence of predatory mites and alternative food could not be disentangled formally in the experiment, as neither was tested in isolation. As mentioned in the introduction, this Ph.D. is part of a bigger project, a part of which was focused on the formulation of alternative food and its effect on predatory mites. Despite most information being private on these studies, we may share some insight to better understand why we conclude that the combination of alternative food and predatory mites in itself is responsible for our results. During this project, many dirt sampling in maize fields were done. The first type of sampling was to control the natural fauna in the soil and the conclusion is that the natural density of predatory mites is extremely low. On 650 dirt samplings of approximately 150g of wet dirt in 3 maize fields over 2 years, we collected only 132 predatory mites. A second type of sampling was realized to assess the effect of food on the natural predatory mites fauna. Alternative food was introduced and samples were made over a month. Despite this additional source of food the natural predatory mites density did not improve when alternative food was introduced alone. When *G. aculeifer* was introduced without alternative food according to Prischmann-Voldseth and Dashiell, (2013) protocols, we get comparable results. Mites were not retrieved in the dirt sample made over a month after their introduction. However, when mites and alternative food were introduced together at sowing, predatory mites were

retrieved at the exact location of *A. ovatus* eggs introduction. These results developed during the MAD project showed that the combination of alternative food with the predatory mites is the origin of the biological control agent efficiency. It implies that the predatory mites population will spend weeks in the soil feeding on the alternative food while WCR eggs matures. This strategy called Predator-in-first, first suggested by Ramakers, (1990) aims to introduce and maintain the predator before the pest population occurs. This first work already focused on predatory mites, especially *Amblyseius swirskii* (Ramakers and Voet, 1995) to control thrips on the aerial part of the plant in the greenhouse. In this work, they highlight the need to use polyphagous predators, being able to feed either on alternative food sources in addition to the pest. Previous Predator-in-first studies counted on plants, especially pollen to provide this alternative food (Kumar et al., 2020, 2015, 2014; Kutuk and Yigit, 2011; Nomikou et al., 2010, 2003; Ramakers, 1990; Ramakers and Voet, 1995) on the aerial part of the plant. However, pollen supply stops *G. aculeifer* oviposition (Navarro-Campos et al., 2016) and pollen introduction in the soil would decay too fast to provide a qualitative source of food over weeks. Bioline agrosiences develop a product called predafix consisting of sterilized *A. ovatus* eggs whose formulation has been adapted to prevent decaying in the soil. However, composition is confidential and will stay unexposed in this work. The only information we can provide here is that *A. ovatus* are sterilized that prevent living dust mites from being released in the field and they provide good quality of food over weeks even in the soil. This product is essential in the predator-in-first strategy because it will provide the food source essential for the population to grow. This strategy is of main advantage for the farmer. We mentioned earlier that the main farmer concern to apply biocontrol products in field crops are the prices and the ease to apply it on a large surface (IBMA, 2019). In the previous work, a mean of 12.5 mobile mites per plant has been necessary to protect it against WCR which reduce substantially the 150 or 300 mites that fail to protect the plant in Prischmann-Voldseth and Dashiell, (2013) and the 100 mites necessary to protect the maize in our greenhouse trial. Even if the mites density necessary to protect the plant has not been determined due to technical issues and could be lower than 100, the predator-in-first strategy could be the origin of an important economy for the farmer within the condition that alternative food quantity necessary in this process does not surpass predatory mites prices. Moreover, our trial showed that predatory mites, alternative food and maize seeds can be introduced simultaneously at sowing. This is of a major

importance for the farmer as it can reduce the number of interventions in the field and ease this biocontrol solution compared to traditional biocontrol product release at pest emergence.

The predator-in-first strategy looks promising, but the necessity for the mites' population to grow to reach a density high enough to protect the plant brings even more questions. Indeed, alternative food and mites will spend weeks in the soil before larvae emergence. This long duration has many impacts on predatory mites population dynamics because the multiplication will depend of environmental parameters such as temperature (Amin et al., 2014; Heckmann et al., 2007; Lobbes and Schotten, 1980), humidity (personal observation, Amin et al., 2014), food quality (Heckmann et al., 2007) and quantity (Ajvad et al., 2018). This highlights the need to assess the impact of these parameters on population dynamics.

CHAPTER 3 : THE EFFECT OF ENVIRONMENTAL CONDITIONS ON THE PREDATORY MITES POPULATION AND ITS IMPACT ON BIOLOGICAL CONTROL EFFICIENCY.

As previously mentioned, the particular biological control method called Predator-in-first requires the introduction of predators and an alternative food prior to the prey's presence (Ramakers, 1990) to reduce costs for the farmer and simplify its labour. Concerning WCR, the predatory mites population will spend weeks in the soil before being able to attack WCR first instar larvae (Vidal et al., 2005). In that strategy, we rely on a small population multiplication to reach a density high enough to control WCR population and prevent damages to plants (Pasquier et al., 2021). However, predatory mites population dynamics in real conditions are poorly described, in particular due to the difficulty to count the mites in the soil a while after their release. This issue does not permit the assessment of mites multiplication, especially since this population dynamic is a multifactorial parameter depending on temperature, population density, food quantity and quality. In the next part, we attend to assess the effect of these parameters on the population dynamic.

1. Factors impacting *G. aculeifer* development

The PIF strategy requires that population growth is high enough to provide high numbers of predators, at the precise timing of pest emergence. It is necessary that the individual introduced at sowing matures and that females lay eggs to let the population grow enough to control WCR larvae later. Despite the lack of knowledge on soil-dwelling predatory mites population development in natural conditions, the impact of the environment has been studied in the laboratory. Development and oviposition are the two main factors in population. Amin et al., (2014) showed that there is a significant effect of temperature on each stage of *G. aculeifer* individual growth and the life cycle is not fully achieved below 9°C and above 32°C with *Rhizoglyphus echinopus* as food. Oviposition is also impacted by temperature with 3.76 eggs per female per day at 30°C whereas only 0.49 eggs per female per day at 16°C fed with *Rhizoglyphus echinopus* (Fumouze & Robin) (Amin et al., 2014). In addition to the temperature, we observed a strong effect of humidity on the survival of *Gaeolaelaps aculeifer*. In rearing units, a

lack of sufficient humidity will cause an important population decline. Moreover, when rearing units become dry, adding a few drops of water at a corner of the unit will induce a rush from most of the individuals toward this newly humidified area even if food is not present. Even if low humidity strongly influence *G. aculeifer* survival, few studies showed that *G. aculeifer* could develop at 60 +/- 5% RH (Amin et al., 2014), 90 +/- 5% RH (Navarro-Campos et al., 2016) or 100% RH (Kasuga et al., 2006). Despite being an important factor for the rearing, the annex provided in Pasquier et al., (2021) showed that humidity in the maize field used for the field trial never goes below 60% RH. Furthermore, as individuals have the capacity to actively search for suitable areas, we assume that most of the time conditions for *G. aculeifer* survival will be met in the field.

Food quality and quantity are other factors influencing *G. aculeifer* development. Some studies showed that in the exact same laboratory conditions of humidity, temperature and food accessibility, *G. aculeifer* developed differently depending on the type of food provided (Heckmann et al., 2007; Lobbes and Schotten, 1980). This means that *G. aculeifer* development in the soil will be specifically determined by the presence and quantity of *A. ovatus* eggs as a source of food. However, no information is available on *G. aculeifer* development on this specific prey. To get multiplication information, life tables are usually required in order to determine growth development and oviposition rate. However, *G. aculeifer* showed a negative density dependent reproduction rate (Ruf, 1991). Females will lay less eggs if other females are around and the life table does not provide information on this mechanism. To assess this multiplication while still considering this negative density dependent parameter, we set up an experiment to focus on small population multiplication. We isolated adults, provided regular *A. ovatus* eggs in a small arena at constant temperature and humidity and observed multiplication after 30 days. Material, methods and results are presented below.

Material and Methods

Gaeolaelaps aculeifer used in this experiment comes from Biological services® (Australia). They have been reared in the Bioline laboratory (Valbonne, France) since their arrival. Rearing units are bug dorms L21 x W21 x H6 cm filled out with 1,5 cm of plaster of Paris. The lid was pierced and a 56µm mesh nylon tissue was placed to let

the air pass through. Rearing units were placed at a controlled temperature ($25 \pm 0.5^{\circ}\text{C}$) and moisture ($80 \pm 10\%$ RH) room, fed twice a week with *Aleuroglyphus sterilized* eggs, at the same time the plaster was rewetted with distilled water. Food used to rear *G. aculeifer* are *A. ovatus* eggs. *Aleuroglyphus ovatus* were reared in a controlled temperature ($25 \pm 0.5^{\circ}\text{C}$) and moisture ($80 \pm 10\%$ RH) room in the Bioline laboratory (Valbonne, France). Eggs were isolated from the older development stage using appropriate sieves. Isolated *A. ovatus* eggs is a Bioline agrosiences product called Predafix.

The experiment boxes were petri dish cylinders filled out with plaster of Paris. The cylinders are 2.5 cm high and 5 cm diameter. We poured 20 ml of plaster of Paris from a mixture with 1 kg of Planodis® plaster powder with 800 mL of water. Once the plaster of Paris dried out, boxes were sterilized using an UV lamp in order to avoid contamination and delay the emergence of undesirable moulds.

Gaeolaelaps aculeifer unsexed adults were placed into experimental boxes one by one with a brush. To ease this manipulation, rearing units of *G. aculeifer* were passed through a $250\mu\text{m}$ mesh sieve to roughly isolate adults from the other stages and make it easier to set up the experiment. Sieve contents were placed on plaster of Paris and adults were picked up one by one and placed in the experimental boxes unit. 120 boxes were prepared. 100 of them followed a uniform probability distribution from 2 to 100 mites. In order to avoid the impact of a biased male/female ratio in a small population, we also prepared 5 boxes with smaller populations : 2,3,4,5 mites.

Experimental boxes were randomly (R programming software) placed in a HPP260 (memmert®) heating chamber with controlled moisture ($80 \pm 0.5\%$ RH) and 25°C temperature. They were placed in the heating chamber for 30 days. Twice a week the experimental boxes were pulled out from the heating chamber in order to feed the *G. aculeifer* and moisturize the plaster of Paris. 1500 ± 73 *A. ovatus* eggs from the eggs solution were added. At the end of the experiment, *G. aculeifer* received 12000 ± 583 *A. ovatus* eggs. The plaster was moisturized with distilled water until saturation.

The number of individuals were counted in each box after 30 days of experimentation. We differentiated individuals according to their development stages (egg, larvae, nymph and adult).

Results and discussion

Unfortunately, the main question, the effect of the negative density dependent on the multiplication in a small population, cannot be answered. Despite sterilization using an UV lamp in order to avoid contamination and delay the emergence of undesirable moulds, we observed an important development of mould in the petri dish regardless of the initial population size. We assume that the introduction of eggs in water solution in addition to the humidity ($80 \pm 0.5\%$ RH) and temperature ($25 \pm 0.5^\circ\text{C}$) are ideal conditions for mould development. We observed that it can be an important cause of food unavailability leading to a decrease of population development and to very heterogeneous results. Despite this failure in the experimental design few conclusions can still be made (Figure 3).

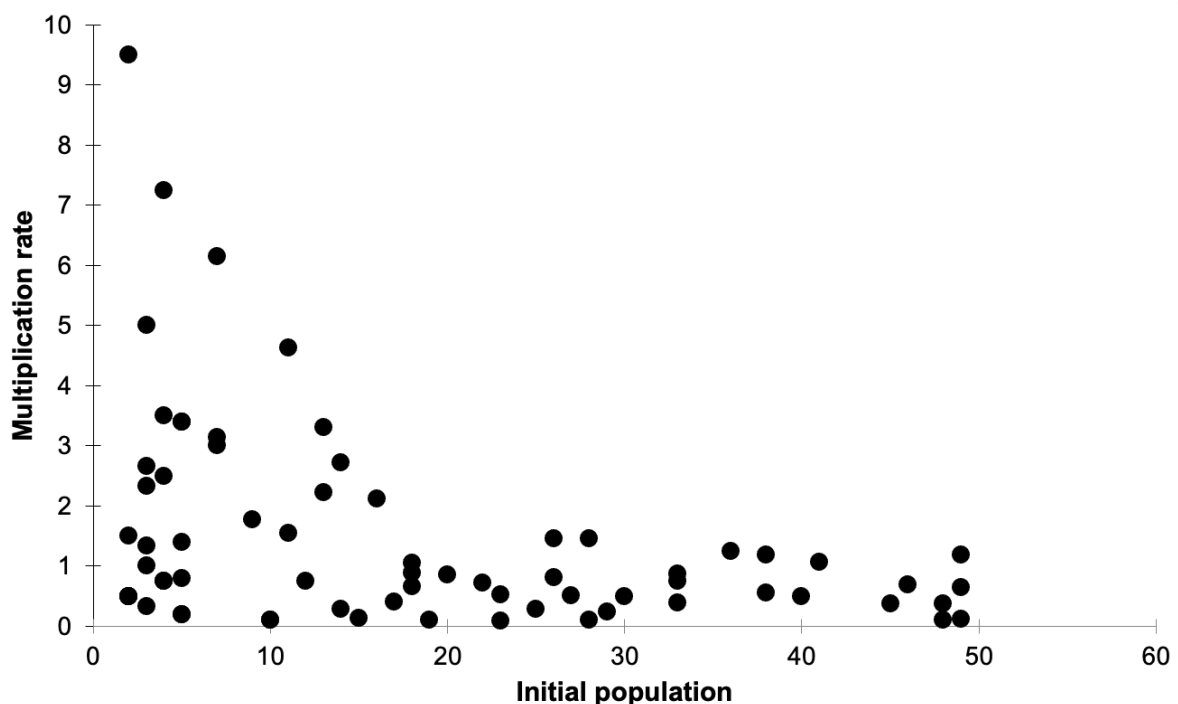


Figure 3: Multiplication rate

An initial population of two individuals has been able to multiply by 9.5 within the 30 days which confirmed the potential of *G. aculeifer* multiplication on *A. ovatus* even if it is necessary to confirm this single observation (Figure 3). The second observation highlights the trend to larger multiplication rate for smaller multiplication which could confirm the negative density dependent population multiplication suggested by Ruf, (1991). However, a doubt is still present if this decrease in multiplication rate originated

from the lack of food access for the larger population due to mould development or to an actual inter-individual phenomenon. These results showed that multiplication on *A. ovatus* is possible and can reach high peaks, but the experimental design does not seem suitable due to the apparition of the mould. Moreover, the experiment start has been delayed due to the covid pandemic in addition to being very time consuming. Indeed, the initial plan was to assess the effect of environmental conditions mentioned previously (food quantity, quality and temperature) following this experimental set up. However, Bioline agrosience owns one heat chamber and assessing the effect of each of these parameters and their interaction seems to be way too ambitious if it takes 1 month per condition to test. We needed to find a better solution to test all these parameters and their effect on the mites population. Instead of an empirical approach, we decided to use a modeling tool based on the information and data already available within Bioline agrosience and the bibliography to assess the effect of temperature, food quantity and food availability on mites multiplication in the maize field.

2. Modeling environmental factors impact on *G. aculeifer* development

The previous part showed that the *G. aculeifer* population can be multiplied by almost 10 in 30 days. However little information is available on (i) the nutritional value of *A. ovatus* and its effect on the population, (ii) the *A. ovatus* quantity required to reach sufficient *G. aculeifer* density and, (iii) the impact of temperature combined with these factors. Bioline agrosiences being the producer of Predafix, it owns precise value and information on *A. ovatus* composition and nutritional value. In addition, *G. aculeifer* individual development has been widely studied on different types of prey including *A. ovatus* at a single temperature (Rueda-Ramirez et al., 2018), and other prey at different temperatures (ALI and Brennan, 1997; Amin et al., 2014; Heckmann et al., 2007). Although these works are mostly life tables which show individual development, survival and reproduction through time, we decided to use these data to build an Individual based model to represent the mites population growth. To consider *A. ovatus* food quality and its effect on mites population dynamics, we used the data available on its nutritional value to create an energy budget model that is described below and which will be the subject of a scientific article.

Among all the available models, we chose an Individual Based Model (IBM) because (i) most of the data available on the bibliography on the impact of these parameters are individually recorded (life table) (Ajvad et al., 2018; Hassan et al., 2017; Rueda-Ramirez et al., 2018; Ydergaard et al., 1997), and (ii) a sex or a stages difference in the small predatory mites population introduced (estimated to 17 mites per plant according to Pasquier et al., (2021)) could highly impact the population multiplication (iii) we imagine this model improving outside the scope of this thesis and a high level of details could be incorporated (DeAngelis and Mooij, 2005) later on. An IBM is especially used to represent these individual mechanisms and their consequences at the population level (DeAngelis, 2018). In *G. aculeifer*, the type of food influences their development and reproduction which, in turn, impacts population dynamics (Heckmann et al., 2007; Navarro-Campos et al., 2016). We consider this aspect using an Energy Budget Model (EBM) approach. EBM theory says that energy is accumulated from food in the organism and is allocated to survival, growth, reserve or reproduction (Martin et al., 2013). We used this method to consider food nutritional value and temperature impact on multiplication. This EBM-IBM model purpose was to simulate the field trial detailed in chapter 2 to fully understand what happens in the soil in the condition of experimentation. This model will be the subject of an article still in preparation.

Material and methods

Initial population

In Pasquier et al., (2021), a mean of 12.5 mobile mites per plant were introduced at sowing to protect the crop. According to Baatrup et al., (2006), a *G. aculeifer*, a stable population is made of 40.8% eggs, 9.9% larvae, 18.8% protonymph, 12.2% deutonymph, 12.7% female and 5.7% male. We picked up 12 mobiles in addition to 5 eggs according to these proportions to create the initial population.

Environmental conditions

Environmental conditions used were based on field trial temperature provided in Pasquier et al., (2021).

Energy Budget model

The EBM-IBM architecture is presented in figure 4 and each part is detailed below. This model has mostly being inspired from van der Vaart et al., (2016).

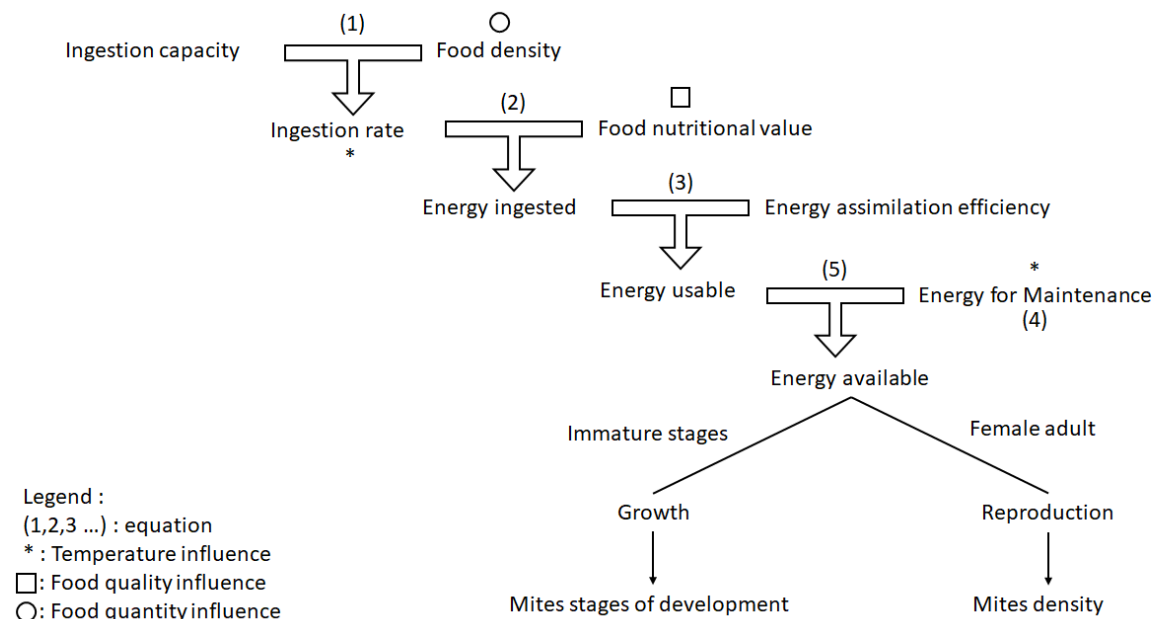


Figure 4 : EBM-IBM architecture.

Ingestion capacity is the maximal quantity of food ingested in a given time. (Azevedo et al., 2019) explored this parameter and showed that larvae, protonymph, deutonymph and adults consume 0.036, 0.066, 0.11 and 0.22 g of prey per day at 28 +/- 0.3°C, 90 +/- 5% RH. 28°C being the pick of *G. aculeifer* activity, we considered they will have the most feeding activity at this temperature and considered these values as the ingestion capacity.

Food density is the quantity of food available per unit of surface or volume. In this work, we consider that the mites population will be present in a single unit of volume. The alternative food introduced in the predator-in-first trial was composed of 0.11g of *Aleuroglyphus ovatus* eggs per plant (Pasquier et al., 2021).

Ingestion rate computes the quantity of **food ingested** per unit of time. It depends on the individual ingestion capacity and the food available in the environment. *I* corresponds to the **slope intensity** which parameter at which speed the individual will reach its Ingestion capacity. The equation given the relation to these parameters is presented below (1).

$$(1) \text{ Ingestion rate} = \text{Ingestion Capacity} \times \text{Food density} / (\text{Food density} + I)$$

Food nutritional value. *A. ovatus* eggs are made of 11.2% (**LR**) of lipid and 29.6% (**PR**) of proteins (Bioline agrosiences communication) which are main sources of energy for predators. Proteins and lipids provided respectively 4 and 9 calories per gram consumed. **Energy ingested** comes from the quantity of food ingested considering its nutritional value and corresponds to the equation (2).

$$(2) \text{ Energy ingested} = \text{Ingestion Rate} (\text{LR} \times \text{Calories per Lipid} + \text{PR} \times \text{Calories per protein})$$

Energy usable. However, even if the energy quantity is absorbed, a part of it is unused or wasted. According to Peters, (1983), only 80% of the energy absorbed is used for predators (3).

$$(3) \text{ Energy usable} = \text{Energy ingested} \times 0.80$$

Energy for maintenance. The Energy Budget Model theory allocates energy usable to maintenance, growth, reproduction or to the individual reserve. Prior to energy used for growth or reproduction, we consider that the individual will allocate the energy to its survival. This energy is classically called **metabolic rate** and is represented by the following equation (4).

$$(4) \text{ Metabolic rate} = B_0 \times m^\alpha \times e^{-E/(k \times T)}$$

B₀ is a constant of proportionality, **m** the individual weight, α , the scaling coefficient **E**, the activation energy, **k**, the Boltzmann's constant, and **T** the temperature in degree Kelvin.

Once the metabolic rate is computed, it is subtracted (5) from the energy usable to determine the **energy available** for the reserve, for growth or for reproduction.

$$(5) \text{ Energy available} = \text{Energy usable} - \text{Metabolic rate}$$

Little is known about predatory mites reserve allocation even if they are visible on female adults. *G. aculeifer* is able to spend weeks of starvation and still survive. We assumed that reserves play a role in this survivability and starvation resistance. However, the scope of the study being inferior to a few weeks, with initial food originated from the rearing in addition to the food provided in the field, we considered that energy reserve management can be ignored. Then, the energy left will be allocated to growth or reproduction. We also considered that immature stages will use their energy to grow whereas females will allocate it to oviposition. In both cases, there is a need to create flesh. Poikilotherms have shown to require 0.0036 J to synthesize 1 microgramme of flesh. In the discrete model, each time an individual reaches this threshold, it grows by 1 microgramme. We pooled 100 female adults together and weighed them using a precision scale, we obtained a mean of 52 microgramme per female. We used this threshold to consider that deutonymphs become adults and sex them following the 63% female/37% male ratio observed in Ajvad et al., (2018). *Gaeolaelaps aculeifer* eggs are estimated to weigh 3 microgrammes. A female adult has to accumulate 0.0108 J of energy to be able to lay an egg. Once this threshold is reached, a new individual is added to the list and is subjected to the same conditions presented. Larvae, protonymphs and deutonymphs will develop and reach the next stage once they weigh 8.16 and 40 microgrammes respectively (Bioline agrosience personal communication). We added a probability of survivability based on Kasuga et al., (2006) work. Eggs to larvae survivability is 94% and larvae to adult 92%.

Model calibration

We used Rueda-Ramirez et al., (2018) work to calibrate the model. Indeed, they observed *G. aculeifer* growth and oviposition fed with *A. ovatus* at a single temperature (21 +/- 1°C). We set up a simulation at this temperature and determine the only factor we did not determine from expertise, bibliography or observation : the **slope intensity** used in the **ingestion rate** equation (1). To calibrate this parameter, we consider that egg to adult development happens in 18.5 days at 21 +/- 1°C. The slope intensity value given this development speed is 1.

Model sensibility

The model will be part of an article and still require some draft in its architecture. Model sensibility will be conducted once the final parameters will be definitely identified.

Results and discussion

Alternative food

First of all, alternative food has not been a limiting factor in the simulation (mean food remaining for 20 simulations : 109917.53 +/- 37.26 microgrammes). After the 30 days period of estimated absence of WCR larvae, a majority of food was still available for the population (Table 1). However, these results have still to be mitigated because alternative food in the field conditions can be consumed or degraded by other organisms. This degradation rate is unknown at the moment and could impact heavily the population dynamic especially at the end when the population rises and needs more food quantity despite the food quality decreases. However, in the WCR population control scope, this decrease in food availability could be an advantage so the predatory mites would multiply during the period of WCR absence but they would spread and starve for a few days which could enhance biological control efficiency (Rueda-Ramirez et al., 2018).

Total population

Among 20 simulations, we noticed an important heterogeneity with a mean of 754.05 +/- 321.75 total individuals. The lowest total population reached 313 individuals whereas the largest population counted 1501 individuals. This confirms that the environmental conditions (food and temperature) allow the population to grow and multiply but still conduct a very heterogeneous population dynamic. The only source of heterogeneity in the simulation comes from the initial population construction and in a minor effect of mortality at eggs hatching and deutonymph to adult path. This means that the initial population constitution is of major importance to protect with efficiency the maize plant. This leads to major consequences to biocontrol agent release. In the field trial, we counted mites density right before introduction and the transport was shortened by the R&D process. However, a slight population mortality increase due to transportation or storage could lead to an important decrease of biological control efficiency if it impacts the initial population introduced.

Table 1: Simulation ID, total number of individuals, Number of feeding individuals and food remaining based on EBM-IBM model.

Simulation ID	Total number of individuals	Number of feeding individuals	Food remaining
1	859	182	109920.3226
2	1007	361	109862.6551
3	1274	394	109852.1009
4	556	103	109950.5621
5	441	160	109940.7488
6	488	102	109948.6605
7	596	161	109931.8325
8	851	178	109919.4747
9	1501	398	109841.315
10	485	157	109943.0146
11	671	187	109921.3003
12	628	180	109930.4045
13	444	101	109949.9525
14	835	194	109918.1014
15	735	184	109920.6637
16	1273	321	109860.3321
17	856	288	109894.206
18	313	31	109981.3173
19	389	147	109943.6924
20	879	196	109919.8913

To improve the biocontrol solution efficiency, we could also imagine selecting only the older stages to ensure that the population will contain enough female adults to multiply quickly. However, this method could create a cohort of individuals from the same stage and induce a gap in the plant protection if the older stages die and younger are not yet ready to consume WCR larvae. This risk is still limited since non feeding stages are a short part of *G. aculeifer* development and a month of development should bring heterogeneity among stages.

Beside agronomic application, the impact of this randomness showed the usefulness of EBM-IBM model which highlights the action of one or few individuals and their consequences at larger scale compared to population based model that would smooth population dynamic response (DeAngelis and Mooij, 2005).

Feeding stages

Individuals that reach the feeding stages are of particular importance in the biological control scope because these stages will represent the individual that will attack and control pest populations. This is even more important in the WCR case because the first instar larva is a large prey compared to *G. aculeifer* size. Among the simulations, a mean of 201.25 +/- 100 feeding individuals were virtually present in the environment with a maximum of 398 feeding stages individual and a minimum of 31. This information is of particular importance if we compare to the population we introduced in the greenhouse study at larvae emergence (Pasquier et al., 2021). Indeed, 100 mites were enough to control the pest and the simulation showed that in only 1 case out of the 20 simulations, this density was not reached.

Negative density dependent

An observation showed that negative density dependent reducing female oviposition rate can occur when females are coexisting (Ruf, 1991). These parameters have not been incorporated in the model and could be the origin of an important population decrease compared to the results we provided in Table 1 especially considering that most of the population will grow and reproduce around the food source.

GENERAL DISCUSSION

Through this thesis, we aim to bring elements to respond to the question: can soil-dwelling predatory mites be an efficient and viable biocontrol solution against Western Corn Rootworm ? The general discussion presents main results presented in this work in addition to the impact of these results and their implication for biocontrol in a broader perspective.

Main results

Proof of concept experiment

In the prey/predator couple WCR/*G. aculeifer*, proof of concept predation results in testing prey activity during 13 days (Prischmann et al., 2011). In the first chapter, we showed that observation of *G. aculeifer* predation for a very short time (10 minutes) in a small arena enables us to provide sufficient information to decide if larger scale experiments are worthwhile. Reducing time and resources costs compared to previous work on the same prey and predators allows for a greater number of replications and testing of a greater number of organisms. More than a project management reason, we think that predation will occur shortly after the first contacts between the prey and the predator, especially in a very small arena, which annihilate interest to observe predation on a longer duration. We could consider that the predation rate of a single individual on the prey could be interesting to estimate its efficiency in real conditions. However, either on the aerial part of the plant or in the soil, there are many parameters that can increase or decrease the individual predation efficiency compared to what happens in the laboratory. This method could be generalized to assess the first step of predator biocontrol potential. In this study's scope, this test reveals its usefulness by providing quantitative (predation frequency) and qualitative (operator observation) on the stage to target to explore biocontrol agent efficiency. This mostly made the difference to previous field work that did not release predatory mites at larvae emergence which might be the origin of the trial failure (Prischmann-Voldseth and Dashiell, 2013). This method could be of particular interest at a period of need to research and develop new biocontrol products to balance decrease of pesticide use. We presented here a low cost/low time method to observe the first step of the

biocontrol agent potential but this reflection could be made at each step of a biocontrol agent research and development process. In the scope of this study, we choose to observe multiple factors of plant physiology and WCR population to assess predatory mites impact on WCR dynamics. However, we could have reduced the experimental time by observing the most common factors to assess WCR damages: root damages. Indeed, root damages are made very early in WCR development and are simple to observe. The observation of this single parameter would have been enough to notice predatory mites efficiency to control WCR population. However, farmers claimed that the lack of proof of efficiency comes in 4th position (IBMA, 2019) when they are asked why they do not use biocontrol products. In the greenhouse trial, we thought it was important to observe multiple factors to convince even more any counterpart that would doubt the potential of some “never heard of” soil-dwelling predatory mites to control a “beetle”. Balance between R&D resource investment and proof weight will be an important challenge for the biocontrol industry that is expected to grow considerably in the next few years and decades.

Change of scale and optimal mite density

More than a proof of concept, we confirmed in the greenhouse then in a field trial that soil-dwelling predatory mites were able to control with efficiency WCR population. In this study’s scope, we decided on purpose to go through each step of research and development as fast as possible to better mark the development of the project. However, work will be necessary to repeat the trials set up in the greenhouse to sharpen the precision of mites density necessary at larvae emergence to control the WCR population. This information is of a particular importance if the biological control by inundative strategy is chosen by the farmer to preserve its crops. Moreover, only one field trial is insufficient to trigger a production or a biological control strategy at large scale. The EBM-IBM model may, however, enable to determine more precisely the densities that should be evaluated in field trials to confirm this work and determine the food and predatory mites initial population needed to introduce at sowing.

Innovation based on the past studies

If the biocontrol is expected to take a larger part of the plant protection market, it will be required to provide solutions to all kinds of pest and cultural practices. In the present

work, we showed that soil-dwelling predatory mites could control a pest a few centimeters deep in the soil in field crops whereas soil pest was traditionally fought with pesticide or GMOs. The challenge to spread these predatory mites on large surfaces has still to be assessed but it shows that organisms, and in this case predators, that have been identified for decades in the literature have still to be explored to use as biocontrol agents against common pests. Within MAD project scope, it was planned to explore predatory mites soil diversity to identify a new species of biocontrol agent. This work is important at a long term to continue to aggregate knowledge on soil diversity but from a biological control perspective scope to bring pesticide alternative solution at short or medium term, the present work induced we could start to match well identified pests and biocontrol agents. This thought might be specific to generalist organisms that can control multiple species of pests.

A biological control agent to control multiple pest

Gaeolaelaps aculeifer particularly showed its potential to control population of various species and we added one to the list within this work. *Agriotes sordidus* is recognized as an important agricultural pest (Ritter and Richter, 2013) impacting many crops. Despite economic loss on potato, sugar beet, corn, tomato, onion, watermelon and melon (Burgio et al., 2012) efficient management methods are limited (Poggi et al., 2021). We observed predation in very controlled conditions and observed that *G. aculeifer* was the only predatory mite tested able to attack the wireworm larvae. Unfortunately, we did not record the interaction between predator and prey but the simplest way to describe the situation is to compare it to a rodeo. Once *G. aculeifer* inserts its chelicerae in the larvae, wireworm would shake and wriggle as much as possible to escape from the predatory mites. This movement discouraged *S. scimitus* and *M. robustulus* which did not even intend to attack the pest but that was not the case for *G. aculeifer*. It stayed connected to the larvae despite being shackled. The potential of *G. aculeifer* to control *A. sordidus* has still to be explored in realistic conditions but this opens a new management perspective using this generalist predatory mites.

Gaeolaelaps aculeifer environmental risk

Generalist predators such as *G. aculeifer* can be interesting for a Biological control company because it requires the production of only one organism to control multiple pests and cover multiple markets. However, their use can also present disadvantages. Indeed, *G. aculeifer* showed being able to prey upon Collembola, dust mites, nematodes and in our case : coleopteran. This prey diversity could be harmful for the environment and this issue will have to be assessed before considering introduction at large scale in field crops. However, we think that multiple factors minimize *G. aculeifer* impact risk on the environment. First of all, this organism is cosmopolite and has been found in the soil in a maize field (1 individual in a dirt sample) meaning it is already able to maintain a population without harm to the environment. Second, the population was able to regulate itself in the natural conditions because we observed it at low densities. We assume that regulation happens during the winter when food availability and temperature limit the species development and reproduction in addition to mortality increase (Jensen et al., 2019). It is also important to note that this predatory mite develops mainly at more than 10 cm in the soil whereas the free-living organisms beneficial to ecosystem functioning (bacteriovores, fungi and predators) are concentrated in the first 10 cm of the soil with a maximum in the 0-3 cm horizon (Jess and Bingham, 2004). In particular, studies combining the use of predatory mites and entomopathogenic nematodes have demonstrated a synergistic effect of the use of these two predators in the control of populations of dipteran pests of the family Sciaridae and Phoridae as well as thrips (Acharya et al., 2019; Ebssa et al., 2006; Jess and Bingham, 2004).

Classical biological control : a transition toward conservative biological control

Moreover, maize monoculture and pesticide use considerably decrease maize soil diversity but we often observed that alternative food use in this work act as a micro-environment for collembola or dust mites. We assume that this addition of biomass in the soil to multiply predatory mites could enable a more complex food web that has been lost due to cultural practice. The gap between the pesticide use interest and predator-in-first would be even larger to preserve the environment because we would have on one side, a pest solution that weakens the environment to face new pests and

decrease biodiversity and on the other side, a pest solution that promote complex food web, increase soil biomass and environment resilience. If *G. aculeifer* and alternative food presence in the maize field seem positive, a particular attention has to be kept about mites spreading away from the maize field and impact adjacent areas.

Spread as essential factor of biological control efficiency

Predatory mites movement is of particular importance to assess its potential impact on the surrounding maize field environment but we also showed in the present work that it might be the reason for maize field failure in Prischmann-Voldseth and Dashiell, (2013). Indeed, releasing mites without alternative food at sowing results in absence of plant protection which could have originated from mites' death or dispersion. Little information is provided about predatory mites spread except that they reach an average velocity of 1 mm.s⁻¹, they move at least 50% of the time (Baatrup et al., 2006) and will go toward plant defense chemical signs (Hall and Hedlund, 1999). Even if information is scarce, they are of particular interest. 1mm.s⁻¹ speed happening 50% of the time considering the delay between mites introduction at sowing and WCR larvae emergence 1 month later would represent a total distance of 1.296 km highlighting the need to find a way to keep the individuals where they are introduced. The formulated and sterilized *A. ovatus* eggs seem to have fully filled this role.

Alternative food, ally or enemy ?

If *A. ovatus* eggs seem to provide an interesting tool to enhance predatory mites to control WCR population we could also consider them as a competitor to protect WCR larvae. In this case, the alternative food would prevent predatory mites from attacking the focus pest. However, by providing alternative food before larvae emergence, we consider that the population will feed and multiply on the food stock while WCR eggs mature. Once the larvae emerge, the mite population will have grown and the food stock will have decreased. Moreover, Rueda-Ramirez et al., (2018) observed *A. ovatus* mix stages effect on a pest consumption *Frankliniella occidentalis* Pergande (Thripidae). It decreases the quantity of prey consumed but they emphasize that predation decrease can be a good trade off if this prey provision compensates for another benefit such as having the predator at the location and time of pest presence (van Baalen et al., 2001).

Predator-in-first strategy toward a more applicable biological control solutions

Pest presence at larvae emergence seems to be the parameter that was missing for Prischmann-Voldseth and Dashiell, (2013) and this problem seems to be solved with the predator-in-first strategy we used in this work. This strategy is promising to control WCR in the maize field due to its facility to use and introduce in the maize field in addition to reducing the biocontrol solution cost. This type of strategy has already been used for predatory mites living on the aerial part of the plant and it also shows a good efficiency with predatory mites density comparable to what we use in the soil (Kumar et al., 2020). We think this predator-in-strategy could be an important step towards a better integrated biocontrol strategy using generalist predators and this could be used out of the predatory mites group. Apart from economic and technical interest, predator-in-first strategy could be used as a preventive solution to avoid pest-pick populations that are the most impacting reason for crop loss. This is of main concern because it is often difficult for farmers to identify pest outbreak early enough to introduce biocontrol agents and control it before the pest significantly impacts the plant.

A global solution for a global threat ?

We have shown at the very beginning of this document how WCR became a pest on different continents and how it was susceptible to spread according to the global trade and global distribution of its main host plant. All along this work, we presented that *G. aculeifer* brings a potential solution to control WCR population. This could be of a particular interest worldwide since this predatory mites species has been identified in South America (Rueda-Ramirez et al., 2018), in North America (Kevan and Sharma, 1964) and in Europe (Kevan and Sharma, 1964). The species presence in the two main areas of WCR nuisance could facilitate its use globally.

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