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Bio-based control strategies of *Bruchus rufimanus* in faba bean crop

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À ma famille,

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Abstract

This thesis broaches the promotion of faba bean cultivation (*Vicia faba* L., Fabaceae) for food uses in Wallonia and the challenge posed by the broad bean weevil, *Bruchus rufimanus* Boheman 1833 (Coleoptera : Chrysomelidae). Faced with no effective control methods against this pest, biocontrol strategies and alternatives to pesticides were investigated. Five approaches were adressed, including the improvement of the pest bio-ecological knowledge, the identification of optimal varieties for seed valorization in food industries, the evaluation of most effective semiochemical traps, and the development of specific control methods based on entomopathogenic fungi and RNA interference.

Chapter I provided an overview of faba bean cultivation and the challenges posed by *Bruchus rufimanus* (BBW) infestations in Europe. The chapter reviews the biology and chemical ecology of BBW, identifies knowledge gaps, and explores potential improvements to semiochemical-based approaches and alternative control methods. This chapter lays the groundwork for the subsequent research areas of the thesis.

Chapters II focused on the ecology and the biology of BBW in Wallonia. Field monitoring studies were conducted to gather comprehensive data about adult infestations, addressing the lack of region-specific information. The diversity of bruchids and their parasitoids in faba bean crops are described, including their spatial distribution and the damage they cause to seeds. *Bruchus rufimanus* was identified as the predominant bruchid species, along with four larval parasitoids: *Triaspis luteipes*, *T. thoracica*, *Pteromalus sequester*, and *P. fasciatus*. Infestation rates and emergence timing of BBW varied across different bioclimatic areas. **Chapter III** provides a methodological description of rearing *Bruchus rufimanus* and models the influence of temperature on the pest's embryonic and post-embryonic development.

Chapter IV evaluated faba bean varieties and the combined influence of climate on crop productivity, seed quality, and *Bruchus* spp. infestations. The aim was to identify promising varieties for local protein sourcing in the food industry. Fourteen varieties were assessed over two seasons, taking into consideration parameters such as protein production and bruchid infestations. A ranking methodology based on

principal component analyses is presented, along with the varieties most suitable for the Walloon agro-climatic context. Factors promoting varietal resilience in European contexts were also identified.

Chapter V focused on the semiochemical control of BBW, aiming to identify efficient traps for capturing BBWs, assess the traps' impact on BBW sex ratios, evaluate collateral effects on crop benefits, and examine the influence of crop stage on trap captures. White pan traps combined with floral kairomones were found to be the most effective. Crop phenology strongly influenced trap attractiveness, and no significant sex ratio trends were observed. However, semiochemical traps had a significant impact on beneficial insects, requiring further adaptation to minimize collateral effects, particularly for threatened species. Recommendations are provided for minimizing impacts on beneficial entomofauna.

Chapter VI investigated in laboratory potential new biopesticides against BBW. Firstly, five strains of entomopathogenic fungi were screened for their lethal and sublethal effects on bruchid species. *B. bassiana* (GHA) exhibited the highest virulence in terms of TL50. Additionally, the study explores RNA interference with *C. maculatus* as a biotechnological tool for bruchid control. All the necessary proteins for gene silencing and the *laccase 1* gene were successfully identified. Micro-injection of *laccase 1* dsRNA resulted in a significant decrease in gene expression in treated adults. Although no significant mortality was observed, further research should focus on the larval stage or explore alternative target genes to induce lethal effects. The study demonstrates successful gene silencing in a bruchid species and highlights RNAi as a potential control method.

Chapter VII summarizes the different control levers evaluated in the thesis for the development of integrated biocontrol strategies against *B. rufimanus*. The most suitable crop itinerary for minimizing BBW impacts is presented as well as areas for future research to further develop the main conclusions of the thesis.

Key words : Broad bean weevil ; Bruchids ; biocontrol ; IPM ;bioecology ; thermal development; semiochemical control ; Entomopathogenic fungi ; RNA interference ; RNAi ; Varietal control; New genomic technologies

Segers Arnaud (2023). Stratégies de contrôle bio-basées de *Bruchus rufimanus* en culture de féverole. Université de Liège, Gembloux Agro-Bio Tech, Belgique.

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Résumé

Cette thèse aborde le développement des stratégies de biocontrôle et les alternatives à l'usage des insecticides contre la bruche de la fève (BF), *Bruchus rufimanus* Boheman 1833 (Coleoptera : Chrysomelidae) en culture de féverole (*Vicia faba* L. – Fabaceae). Cinq leviers de lutte ont été évalués, notamment l'amélioration des connaissances bio-écologiques du ravageur, l'identification de variétés optimales pour la valorisation des graines en alimentation humaine, l'évaluation d'efficacité de pièges sémiochimiques et le développement de méthodes de lutte spécifiques basées sur les champignons entomopathogènes et l'interférence ARN.

Le chapitre I fait l'état des lieux de la problématique des bruches en culture de féverole en Europe. Le chapitre passe en revue la biologie et l'écologie chimique du ravageur, identifie les lacunes des connaissances et explore les améliorations potentielles de l'utilisation des sémiochimiques et des méthodes de lutte alternatives. Ce chapitre pose les bases des domaines de recherche et des objectifs abordés ultérieurement dans cette thèse thèse.

Le chapitre II se concentre sur l'écologie et la biologie de BF en Wallonie. Des études de monitoring ont été menées pour collecter des données décrivant les infestations d'adultes, répondant au manque d'informations spécifiques à la région wallonne. La diversité des bruches et de leurs parasitoïdes dans les cultures de féveroles y sont décrite, y compris leur distribution spatiale et les dommages qu'ils causent aux graines. *Bruchus rufimanus* a été identifié comme l'espèce de bruche prédominante, ainsi que quatre parasitoïdes larvaires : *Triaspis luteipes*, *T. thoracica*, *Pteromalus sequester* et *P. fasciatus*. Les taux d'infestation de BF et les périodes d'émergences variaient selon les différentes zones bioclimatiques. Le **chapitre III** fournit une description méthodologique de l'élevage de BF et modélise l'influence de la température sur le développement embryonnaire et post-embryonnaire du ravageur.

Le chapitre IV aborde l'effet variétal et l'influence combinée du climat sur la productivité des cultures, la qualité des graines et les infestations de *Bruchus* spp. L'objectif était d'identifier des variétés les plus prometteuses pour l'approvisionnement local en protéines dans l'industrie alimentaire wallonne. Quatorze variétés ont été évaluées sur deux saisons, en tenant compte de paramètres tels que la production de protéines et les infestations de bruches. Une méthodologie de classement basée sur des analyses de composantes principales est présentée ainsi que les variétés les plus favorables au contexte agro-climatique wallon. Les facteurs favorisant la résilience variétale dans les contextes européens ont également été mis en évidence.

Le chapitre V aborde le contrôle sémiochimique de BF, plus particulièrement l'identification des pièges les plus efficaces vis-à-vis de BF, l'évaluation de l'influence des pièges sur le sexe-ratio des captures, l'évaluation des potentiels effets collatéraux sur les auxiliaires ainsi que l'influence de la phénologie des cultures sur sur les captures. Les pièges blancs combinés à des kairomones florales se sont avérés les plus efficaces. La floraison des cultures exerçait une forte compétition sur les captures de bruches, et aucune tendance significative des sexe-ratios n'a été observée. Cependant, les pièges sémiochimiques ont eu un impact significatif sur les insectes bénéfiques, nécessitant une adaptation supplémentaire pour minimiser les effets collatéraux, en particulier pour les espèces menacées.

Le chapitre VI a étudié de nouveaux biopesticides potentiels contre BF, en deux temps. Premièrement, cinq souches de champignons entomopathogènes ont été testées sur deux espèces de bruches pour leurs effets létaux et sublétaux. *B. bassiana* (GHA) était la souche la plus virulente en termes de TL 50. Ensuite, une nouvelle technique biotechnologique pour le contrôle des bruches est étudiée, l'interférence par ARN. L'ensemble des protéines nécessaires au mécanisme de mise sous silence génétique, ainsi que le gène de la *laccase 1* ont été identifiées par analyse phylogénétiques. Ensuite, des micro-injections d'ARN doubles brins (ARNdb) de la *laccase 1* ont été effectuées. Une diminution significative de l'expression génétique de ce gène a été mise en evidence via RT-qPCR chez les adultes traités. Bien qu'aucune mortalité significative n'ait été observée, ces résultats rapportent pour la première fois une mise sous silence génétique chez une espèce de bruche. Les recherches futures devraient se concentrer sur le stade larvaire ou cibler d'autres gènes pour induire des effets létaux.

Le **chapitre VII** effectue la synthèse des différents leviers de lutte évalués pour le développement de stratégies de biocontrôle intégrée contre *B. rufimanus*. Ce chapitre explore l'itinéraire de culture le plus adapté pour minimiser les impacts de BF et identifie les domaines de recherche future pour approfondir les principales conclusions de la thèse.

Mots clefs : Bruche de la fève ; bruches ; féverole ; futte biologique ; IPM ; bioécologie ; développement thermique ; lutte sémiochimique ; champignons entomopathogènes ; interférence ARN ; RNAi ; lutte variétale ; parasitoîdes, nouvelles technologies génomiques

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List of abreviations

AA: Amino acids
AC: Ash content
AGDF: AgriOdor lure from flower type
AGDG: AgriOdor lure from pod type
Ago-2: Argonaute 2
Arg-K: Arguinine-kinase
Bactin: Beta-actin
BBSV: Broad bean stain virus
BBTMV: Broad bean true mosaic virus
BBWs: Broad bean weevils
BC: Before Christ
BLAST: Basic Local Alignment Search Tool
C: Convicine
CAP: Common agricultural policy
cDNA: complementary DNA
CFU: Colony forming unit
CI95%: Confidence interval at 95%
CL: Crude lipids contents
CO2 : Carbon dioxyde
CP: Crude protein
Cq: Cycle of quantification
dbRDA: Distance based redundancy analysis
Dcr-2: Dicer-2
df: Degrees of freedom
Dim1: First dimension
Dim2: Second dimension
DNA: Desoxyribonucleic acid
dpi: Days of post-injection
dsRNA: double-stranded ribonucleic acid
DW: Dry weight
EAG: Electro-antennographic
EF: Entomopathogenic fungi
EFA: Environmental focus areas
EH: Seeds presenting BBWs emergence hole
EIL: Economic injury level
ET: Economic threshold

EU: European Union
FAO: Food and Agriculture Organizarion
FY: Field yield
G6PD: Glucose-6-posphate dehydrogenase
GC-MS: Gas-chromatography-mass spectrometry
GFP: Green fluorescent protein
GLMMs: Generalized Linear Mixed-Models
GS105: Growing stage vegetative of faba bean presenting five complete leaves
GSP: Generalized spring parcels
GST: Gluthiatone S-transferase
GWP: Generalized winter parcels
HCl: Chloridric acid
HEF: Hypocrealean entomopathogenic fungi
HIGS: Host induced gene silencing
HKG: House Keeping gene
HMP: Host marking pheromone
HS: Healthy seeds
INRA: Institut national de recherche agronomique
INRAE: Institut national de recherche pour l'agriculture, l'alimentation et l'environnement
IPM: Integrated pest management
IPSF: International Pheromone System lure from flower type
IRM: Institut Royal Météorologique de Belgique
JHIII: Juvenile hormone III
L1: First larval instar
L2: Second larval instar
L3: Third larval instar
L4: Fourth larval instar
Lac 1: Laccase-1
Lac2: Laccase-2
LMMs: Linear Mixed-Models
LT50: Median lethal time
Mbp: Mega base-pairs
MCA: Microbial control agent
MCOs: Multicopper oxidases
MIQE: Minimum Information for Publication of Quantitative Real-Time PCR Experiments
miRNA: micro-RNA

MLPA: Multi-level pattern analysis
mRNA: messenger RNA
N: Nitrogen
N2O: Nitrous oxyde
NCBI: National Center for Biotechnology Information
ND: Not determined
NGT : New genomic technologies
NTOs: Non-target organisms
nts: nucleotides
NVOCs: Non-volatile organic compound
ODP: Oviposition deterring pheromone
ORF: Open reading frame
ORNs: Olfactive receptors neurons
PB: Piège blanc (i.e., white trap)
PC: Principal component
PCA: Principal component analysis
PCoA: Principal coordinates analysis
PCR: Polymerase chain reaction
Pcum: Cumulated precipitations
PDA: Potato dextrose agar
PEMV-1: Pea enation mosaic virus-1
piRNA: piwi-RNA
PPS: Protein productions
PV: Piège vert (i.e., green trap)
R2adj: Adjusted determination coefficient
RdRp: RNA-dependant RNA polymerase
RG: Reference gene
RISC: RNA induced silencing complex
Rlv: Rhizobium leguminosarum var. viciae
RNA: Ribonucleic acid
RNAi: RNA interference
RT-qPCR: Real time quantitative polymerisation chain reaction
SD: Seeds presenting surface damage
sd: Standard deviation
SDA: Sabouraud Dextrose Agar
SDB: Sabouraud Dextrose Broth
SE: Standard error
SFB: Spring field bean

SIGS: Spray induced gene silencing
siRNA: small-interferring RNA
SO: Specific objective
T: Tannin
Tavg: Average of temperatures
Tbp: tata binding protein
Tcum: cumulated temperatures
TSA: Transcriptome shotgun assembly
TSW: Thousand seed weight
Tuba-1: alpha-tubuline 1
UK: United Kingdom
UV: Ultra-violet
V: Vicine
Var.: Variety
Vc-: mutant allele coding for low vicine or convicine contents
VOCs: Volatile organinc compound

Chapter I

Introduction and objectives

1 Grain legumes crops in a context of global change

One of the biggest challenges facing modern agriculture is ensuring food security while simultaneously reducing its environmental impact (Foley *et al.* 2011; Bedoussac *et al.* 2015). In Europe, a particular concern is observed over last decades concerning the plant protein production. Since the Blair House agreements between US and Europe in 1992 that implemented non taxable protein imports from American countries and limited cropped surfaces for grain legumes in the EU, the EU production of plant protein became competitively disadvantaged and entered in a period of dependence on soybean American producing countries (Cernay *et al.* 2015). Today, this dependence on vegetable protein importations continues to prevail. The European cropped areas with legume crops have witnessed a significant decrease over the past few decades. Specifically, the area of land dedicated to growing legumes has shrunk from 5.8 million hectares in 1961 to 1.8 million hectares in 2013. This decrease amounts to a mere 1.7% of the total arable land in the EU-27 nations. In comparison, the proportions of land used for cultivating legumes were 14.5% in North America and 25.5% in South America in 2013 (Preissel *et al.* 2015; Cernay *et al.* 2015). This decrease in European grain legume-cultivated areas was coupled to a constant increase in the plant proteins demand for livestock feed, which lead to a deficit of about 70% in proteic material that has been fulfilled by 87% of unsustainable soybean imports, increasing in consequence the carbon footprint of cropping systems (Nemecek *et al.* 2008; Watson *et al.* 2017). This dependence created a multifaceted impasse that the European Union is attempting to address by promoting the cultivation of indigeneous grain legume crops.

Grain legumes are members of the Fabaceae family and are worldwidely cultivated for their highly nutritive seeds. Major agricultural grain legumes in the world may be divided into two groups, the warm-weather group including the genera *Vigna, Phaseolus, Cajanus, and Glycine*, and the cold-season group that includes the genera *Vicia, Pisum, Trifolium, Lotus, and Lupinus* (Link *et al.* 2009).

In Europe, farmers seldom incorporate leguminous plants in their cropping systems. Beyond the unfavorable trade policy, supplementary factors contribute to the devaluation of these crops, such as yield uncertainties that have led farmers towards more economically reliable crops like grains, potatoes, or sugar beets (Von Richthofen *et al.* 2006). This situation is moreover endorsed by lock-ins of sociotechnical systems that have occurred when investments in varietal selection, experimentation, production system setup, processing industries and sectors, were planned without considering the potential of legume crops (Voisin *et al.* 2014; Reckling *et al.* 2016). Today, legume grain crops remain subject to numerous climatic and biotic hazards that make the quantity and quality of legume seeds uncertain (Vadez *et al.* 2012).

The introduction of grain legumes in crop rotations however provides numerous environmental, health, and ecological benefits (Voisin *et al.* 2014). Aware of these benefits, the European Union is actively promoting the introduction of grain legume in crop rotation to simultaneously address the protein self-sufficiency gap and to promote regenerative, ecofriendly and climate-resilient feed/food systems with less fossil energy requirement (Puma *et al.* 2015). In 2018, a *strategy for the promotion of protein crops encouraging the production of protein and leguminous plants in the European agriculture sector* was published. One of the legume crops suggested by this strategy is an indigenous crop commonly named faba bean (*Vicia faba* L.) which is the most yielding of all cold season legume crops (Cernay *et al.* 2016; European Parliament 2018). The Green Deal and the latest reforms of the Common Agricultural Policy also support the promotion of faba bean crops.

This thesis focuses on the faba bean crops in Wallonia (Belgium). The sustainable introduction of faba bean, like other legume crop, is facing technical, logistical, and cultural barriers (Simmen 2020). The first issue that should be managed is the high variability in quality and quantity of seeds production. More particularly, the qualititative issue caused by an increasing

pest pressure, commonly named bruchids (Coleoptera : Chrysomelidae) and the restriction of pesticide necessitate the research of alternative methods of control to strengthen the seeds quality and the economic reliability of faba bean seeds production which would simultaniously beneficiate crop rotations with multiple agro-systemic services.

2 Bruchids, a specific group of leguminous seeds pest

Bruchids or seed beetles are important pests of leguminous seeds. This general denomination refers to small Coleoptera developing in forming and/or stored legume seeds, causing significant economic losses (Southgate 1979; Borowiec 1987). The ravaging stage is caused by the endophytic development of the larvae in the grains that consume the reserves of the cotyledons (*i.e.* seminovorous larvae) (Hoffmann 1945).

The taxonomy of bruchids considerably evolved during last decades which has led to a confusion in the designations of pests (Borowiec 1987; Kergoat *et al.* 2007; Kergoat 2011). First works classified these small Coleoptera in the Curculionidae until the XIXth Century. Then, these Coleoptera were considered from the middle of the XIXth Century as a specific family named Bruchidae. At the beginning of the nineties, systematicians classified bruchids within the family of Chrysomelidae, as one of the 13 subfamily named *Bruchinae* (Kergoat 2011). This group includes 1700 species belonging to 64 genera in six tribes, *Amblycerini*, *Bruchini*, *Eubaptini*, *Kytorhinini*, *Pachymerini* and *Rhaebini* (Kergoat *et al.* 2007; Ghahari and Borowiec 2017). *Bruchini* represents the majority (about 80%) of seed beetles with 177 recognized species and 16 genera (Johnson 1989; Kergoat *et al.* 2007).

All bruchid species present similar global life cycle. Gravid female lay eggs on pods or seeds, larvae develop inside one or several seeds through four larva stages and mostly pupate inside the seed. However, some ecological differences in this general life cycle led to the distinction of three ecological groups established on the basis of the laying behaviour of females and the ability of larvae to develop in dry seeds (Huignard *et al.* 2011). The first

group refers to females laying eggs on forming pods, *i.e.*, larvae developping in forming seeds. Those insects greatly depend on host plant phenology and can realize only one generation per year. Species from *Bruchus* or *Bruchidius* genera belong to this group (Howe and Currie 1964). Species of special agronomic importance in Europe are *B. rufimanus* impacting *V. faba*, *Bruchus pisorum* (L., 1758) impacting *Pisum sativum* L., and *Bruchus lentis* Frölich, 1799 impacting *Lens culinaris* Medik. (Huignard *et al.* 2011; Yus-Ramos *et al.* 2014).

The second group corresponds to bruchids whose larvae can develop both in forming seeds and mature/dry seeds. They will be able to make several generations per year and develop in stored seeds (Johnson 1981). This group is therefore economically more important than the first one and is essentially present in tropical areas (Southgate 1979). Species from the genus *Callosobruchus*, *Zabrotes* and *Acanthoscelides* are notable because of their damage to crops and stored legume seeds. In Europe, only *Acanthoscelides obtectus* (Say, 1831) is able to support external climatic conditions and develops on vetches, beans and other leguminous plants (Yus-Ramos *et al.* 2014).

The third group, which is more anecdotic, includes bruchids whose larvae develop in mature seeds. Eggs are laid on pods at the end of the host plant fructification and the fourth larval stage moves out from the seed and pupates in a silk cocoon at the external surface of the pod (Southgate 1979; Johnson 1981). Species of the *Pachymerini*'s tribe are part of this group, more particularly from the genus *Careydon* which are pests of stored legume seeds (Huignard *et al.* 2011). Besides damage caused to legume crops seeds, other bruchid species may also impact some Acacias trees and decrease their regeneration (Southgate 1979; Kergoat 2011).

The particularity of bruchids is their high trophic specialization level to their host plants that was relatively constant over their evolution and referred as "*taxonomic conservatism in host-plant use*" (Janzen 1980; Jermy and Szentesi 2003; Kergoat *et al.* 2007). As an example, almost all species from the genus *Bruchus* are related in plants from the *Vicieae* tribes (*Fabaceae*) (Delobel and

Delobel 2006). The phylogenetical relationship observed between some plants taxas and a single bruchid genus is presented in **Figure 1** with a bilateral interaction network including 22 *Bruchus* species whose larvae develop in seed of *Vicia*, *Lathyrus*, *Pisum* or *Lens* host plant species. Some species are strict monophageous such as *B. lentis* or *B. pisorum* while others are oligophageous such as *B. rufimanus* or *B. atomarius* (Linnaeus, 1760).

Association patterns between a phylogenetically related group of bruchids and another phylogenetically related group of host plant may be explained by the complex diversity of secondary compounds produced by leguminous plants. A given bruchid species has specific affinity for its host plant regulated by (i) the diversity of surface compounds that allow the recognition of the host plant for ovisposition (*i.e.*, the complex surface chemistry of plants, often shared phylogenetically), (ii) the multiple adaptation at the level of post-embryonic physiology that allow the by passing of physical and chemical defense of seeds (*cf.*, the detoxification of secondary compounds produced by plants during larval development) (Kergoat 2011).

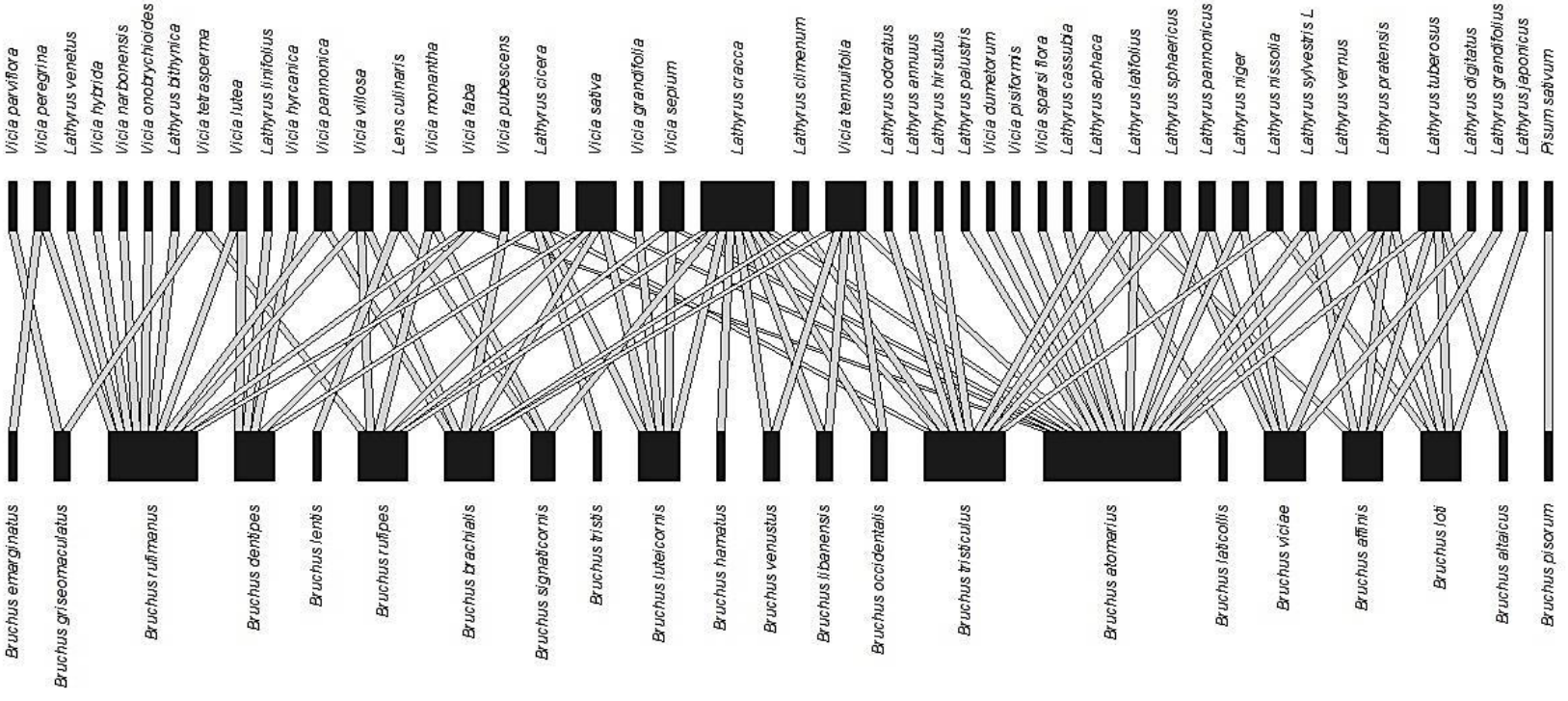


Figure 1: Bilateral trophic network illustrating the "*taxonomic conservatism in plant use"* concept with 22 species from the genus *Bruchus* associated with four genera of *Vicieae* Tribe host plants. The network's data comes from literature review about feeding of *Bruchus* larvae (Kergoat *et al.* 2007) and was transcripted on a bilateral network with Rstudio ® - version 1.1.463 – package bipartit

3 Faba bean (*Vicia faba* L.)

3.1 Origin, taxonomy, botanical description and physiology

Faba bean, also known as field bean, horse bean, and tic beans, belongs to the species *Vicia faba* L. (Fabaceae). This plant is part of the subfamily *Faboideae* and to the tribe *Fabeae* (also named the tribe *Vicieae*) and constitutes one of the earliest domesticated crops cultivated as grain legume for food and feed uses (Cubero 1974; Link *et al.* 2009). The name *faba* ethimologically refers to a Greek verb meaning "to eat" attesting the common uses of seeds in human and animal alimentation by ancient Greeks and Romans (Muratova 1931; Duc *et al.* 2010). The crop is cultivated under temperate climates in Asia, Europe, North and East Africa and in the northwest of America (Duc 1997). Despite the crop global importance, no wild progenitor could be identified yet, and the plant origin is still debated. The discovery of wild specimens in Israeli Carmel Mount dating from 14,000 years ago supports that the geographical origin is located in the Near East, where the first evidence of faba bean domestication is dated to 10,200 BC (Tanno and Willcox 2006; Caracuta *et al.* 2016).

The long history of faba bean domestication under a wide range of latitude and longitude resulted in great genetic diversity offering a wide range of growth habits, stress tolerances abilities and seed sizes traits (Cubero and Suso 1981; Duc *et al.* 2010). This genetic diversity is stored 37 *ex situ* genbanks collections that include approximately 38,000 accession of *V. faba* (Duc *et al.* 2010). Such collection of faba bean genetic accessions constitutes an opportunity for selection processes and cultivar adaptation to numerous environments.

The different cultivars of *V. faba* are generally classified according to the size of the seeds. Four "varietal groups" or "botanical types" are considered, namely *major*, *minor*, *equina*, and *paucijuga* (Cubero 1973). None major reproductive barriers are observed between these groups, even though some rare unilateral incompatibilities could exist in certain sexual cross (Guen *et*

al. 1983; Jayakodi *et al.* 2023). *Major* varieties are commonly called “broad beans”, they include varieties with large flattened seeds weighting from 1.0 to 2.0 g per seed, and are mainly consumed as fresh legumes in Europe notably (Duc 1997; Mínguez and Rubiales 2021). *Equina* varieties are commonly called “horse beans” or “field beans” and present smaller flattened seeds weighting from 0.6 to 1 g per seeds. The *minor* group corresponds to varieties commonly named “tic beans” or “pigeon beans” and present ellipsoidal seeds weighting from 0.3 to 0.6g per seeds (Ward 2018). Finally, the *paucijuga* group is a more anecdotic group related with supposed wild progenitor of *V. faba* (Cubero and Suso 1981; Guen *et al.* 1983; Duc 1997).

The discrepancies in language designations for botanical groups of *V. faba*, coupled with differences in seed use or political registration processes between countries, have contributed to the confusion in the taxonomic consideration or classification of *V. faba* cultivars. As an example, this thesis focuses on the crop named in Frensh "*féverole*," which Guen *et al.* (1983) reports as being integrated into the *equina* type ("*féverole à gros grains*") and the *minor* type ("*féverole à petits grains*"). However, the Common Catalogue of European Agricultural and Vegetable Species includes this crop only in the *minor* type and refers to it as "field beans", which is the common name of the *equina* type. It is therefore essential to clarify the taxonomy, botanical type and associated common name to avoid further confusion. In this work, the French crop denomination "*féverole*" is defined as belonging to both *equina* and *minor* botanical types, and is commonly named "field beans," such as proposed by Link *et al.* (2009) to be consistent with EU texts. The denomination “faba bean” is a general denomination that addresses all the four botanical type. **Table 1** summarize different type of *V. faba* varieties, their common names, uses and area of domestication (Guen *et al.* 1983; Duc 1997; Mínguez and Rubiales 2021).

Table 1: Botanical groups of *V. faba*, common denomination, area of selection and seed uses according to Guen *et al.* (1983), Duc (1997), Mínguez and Rubiales, (2021).

Species name	Botanical type	Common English denomination	Common Frensh denomination	Area of selection	Uses
Vicia faba L.	*major*	Broad bean	Fèves/Fèves des marrais	South mediteranean countries China Expansion toward Mexico and South America	Fresh legume
	equina	Field bean/horse bean	Féveroles	Middle east and North Africa (Egypt)	Dry seeds: Staple food and feed
	minor	Tic bean	Féveroles	Ethiopia North Europe	Dry seeds: Staple food and feed
	paucijuga	ND	ND	Not applicable (wild hypothetical form)	Not applicable (wild hypothetical form)

Beyond the four botanical groups based on seed size, different genotype-by-environment adaptations lead to differences in physiology and environmental resistance of faba bean across Europe. In consequence, faba bean can also be divided into five ecotypes or gene pools across Europe: Mediterranean faba bean, spring-sown faba bean within the oceanic zone, autumn-sown faba bean (*i.e.*, winter faba bean) within the oceanic zone, spring-sown faba bean within the continental zone, and autumn-sown faba bean within the continental zone (Flores *et al.* 2012, 2013). These different groups distinguish by tolerance to winter condition, the duration of crop-growth, the optimum of flowering time, the photoperiod requirement and the disease resistance (Patrick and Stoddard 2010; Duc *et al.* 2015).

Vicia faba has a strong tetragonal erected stem. Several stems may branch at the base of the plant, especially with winter cultivars that present four to six stems per plants, while the spring cultivars present one to two stems per plants (Duc 1997; Link *et al.* 2009). The plant height ranges from 10.3 cm to 201.5 cm, with an average height of 78cm (Duc *et al.* 2015). The growth of faba bean is indeterminate, consequently, the number of nodes per plants, the number of nodes bearing flowers or pods are unpredictably resulting from environmental and intra-plant factors (Patrick and Stoddard 2010).

Leaves insertion on the stem is alterne and one pinnate leave with two to six leaflets is observed per node (Mínguez and Rubiales 2021).

The flowering of faba bean is mainly regulated by thermal time with 830 – 1000 degree-days above 0°C required for flowering (Patrick and Stoddard 2010). Racemeal inflorescence with two to eight flowers are observed from the fourth node of the plant (Link *et al.* 2009). Flower length 2-3 cm and their morphology is typical from *papillonaceous* structures (Duc 1997). The flower colors vary according to varieties. White flowers with pink traces are the wild type flowers but other color gradients are observed such as pink or brown petal. In most case, flowers present a black spot on wing petals, totally white flowers is a pleiotropic trait corresponding to the allele of zero tannins in seeds coat content (Duc 1997; Link *et al.* 2009; Duc *et al.* 2015).

Faba bean has a mixed breeding system with autopollination and insect pollination (Stoddard and Bond 1987). The transition of self/allo-pollinated flowers into pods is subjected to three factors including (i) the presence of pollinating insects, (ii) the environmental light and water resource availability during flower fertilization (*i.e.*, transient environmental stress), and (iii) the intra-plant competition between reproductive and/or vegetative structures for photoassimilates (Patrick and Stoddard 2010). The flower production of faba bean is excessive to increase the plant attractiveness on pollinating insects, and increase therefore their fitness (Schoonhoven *et al.* 2005). The amount of pods produced per plant range from 1.1 to 93.7. In general, faba bean yield more than twelve pods per plant containing up to six seeds (Link *et al.* 2009; Duc *et al.* 2015).

The roots *V. faba* form a tap root system as it can be observed with other leguminous species. Secondary roots carry nodules hosting nitrogen fixing bacteria that mostly belong to the species *Rhizobium leguminosarum* var *viciae* (Rlv) which are diverse and ubiquitous soil bacteria (Allito *et al.* 2021). These root symbioses of *V. faba* are therefore naturally occurring and do not require an inoculation of rhizosphere. They are reported as plant growth-promoting rhizobacteria (PGPR) as they exert a direct benefit on the plant growth by

the supply of an uptaking form of Nitrogen to the plant (Saïdi *et al.* 2013). Roots of *V. faba* may also form other ecto/endomycorrhizal associations (Duc 1997).

The genome of *V. faba* is one of the largest diploid field crops with 13 000 Mbps. The standard karyotype contain six pairs of chromosoms with five pairs of acrocentric chromosom of approximately 7-9 µm length, and one pair of metacentric chromosoms of about 18µm length. This large pair of chromosome is equivalent to the entire human genome and made progress towards genome assembly very challenging (Duc 1997; Link *et al.* 2009; Duc *et al.* 2015). Despite the identification of some isolated gene involved in the production of antinutritionnal factors in seeds composition, no complete assembly of *V. faba* genome was performed until Jayakodi *et al.* (2023) recently sequenced and completely assembled for the first time the whole the giant genome of faba bean. Such work offers a reference genome necessary for further works of varietal selection.

3.2 Seed composition, plant uses and worldwide production

Faba bean is cultivated for its high-nutritive seeds being mostly harvested dry/mature for food or feed. Immature/fresh seeds may also be used as vegetable, especially with the *major* group. Some varieties that produce a significant amount of biomass can also be used for bioenergy production.

The high nutritive value of seeds is explained by their high content in carbohydrates (starch and fibres) and protein, which respectively surround 40% and 30% of dry weight (DW). The protein content may reach 34% in some cultivars which is more important than other cool-season legume crops such as pea, lentil or chickpea (Duc 1997; Vioque *et al.* 2012). Seeds also contain minerals (~3-4% DW) and lipids (~2%) (Vioque *et al.* 2012). The amino acids composition includes lysine, cysteine, methionine and tryptophan which are important for human and animal alimentation (Crépon *et al.* 2010). Compared with seeds of soybean, the lysine content of faba bean seeds is more important but the sulphur amino-acids content such as tryptophan is poor (Vioque *et al.* 2012). Seeds of *V. faba* also contain anti-

nutritional factors such as tannins (T), vicine (V) and convicine (C). Tannins are polyphenols located in the seed integument at about 5 to 10 g/kg DM and decrease the digestibility of seed proteins (Butler and Rogler 1992; Crépon *et al.* 2010). The tannin content in faba bean seeds includes mainly catechin, epicatechin and syringic acid. Vicine and convicine are glucosidic aminopyrimidine derivative located in the cotyledon at 6 to 14 g/kg DM and cause a disease in human called "*favism*". Aglycone forms, which are divicine (2,6-diamino-4,5-dihydroxypyrimidin) and isoumaril (6- amino-2,4,5-trihydroxypyrimidin) are produced from vicine and convicine by the hydrolysis of the β-glucosidic bond between glucose and the hydroxyl group on the C-5 of the pyrimidine ring. Humans showing a deficit in glucose-6-posphate dehydrogenase (G6PD) are affected by favism. The production of aglycones in the large intestine and cecum are transported in the blood, and form some products causing destructive oxidation of red blood cells in the presence of oxygen leading to potential lethal hemolytic anemia (Crépon *et al.* 2010; Duc *et al.* 2015). Vicine and convicine also impact the production performance in chicken or laying hens (Crépon *et al.* 2010).

Over last decades, mutant alleles involved in the tannins synthesis have been identified in two distinct loci (*zt1* and *zt2*) in *V. faba* genetic resource, which allowed the emergence of low tannins cultivars that present at the same time a total white flower colour (Brun 1991). A mutant allele (vc-) could also be discovered and decreased 10-20 folds the vicine and convicine contents (Duc *et al.* 1999). As a consequence of the presence or absence of antinutritionnal factors (T-V-C), the European catalogue of registered varieties of faba bean (including 207 *major* and 372 *minor* varieties) distinguish four goups of varieties: varieties with high or low content of tannins and varieties with high or low content of vicine-convicine (Crépon *et al.* 2010). The seed uses are related to these groups as field bean seeds with low tannin are favored in the alimentation of monogastric animal diets, low vicine and convicine varieties are favored in the alimentation of poultry and also in the human consumption.

At the worldwide level, faba bean is the seventh most cultivated grain legume crop (Duc *et al.* 2015). In 2021, a total of 7.69 Mt of faba bean seeds was produced in the world, respectively 5.96 Mt of dry seeds and 1.73 Mt of fresh seeds (**Figure 2**). The top five producing countries of dry seeds were China (1.69 Mt), Ethiopia (1.09 Mt), the United Kingdom (0.694 Mt), Australia (0.509 Mt), and Germany (0.235 Mt) ("FAOSTAT" 2021).

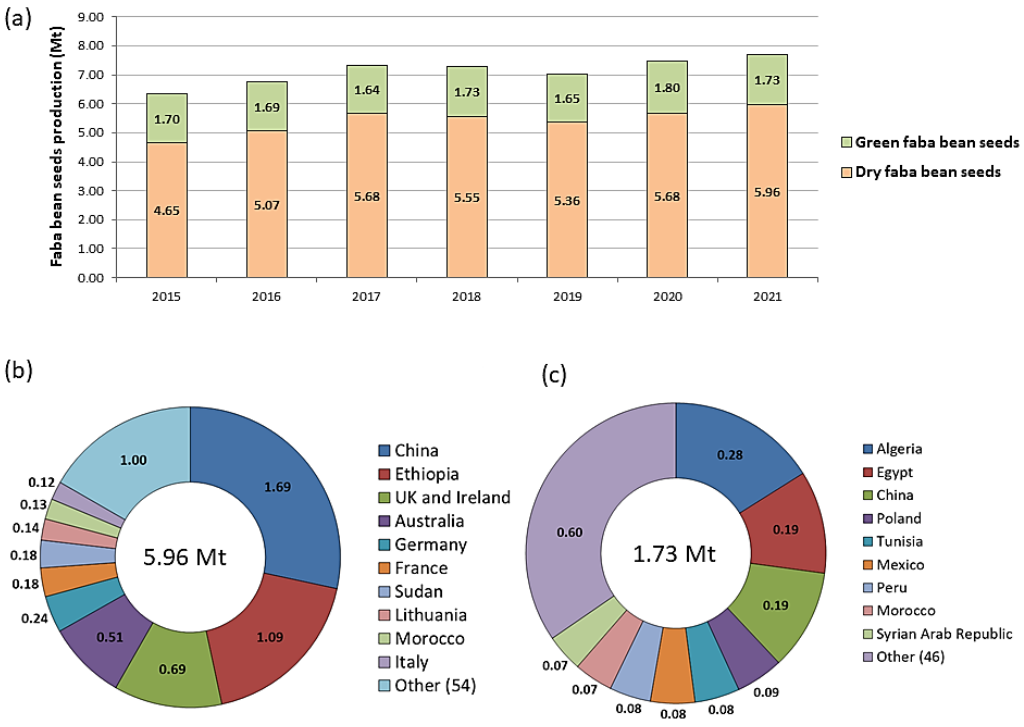


Figure 2: Worldwide annual production of faba bean seeds (Mt) between 2015 and 2021 (a); Worldwide production dry faba bean seeds (Mt) in 2021 (b); Worldwide production of green faba bean seeds (Mt) in 2021 (c) (FAOSTAT 2021) ©A. Segers 2023

In Europe, faba bean is the third most cultivated grain legume after the pea and the soybean (Sepngang *et al.* 2020). United Kingdom was the first producer of European countries before the brexit with 0.55 Mt of estimated seed production in 2020 (**Figure 3**). Germany, Lithuania and France are the three following countries with respective productions of 0.250 Mt, 0.210 Mt and 0.158 Mt in 2022 ("EUROSTAT" 2022).

Faba bean is rarely used as food in Europe, unlike in Mediterranean countries from north Africa which use seeds in staples dishes (Crépon *et al.* 2010). In central Europe, faba bean seeds are employed as animal feed. The utilization of these seeds hinges on their attributes within the diets of ruminants, pigs, and poultry, providing a local plant protein source that contributes directly to proteic autonomy (Abras *et al.* 2015). In countries like Spain, *V. faba* is locally harvested fresh and canned for human consumption. It can also be used in bread or in processed products such as falafel or hummus (Sepngang *et al.* 2020). Technico-functionnal characteristics of faba bean seeds offer a promiscuous resource for the formulation of new products in the food industry as an alternative to traditional animal proteins. This food resource is low cost in energy and water resource for the fractioning of proteins from carbohydrates and respond to the growing demand for plant based food products (Dumoulin *et al.* 2021). Globally, the main export markets for *V. faba* are destined to the Middle East and North Africa with Sudan and Egypt that import respectively 331 and 56kt (Mínguez and Rubiales 2021). Thirteen percent of the European production is exported and 80% of these exports are to countries outside the European Union (Sepngang *et al.* 2020). The rest is intra-Europe traded with new export markets emerging such as dehulled seeds for fish farming in Norway that settled last decades as a substitution of the export market to Egypt for food uses which collapsed due to quality issues (Lacampagne 2021).

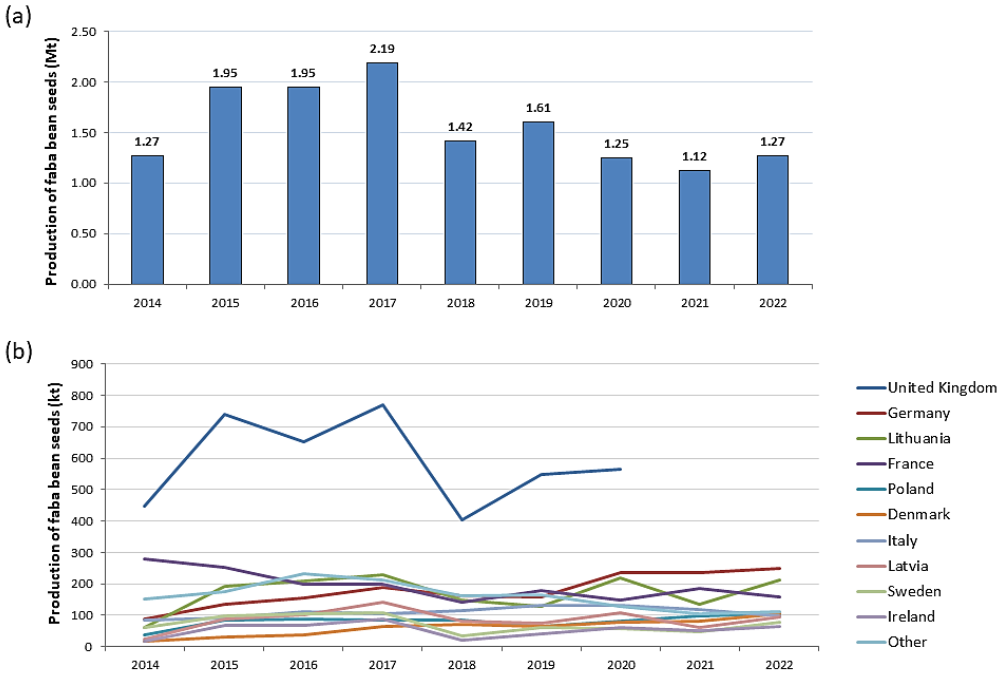


Figure 3: EU28 annual production of faba bean seeds (dry and fresh) during the period 2014-2022 (a); Top ten of EU28 countries production of faba bean seeds during period 2014-2022 (b) (EUROSTAT 2022) ©A. Segers 2023

3.3 Cropping practices, cultural advantages and drowbacks

Vicia faba presents the highest yields of all grain legumes with an average of four tons of seeds per hectare but the production is highly variable compared to non-legume crops (Cernay *et al.* 2015, 2016). This culture is usually cropped in Europe as pure crop, sown either in autumn or in spring (Biarnès *et al.* 2018). Winter cultivars are more productive than spring cultivars and seed are sown at different densities regarding the difference in stem branching per plant. Winter faba bean cultivars are usually sown at 20 to 30 seeds/m² while spring cultivars are sown at 35-60 seeds/m² (Link *et al.* 2010). A six year period is advised between two faba bean crops, previous crops with high nitrogen fertilizer input should be avoided because they would cause a strong vegetative development of the crop rather than seed production and they would reduce the plant abilityto fix atmospheric nitrogen (Abras *et al.* 2015). Compared with other legume crops such as pea, faba bean presents the advantage to be resistant to *Aphanomyces euteiches*

Drechsler, 1925, an important root disease of other leguminous plants (*eg.*, pea crops) that may remain in soil up to thirteen years (Abras *et al.* 2015; Biarnès *et al.* 2018).

Faba bean is particularly interesting as previous crop of cereals due to its contribution to the nitrogen soil content. This crop is suitable for clay or stony soils and can be grown in a coarse seedbed (Biarnès *et al.* 2018). The seeding depth must take into account possible winter conditions. The rigid stem of the plant allows sowing with a large spacing which allows a weeding by hoeing. The sowing dates are different of cereals or rapeseed crops which allows a spread out work. Tilling is not necessary after the harvest.

Faba bean may also be cropped as intercrop, especially with cereals. The association of faba beans with other crops is becoming more popular with the development of organic farming and low inputs systems (Mínguez and Rubiales 2021). This legume can also be used as a green manure in crop rotations. Frosty cultivars such as spring beans are then favored to cover the soil and enrich it with nitrogen during winter periods (Jensen *et al.* 2010). All these cropping itineraries (pure crop, intercropping, green manure crops) aim to maximize the multiple agro-systemic services of faba bean including (i) the ability to fix atmospheric nitrogen, (ii) the decrease in greenhouse gases emissions, (iii) the favoring of a benefic entomofauna and (iv) the high nutritive seed production – see previous section (Köpke and Nemecek 2010).

Faba bean is one the most efficient legume crop for nitrogen fixation before lentils, common bean or peas, with up to 76% of N crop content issued from biologically fixed nitrogen (Walley *et al.* 2007; Herridge *et al.* 2008; Liu *et al.* 2019). Other studies such as Silim and Saxena (1992) reported that the Nitrogen fixation was varying from 82% to 88%. An important finding from this study was the fact that faba bean present a positive N contribution for the successive crop as far as roots and aerial plant residues remain in the soil after harvest. When only roots are left in the soil, the N contribution for the subsequent crop can be negative at -18.3 kg N per hectare (Senaratne and

Hardarson 1988). Natural nitrogen fixation with root symbioses offers two main advantages to cropping rotations. Firstly, the crop is self-sufficient in nitrogen nutrition and secondly, it enriches the soil for the following crops or for the plant species grown in association (Jensen *et al.* 2010). A wheat crop following a faba bean crop can benefit from approximately 30 kg N per hectare (McEwen *et al.* 1990), and a yield increase ranging from 500 to 750 kg per hectare (Abras *et al.* 2015; Biarnès *et al.* 2018). Crop rotations and agronomic practices must be arranged so that the following crop uses a maximum of biologically active N to reduce the possible environmental risks related to leaching losses or to the emission of nitrous oxide (N_2O) into the atmosphere (Jensen *et al.* 2010).

The natural N enrichment of soil allows at the same time a reduction in greenhouse gases emissions. Indeed, the production of 1 kg of synthetic N fertilizer in crops require about 42 to 90 MJ and their application on crops results in greenhouse gas emissions ranging from 2.8 kg to 16.1 kg of carbon dioxide (CO_2) equivalent (Köpke and Nemecek 2010). The introduction of faba bean therefore decreases greenhouse gases emissions and attenuates the carbon footprint of cropping rotations.

Faba bean crops are also greatly attractive for beneficial insects among cropping systems. This crop plays an important role in maintaining the diversity and abundance of many pollinators by providing a source of pollen and nectar (Köpke and Nemecek 2010). Moreover, the presence of pollinators increase the crop yields as this plant is partially allogamous. Cross-pollination can increases the number of pods per plants, the number of seeds per pods, and the weight of seeds, which may increase total yield by up to 185% compared to self-pollination (Nayak *et al.* 2015). Insect pollination also stabilizes the faba bean seeds production (Bishop *et al.* 2016; Bailes *et al.* 2018). The main pollinators observed in temperate faba bean crops are bumblebees like *Bombus terrestris* (Linnaeus, 1758), *B. hortorum* (Linnaeus, 1761), *B. pascuorum* (Scopoli, 1763), *B lapidarius* (Linnaeus, 1758), *B. hypnorum* (Linnaeus, 1758) and *B. pratorum* (Linnaeus, 1761), honeybees

such as *Apis mellifera* Linnaeus, 1758 as well as some solitary bees belonging to the genera *Lasioglossum* Curtis, 1833 and *Andrena* Fabricius, 1775. However, not all of these insects contribute equally to pollination. Bees with long tong are more probably in contact with anthers rather than short-tongued bees which reach the nectaries at the base of the long corolla tube by incising it at the bottom, and do not come into contact with the anthers. These insects are called "*nectar robbers*" (Leadbeater and Chittka 2008). In addition to pollinating insects, *V. faba* flowers and extra-floral nectaries are also attractive to aphid predators such as hoverflies (Diptera: Syrphidae), lacewings (Neuroptera: Chrysopidae), ladybeetles (Coleoptera : Coccinellidae) or parasitoids (Nuessly *et al.* 2004; Schulz-Kesting *et al.* 2021). These insects found in the semi-natural habitat of faba bean crops serve as natural biocontrol agents against pests that can affect *V. faba* crops and/or other nearby crops. This has the potential to enhance crop profitability over the long term (Rosa-Schleich *et al.* 2019).

Faba bean crops include multiple other benefits such as the increase of crops diversity, the improvement of associated biodiversity (Köpke and Nemecek 2010), the disruption of pest cycle within rotations (Jensen *et al.* 2010), the improvement of the biological characteristics of the soil via the stimulation of microbial activity (Wahbi *et al.* 2016), the improvement of the soil structure (Rochester *et al.* 2001) and the soil chemical characteristics via the mobilization of phosphorus and potassium in addition to nitrogen nutrients (Köpke and Nemecek 2010).

Nevertheless, faba bean crops show high instabilities in yield related to multiple biotical and abiotical factors. The major abiotic stresses in *V. faba* are heat, drought, waterlogging, and frost (Karkanis *et al.* 2018). The impact of these stresses on the seed quality and quantity vary according to the cultivars (*cf.*, the four ecotypes of faba bean), the sowing dates (*i.e.*, the growing stage of faba bean affected by the stress) and region where faba bean is grown (Maalouf *et al.* 2021). Drought and heat stresses are recognized as the primary cause of yield loss in Europe (Karkanis *et al.* 2018),

especially drought periods, *i.e.* period of water deficit (Khan *et al.* 2010). Faba bean is part of the most sensitive legume crops to water deficit (Amede and Schubert 2003). Such stress is greatly impacting during key developmental stage of crops, *i.e.*, the fertilization of flowers and production of pods (Patrick and Stoddard 2010). The rate of symbiotic nitrogen fixation also decreases with water deficit (Vertès *et al.* 2010). However, some cultivars are more tolerant to drought by reducing their leaf transpiration when the soil dries (Girma and Haile 2014; Kibbou *et al.* 2022). Faba bean is also sensitive to heat stress. It tolerates low temperatures relatively well, especially winter cultivars can survive up to -10°C (Abras *et al.* 2015). Frost sensitivity of faba bean is accrue during seedling and flowering stage (Maqbool *et al.* 2010). Conversely, temperatures above 23°C may inhibit the flower initiation (Evans 1959). High temperature may also impact the pollen viability (Lavania *et al.* 2014) and have a negative effect during the pods filling stage (Kibbou *et al.* 2022). Faba bean demonstrates the highest tolerance among cool-season grain legumes to waterlogging. However, when waterlogging occurs during flowering, it imposes limitations on yields, and the associated symptoms persist even after the soil flooding subsides (Solaiman *et al.* 2007; Pociecha *et al.* 2008; Pampana *et al.* 2016).

3.4 Pests and deseases

Faba bean is sensitive to a wide range of biotic stresses including insect pests, nematodes, parasitic weeds, foliar fungal disease, viruses and soil borne pathogens. Among the diseases, the most damaging to the crop are fungal foliar diseases such as botrytis (*Botrytis fabae* Speg., 1898), ascochyta (*Ascochyta fabae* Speg., 1898), rust (*Uromyces fabae* (Pers.) de Bary, 1879) or downy mildew (*Peronospora viciae* (Berk.) de Bary, 1863) and root diseases such as fusarium (*Fusarium* spp.). These diseases are favored by cloudly weather whith high relative humidity. Several agricultural practices can be implemented to manage faba bean disease like adapted crop rotations, delayed sowing, timing of fungicide applications or the use of certified seeds (Torres *et al.* 2006; Mínguez and Rubiales 2021)

A large-scale survey led by Kumari and Van Leur (2011) identified 18 viruses infecting faba bean, including eight persistent viruses causing yellowing/stunting/necrosis symptoms and 10 non-persistent viruses causing mosaic/mottling symptoms. All viruses of major importances are mainly transmited by insects. Concerning European viruses affecting faba bean, only three were indicated, *i.e.*, the broad bean stain virus (BBSV – transmitted by beetles), the broad bean true mosaic virus (BBTMV – transmitted by beetles) and the pea enation mosaic virus-1 (PEMV-1 – transmitted by aphids).

Several pests impacting faba bean are documented in the literature, the most damaging to the crop are the sitona - *Sitona lineatus* L. (Coleoptera : Curculionidae), the black aphids - *Aphis fabae* Scopoli, 1763 (Hemiptera – Aphididae), the stem nematode - *Dytilenchus dispaci* (Kuhn, 1857) and the bruchid beetles - *Bruchus* spp. (Coleoptera – Chrysomelidae). Pigeons are also reported in some countries like France or Belgium as important pests destroying recently sown field bean crops (Abras *et al.* 2015). While the majority of these pests exert a direct influence on crop yields by impacting physiological aspects of the plants, such as photosynthetic capacity, vascularization, and virus transmission, bruchid beetles have no impacts on the seed yields because their larva development within forming seeds which is a photosynthetic sinks rather than sources. Their quantitative impacts are relatively low but their impacts on seeds quality are very important which makes bruchids a major pest of faba bean crops. In Belgium, five species are potentially infesting faba bean seeds according to informations provided by Decelle (1989) and Baugnée *et al.* (2021) addressing a list of the bruchid species recorded in Belgium. When cutting these information with works of Zampetti and Ricci (2012) reporting the larval host plant bruchid species, it can be assumed that five potential bruchid species may infest *V. faba*, including *Bruchus atomarius* (L. 1761); *B. pisorum* (L. 1758); *B. brachialis* Fåhraeus 1839; *B. affinis* Frölich 1799 and *B. rufimanus* Boheman 1833 (Zampetti and Ricci 2012; Baugnée *et al.* 2021). *Bruchus rufimanus* is reported to be the main species infesting *V. faba* in Tunisia (Titouhi *et al.* 2015).

4 Overview of *Bruchus rufimanus* Boheman 1833 (Coleoptera : Chrysomelidae): Biology, chemical ecology and semiochemical opportunities in integrated pest management programs

Taken from the following reference:

Segers, A., Caparros Megido, R., Lognay, G., & Francis, F. **(2021)**. *Overview of Bruchus rufimanus Boheman 1833 (Coleoptera: Chrysomelidae): Biology, chemical ecology and semiochemical opportunities in integrated pest management programs*. Crop Protection, 140, 105411.

Abstract : Promoting the cultivation of native legumes, such as faba beans (*Vicia faba* L.) within the European Union is anticipated to contribute to the sustainability of cropping systems and provide food and feed proteins as alternatives to unsustainable imports. However, efficient alternative control methods to pesticides must be implemented to combat key pests that devalue faba bean seeds (namely, *Bruchus rufimanus* Boheman 1833, Coleoptera: Chrysomelidae). This pest causes significant economic losses in faba bean crops as post-embryonic development (*cf.* seminovorous larvae) occurs inside forming seeds. While there has been extensive research on the biology and chemical ecology of *B. rufimanus*, efficient control methods are lacking. Here, we review this pest species to identify: (i) knowledge gaps on its biology that could enhance management tools; (ii) potential improvements to current semiochemical-based control approaches; and (iii) other method of control based on semiochemicals that could be implemented.

Keywords: Biocontrol; Bruchids; Semiochemicals; Pheromones; Kairomones; Entomopathogenic fungi

4.1 Introduction

Vicia faba L. (Fabaceae) (common names: broad bean, field bean, and horse bean) is a leguminous plant that provides multiple ecological services to agricultural systems, and contributes to their sustainability (Köpke and Nemecek 2010). (Jensen *et al.* 2010) previously listed four benefits of introducing *V. faba* to cropping systems. First, it provides soil with natural green manure by fixing atmospheric nitrogen via root symbiosis (*cf. Rhizobium* bacteria located in nodules), significantly enhancing successive crops yields, particularly cereals. Second, this manure input reduces carbon dioxide emissions generated by the manufacture, transport, and spread of synthetic fertilizers (Wani *et al.* 1991, 1994). Third, *V. faba* diversifies cropping systems, hindering pests and diseases, and promoting biodiversity by providing floral resources that benefit organisms, like pollinators (Jensen *et al.* 2010; Abras *et al.* 2015). Fourth, *V. faba* produces seeds rich in starch and proteins, which are valued in the food and feed market, providing sustainable local alternatives to imports (Duc 1997; Duc *et al.* 2010; Köpke and Nemecek 2010).

Nevertheless, *V. faba* is sensitive to biotic and abiotic threats, which constrain productivity and reduce crop yields (Duc 1997; Torres *et al.* 2006; Jensen *et al.* 2010). Biotic factors include fungal diseases, like *Ascochyta fabae* Speg., 1898, *Botrytis fabae* Sardiña, 1929, or *Uromyces fabae* (Pers.) de Bary, 1879, and pests, like *Sitona lineatus* (L. 1758) (Coleoptera: Curculionidae), *Bruchus rufimanus* Boheman 1833 (Coleoptera: Chrysomelidae), and *Aphis fabae* Scopoli, 1763 (Hemiptera: Aphididae). All these pests may cause direct yield losses by impacting plant photosynthetic ability, except for *B. rufimanus*, whose larva develops in forming seeds without significantly prejudicing the plant physiology (Shearman *et al.* 2005; Roubinet 2016). This pest however quantitatively and qualitatively affects the agricultural products of *V. faba* by (i) reducing the seeds weight due to the endosperm consumption of feeding larvae, (ii) decreasing the seeds nutritional value due to the accumulation of larvae faeces, and (iii) altering the seeds aesthetic quality due to perforations caused by the emergence of adults (Kaniuczak 2004; Khelfane-Goucem and

Medjdoub-Bensaad 2016). Consequently, seeds are devalued from food and feeding markets that have strict quality standards fixed at respectively max. 3% and 10% of infested seeds (Bruce *et al.* 2011; Leppik *et al.* 2016). Germination potential is also lost, and fungal infestation risk increases (Boughdad and Lauge 1995; Titouhi *et al.* 2015). The presence of living insects inside seeds also impact access to domestic and international markets (Roubinet 2016). Bachmann *et al.* (2020) estimated losses of 60 euros/t for faba beans, as damage prevents valuation in food markets, and 176 euros/t, as seed batches cannot be valorized in food and feed markets.

Limited methods exist for controlling *B. rufimanus*, exacerbating seed devaluation. Pyrethroid insecticides are typically sprayed during the flowering and first pod setting stages to target adult pests before oviposition, but the success of this method faces numerous limiting factors. Firstly, active substances approved in the European Union are limited to lambda cyhalothrin, zeta cypermethrin, and deltamethrin, and their uses are either restricted at max. two treatments per crop during the flowering period, either completely banned from "greening measures" foreseen by the Common Agricultural Policy, namely *Environmental Focus Areas.* In addition to these spraying restrictions, *V. faba* dense crop canopy hinders spraying efficiency by preventing a proper penetration into the target plant-parts (*i.e.*, the plant basal nodes, where pods grow and most *B. rufimanus* damage occurs). Also, high temperatures shorten pyrethroid persistence, while it promotes *B. rufimanus* activity (Mansoor *et al.* 2015; Ward 2018). As a result, the little amount of active substances, combined with application inefficiency, is likely to promote the onset of resistance mechanisms, as demonstrated for *S. lineatus* (Ward 2018). These pesticides also negatively impact pollinators through direct contact toxicity and perturbation of foraging behavior, which is exacerbated when combined with fungicides (Sanchez-Bayo and Goka 2014). It is thus essential to identify efficient and cost effective control methods that enhance the seed quality of native plant proteins, particularly in the context of increasing deficit in vegetable proteins

throughout European Union, which is offsetted with unsustainable imports (European Commission 2018).

Many studies have assessed the development of alternative control method over last decades. These studies highlighted five control levers, namely (i) semiochemicals (Ward 1999; Bruce *et al.* 2011; Frerot *et al.* 2015), (ii) selection of resistant varieties (Szafirowska 2012; Seidenglanz and Hunady 2016; Carrillo-Perdomo *et al.* 2019), (iii) vegetal oils, (iv) microbial control agents (e.g., entomopathogenic fungi) (Sabbour *et al.* 2007; Titouhi *et al.* 2017), and (v) an adaptation of cultural practices (e.g., sowing/harvesting dates, sowing density, and crop association) (Ward 1999; Seidenglanz *et al.* 2011; Szafirowska 2012; Bachmann *et al.* 2020). To date, management approaches mitigating *B. rufimanus* damage have yet to be implemented in Europe, and some biological aspects of this pest should still be elucidated to develop effective pest management tools, including overwintering behavior, temperature-dependent development, and quantitative economic thresholds. This review presents existing knowledge on *B. rufimanus* biology based on studies conducted across Europe and Mediterranean countries. Emphasis is placed on semiochemical processes regulating *B. rufimanus* interactions at different phenological stages of *V. faba*. Finally, semiochemical-based control methods are reviewed to highlight future research directions to implement efficient integrated pest management (IPM) strategies of *B. rufimanus*.

4.2 Biology of *Bruchus rufimanus*

4.2.1 Overview of B. rufimanus

Bruchus rufimanus (**Figure 4**) (common name: broad bean weevil or bean seed beetle) is a Coleoptera belonging to the Chrysomelidae family, subfamily Bruchinae. This subfamily contains 1,700 species, called bruchids or seed beetles. About 30 bruchid species cause major economic damage, of which nine are cosmopolitan, belonging to the genera *Acanthoscelides*, *Bruchus*, *Callosobruchus*, *Caryedon*, and *Zabrotes* (Kingsolver 2004).

All bruchids are oligophageous or monophageous species (Huignard *et al.* 2011), with an endophytic ravaging stage, when larvae consume the endosperm during post-embryotic development inside leguminous seeds (*cf.* seminovorous larvae). Two ecological groups exist based on female egg-laying behavior and the ability of larvae to develop in dry seeds (Howe and Currie 1964). The first group includes multivoltine species that breed and develop in crops and in stored dry seeds, destroying large quantities of stored pulses. The second group includes univoltine species that only develop in crops forming seeds, and do not damage stored pulses.

Bruchus rufimanus is an oligophageous species from the second group. It is a major pest of *Vicia spp.* (Hoffman *et al.* 1962). It is globally distributed from the temperate to sub-tropical areas of 27 countries, partly due to the trade of infested seeds, and particularly in areas where *V. faba* are cultivated as dry seeds (Hoffman *et al.* 1962; Bahr 1976; Southgate 1979; Ward 2018).

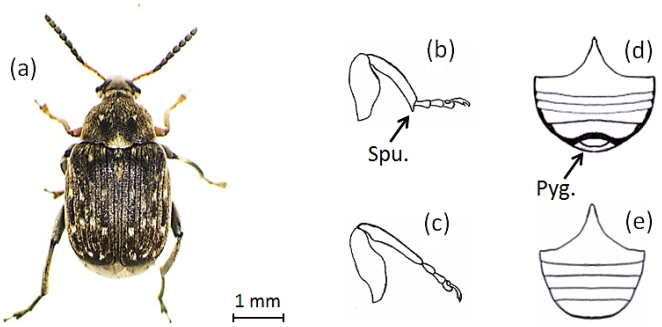


Figure 4: Morphological illustration of Bruchus rufimanus and sexual dimorphism: (a) Adult; (b) Middle leg of males presenting spurs (Spu.); (c) middle leg of females without spurs; (d) Ventral face of males abdomen indented by pygidium (Pyg.); (E) Ventral face of females abdomen without indentation. ©A. Segers

This pest strongly depends on host plant phenology, as adults feed on flowers and larvae feed on forming seeds of host plants along its univoltine life cycle. (Kergoat *et al.* 2007) identified 11 host plants of larvae, including three *Lathyrus* species and eight *Vicia* species. Adults feed on the pollen and nectar of several additional plants, depending on floral resource availability in the environment outside hosts flowering periods (Boughdad 1994;

Medjdoub-Bensaad *et al.* 2007). Pölitz and Reike (2019) detected the pollen of 12 species in the digestive tube of adults, including *Carduus sp.*, *Trifolium repens* L., *Chamaemelum nobile* (L.) All., *Sinapis arvensis* L., *Phacelia tanacetifolia* Benth., *Raphanus sativus* L., *Anethum graveolens* L., *Helianthus annuus* L., *Malva sp.*, *Cyanus segetum* Hill, *Calendula officinalis* L., and *Fagopyrum esculentum* Moench.

Figure 5 presents the life cycle of *B. rufimanus* on faba bean crops. The seasonal population dynamics of this pest are regulated by crop growth and climatic conditions (Ward 2018). Adults appear when crops flower during spring and summer, with sunshine and warmth (23–26 °C) enhancing feeding, reproduction, and oviposition (Leppik and Frérot 2014). Embryonic and post-embryonic developments correspond to the fructification period, from when young pods appear to late-summer, when most adults emerge from harvested seeds. The extent of damage (3–100%) varies across years, depending on cultivar, meteorological conditions, and crop location (Boughdad 1997; Roubinet 2016). Reproductive diapause allows insects to survive winter until trophic resources become available in spring.

4.2.2 Life cycle

When temperatures reach 15 °C in spring (*i.e.,* threshold for adult activity), diapausing adults leave overwintering sites to colonize crops (Hoffman *et al.* 1962; Medjdoub-Bensaad *et al.* 2007; Roubinet 2016). First, males colonize crops at the flower bud stage (Frerot *et al.* 2015) or vegetative stage, and consume nectar from extrafloral nectaries (Pölitz and Reike 2019). Once crops flower, females colonize them. (Tran and Huignard 1992; Tran *et al.* 1993) identified two factors regulating the end of reproductive diapause under laboratory conditions: the increase of the photoperiod to a duration of 16 light hours that allows males to mature sexually, and the consummation of host plant pollen that is additionally required for females sexual maturation. Adults only become sensitive to these two factors after 6–7 months of diapause (*i.e.,* the obligate period of suspension of sexual organ

development), and a latency period of 10–15 days is required to terminate diapause (Tran *et al.* 1993).

Bruchid activity and reproduction is optimal at 20–25 °C (Frerot *et al.* 2015; Roubinet 2016). Mating spans 2–3 weeks, primarily on faba bean flowers (Boughdad 1994). After mating, males leave crops when host plants stop flowering. Females remain on fructifying crops to lay eggs on forming pods (Medjdoub-Bensaad *et al.* 2015; Pölitz and Reike 2019).

Oviposition starts when the first pods are produced at the plant basal nodes, and spans around six weeks, corresponding to the fructification period before pods dry (Boughdad 1994; Medjdoub-Bensaad *et al.* 2015). Females lay eggs on green pods (**Figure 6**), regardless of growth stage; consequently, the first pods are the most ravaged (Medjdoub-Bensaad *et al.* 2007, 2015). Egg laying lasts 1–2 min, with max. 10 eggs laid/pod, and 100 eggs/female (Boughdad 1997; Huignard *et al.* 2011). Eggs are sensitive to rain and low temperature (Roubinet 2016). Degree-day data are unavailable; however, incubation lasts 1–3 weeks, with most eggs hatching after 10 days (Boughdad 1994; Yus-Ramos *et al.* 2014; Roubinet 2016; Pölitz and Reike 2019).

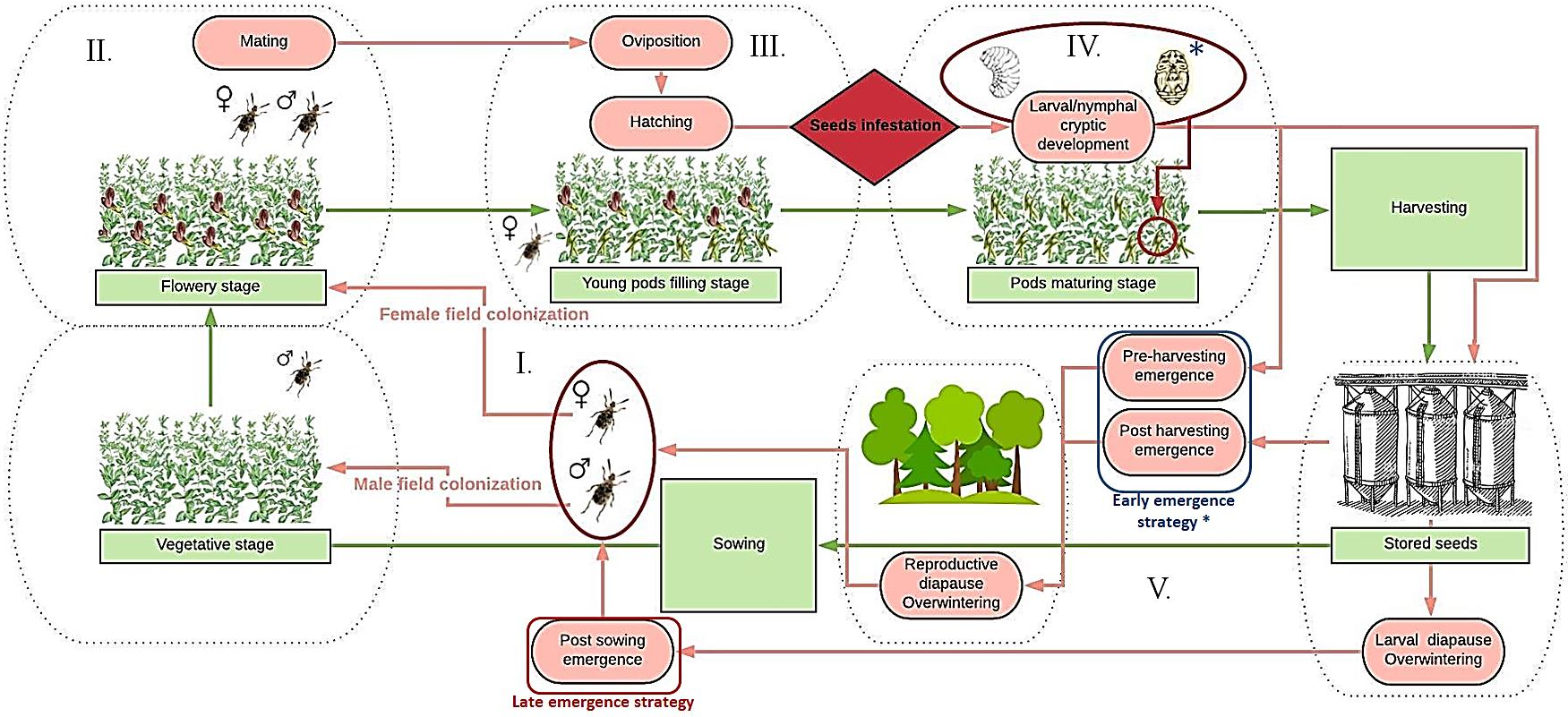


Figure 5: Illustration of the life cycle of *B. rufimanus* (pink arrows) based on *V. faba* phenology and harvesting practices (green arrows). The univoltine development of *B. rufimanus* is described in five steps: I. Field colonization of males as the crop is still at the vegetative stage, then followed by the females colonization as the crop is at the flowering stage; II. Mating in flowers after feeding which allows the achievement of reproductive diapause; III. Oviposition on young forming pods; IV. Eggs hatching, *i.e.* seeds infestation by seminovorous larvae during the maturation of the seeds; V. Adult emergence and overwintering in wooded sites in reproductive diapause (*cf.* early emergence strategy) or larval/nymphal overwintering until the next spring seeding (*cf.* late emergence strategy). © A. Segers.

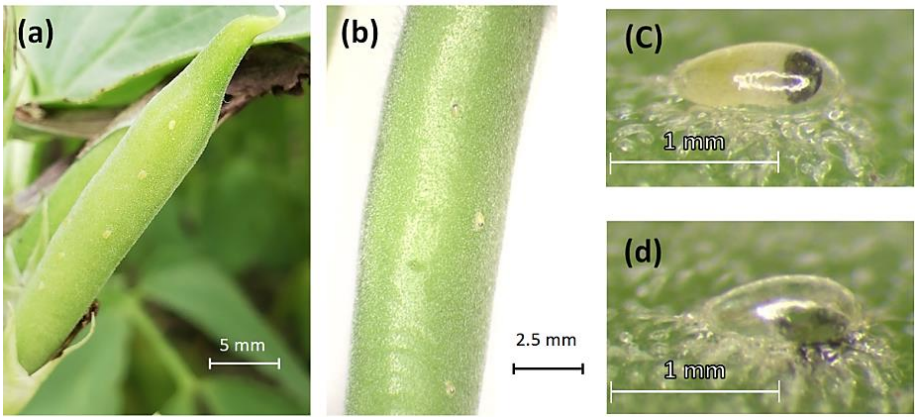


Figure 6: *Bruchus rufimanus* eggs illustrations: (a) Recently yellow laid eggs on *V. faba* young pod; (b) Eggs about to hatch (white eggs presenting one black point); (c) Egg detail containing "black head" L1 larvae inside; (d) hatched egg presenting a penetration hole on the pod pericarp side. © A. Segers.

Hatching larvae (**Figure 7**) directly bore through the pod walls, and remain in the pericarp for about one week before entering a single forming seed, where they consume the endosperm without damaging the embryo (Pölitz and Reike 2019). Two-three larvae may develop inside the same seed (Pfaffenberger 1977). Survival depends on the composition of food resources, including potential antinutritional factors (*e.g.*, tannins and α-amylase inhibitors), initial egg position at the pod surface, and pod/seed coat physical barriers to larval penetration (Lattanzio *et al.* 2005; Seidenglanz and Hunady 2016; Tsialtas *et al.* 2019). Four larval instars occur under different trophic conditions. First larval instars (morphologically recognizable by a prothoracic "H" plate) develop in young forming seeds, with low nitrogen content (0.02 g/100 g). Fourth larval instars occur in nearly dry mature seeds, with 2.88 g/100 g nitrogen content (Boughdad 1997). External factors that influence larval instar development, especially temperature, need investigation. The mean duration of each larval instar is 23.0, 20.8, 22.5, and 35.6 days, respectively (Boughdad 1994). Growing larvae position their heads against the grain tegument before nymphosis.

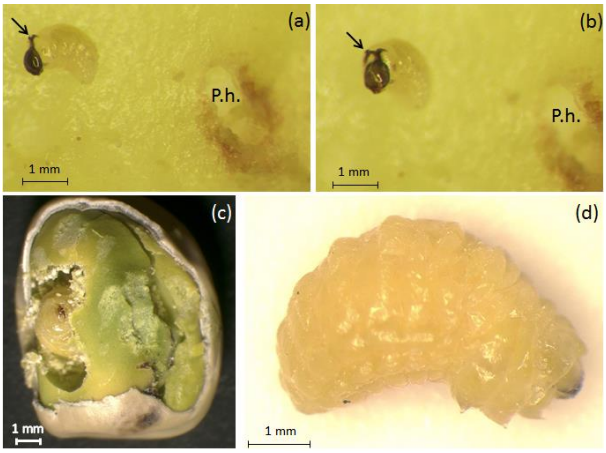


Figure 7: Illustrations of the first and fourth larva instars of *B. rufimanus*: (a) and (b) First larva instar with prothoracic "H" plate (arrow) and the penetration hole through the pod pericarp (P.h.); (c) Fourth larva instar developing inside seed; (d) Lateral view of fourth larva instar ©A.Segers

Two developmental strategies, overwintering stages, and sites are possible depending on the time of nymphosis (Medjdoub-Bensaad *et al.* 2007; Huignard *et al.* 2011). The "*early emergence strategy*" involves continuous post-embryonic development of 90–110 days until imagoes emerge in August-September (Boughdad 1994; Huignard *et al.* 2011). These adults are in reproductive diapause, actively feeding on flowers available in their environment before decreasing temperatures force them to locate overwintering sites such as the underside of bark, lichen on trees, humus, hedges surrounding crops, and storage facilities (Hoffman *et al.* 1962; Bruce *et al.* 2011; Ward 2018). The "*late emergence strategy*" involves decelerated larval development, whereby insects overwinter as larvae in seeds, and pupate the following spring when seeds are sown (Medjdoub-Bensaad *et al.* 2007). Pupation lasts 10–15 days (Boughdad 1994; Roubinet 2016). Data describing the external factors of nymph development, especially degree-day, is required.

Consequently, two colonization patterns are possible the next spring. For the latter strategy, adults directly colonize the crop from sown seeds (Boughdad 1994). Changes to environmental moisture and temperature

caused by sowing trigger the onset of larval post-embryonic development of overwintering larvae inside sown seeds (Medjdoub-Bensaad *et al.* 2007; Huignard *et al.* 2011). For the former strategy, at 15 °C, insects can fly several kilometers at 8–10 m altitude from overwintering sites to crops (Huignard *et al.* 2011). Data on overwintering, field colonization, and temperature effects on *B. rufimanus* are required.

Life cycle timing shifts with the latitude and climate at which populations occur. For example, in Morocco, male and female field infestation peaked on March 20 and April 20, 1990–1991, respectively (Boughdad 1994). In Germany, male and female field infestation peaked on May 22 and June 16, 2018, respectively (Pölitz and Reike 2019).

4.3 Chemical ecology of *Bruchus rufimanus*

The life cycle of phytophagous insects imply three essential steps that are (i) locating trophic resources for adult feeding, (ii) locating sexual partners for reproduction, and (iii) locating adequate spawning substrate for offspring survival (Whittaker and Feeny 1971; Bernays and Chapman 1994; Sauvion *et al.* 2013). Each step is regulated by volatile and non-volatile organic compounds (VOCs and NVOCs) emitted by plants and/or insects, causing chemical signal (*i.e.,* semiochemical) inducing modifications to insects behavior (Kingsolver 2004).

Concerning phytophagous insect like *B. rufimanus* that feed, mate, and oviposit on different parts of host plants, three classes of semiochemicals (or VOCs) regulate the interaction of *B. rufimanus* with *V. faba* and sexual partners (Ward 1999; Bruce *et al.* 2011; Frerot *et al.* 2015; Leppik *et al.* 2016) (**Table 2**). These include: (i) kairomones released by flowers providing food localization signals (Ward 1999; Bruce *et al.* 2011), (ii) sexual pheromones emitted by males to locate sexual partners (Bruce *et al.* 2011), and (iii) kairomones released by *V. faba* pods for oviposition (Frerot *et al.* 2015; Leppik *et al.* 2016). .

Vicia faba volatiles have different emission rates depending on phenological stage (*cf.* vegetative, flowering, and fructification). These VOCs are ubiquitous (*i.e.*, emitted by many species). Signal specificity is based on the ratio of the blend, rather than specific compounds (Bruce and Pickett 2011; Leppik and Frérot 2014; Frérot *et al.* 2017). Adding *B. rufimanus* pheromones to *V. faba* volatiles (*i.e.*, chemical signature) synergistically impacts the attractiveness of sexual partners (Bruce *et al.* 2011; Frerot *et al.* 2015). **Figure 8** shows the dynamic evolution in chemical signature characterizing the interaction of *B. rufimanus* with *V. faba* during feeding, mating, and ovipositing.

Table 2 : Semiochemicals (VOCs) regulating *Bruchus rufimanus* feeding, mating and egg-laying behaviors.

Type of semiochemicals	**Chemical identification (proportion of the active blend)**	**Targeted individuals**	**Laboratory/field efficiency and mode of action**	**References**
V. faba (*var. Sutton dwarf*) flower Kairomone	Myrcene (2.7%) *(R)*-limonene (10.4%) *(E)*-ocimene (1.8%) *(R)*-linalool (27.5%) 4-allylanisole (4.5%) Cinnamyl alcohol (0.6%) Cinnamaldehyde (1.2%) α and β caryophyllene (50.6%)	Male - female	Efficient attractiveness both in laboratory and in the field	Ward 1999; Bruce *et al.* (2011)
Male emitted sexual pheromones	1-undecene	Female	Efficient attractiveness in laboratory but not in the field, attraction synergized by nine floral VOC's	Bruce *et al.* (2011); Frerot *et al.* (2015)
V. faba (var. Espresso) pods kairomone	Cis-3-hexenyl acetate (30.0-40.0%) Limonene (15.0-20.0%) Ocimene (15.0-20.0%) Linalool (10.0-20.0%) α and β caryophyllene (15.0-20.0%)	Gravid female	Efficient attractiveness both in laboratory and in the field	Leppik *et al.* (2016); Frérot and Leppik (2018)

4.3.1 Kairomones triggering field colonization

Volatile organic compounds emitted by plants are important for host-seeking insects. When adult *B. rufimanus* leave overwintering sites in spring to locate food, flowering faba bean crops trigger field colonization **(Figure 8–I)**. Semiochemicals governing this attractiveness were identified by Bruce *et al.* (2011) that collected VOCs from flowering plants and determined nine attractive VOCs: myrcene, *(R)*-limonene, *(E)*-ocimene, *(R)*-linalool, 4-allylanisole (*i.e.* estragol), cinnamyl alcohol, cinnamaldehyde, α and β-caryophyllene. It was also underlined in this study that a combination of only three of these components - *(R)*-linalool (17.7 mg/day), cinnamyl alcohol (0.4 mg/day) and cinnamaldehyde (0.77 mg/day) - were attractive to *B. rufimanus*, and that there is a difference in the attractive response according to sexes, males being more attracted to this scent of flowers than females. Thus, *B. rufimanus* is specifically attracted to *V. faba* flower volatiles, with sex-specific responses corresponding to colonization patterns (*i.e.*, males colonize crops before females).

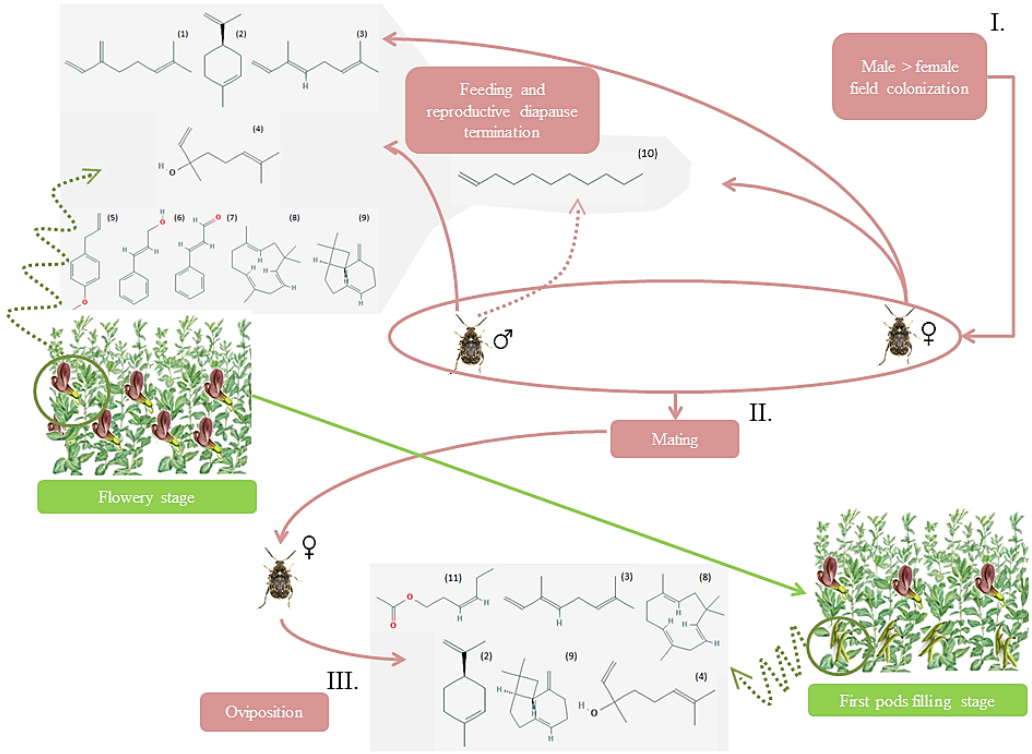


Figure 8 : : Detailed chemical process of the three first developing stages of *Bruchus rufimanus* (field colonization, mating and oviposition; pink arrows) according to current knowledge on the evolution of the chemical signature (dotted arrows) related to the host plant phenology (green arrow) - Male and female attractiveness by the VOCs flowers for feeding and mating (Bruce *et al.* 2011): (1) Myrcene, (2) *(R)*-Limonene, (3) *(E)* Ocimene, (4) *(R)*-linalool, (5) 4-allylanisole, (6) Cinnamaldehyde, (7) Cinnamyl alcohol, (8) β caryophyllene, (9) α-caryophyllene – Female attractiveness by a combination of flowering host plant scent and male sexual pheromones (Bruce *et al.* 2011): (10) 1 undecene – Gravid female attractiveness by filling pods scent (Leppik *et al.* 2016): (11) cis-3-hexenyl acetate. ©A. Segers

Frerot *et al.* (2015) explored how volatile signal evolves with *V. faba* phenological stage. *V. faba* volatile emissions include ~ 20 ubiquitous components, with emission rates evolving with plant phenology, altering odor profiles. Vegetative stage was demonstrated as being unattractive to *B. rufimanus*, whereas flowering odors are strongly attractive, particularly to males, confirming that insects are attracted to specific scents of host plant flowers.

Insights on the attractiveness of *B. rufimanus* to floral scent according to physiological stage (*i.e.* in reproductive diapause or sexually mature) were recently provided by field monitoring study led by Ward (2018) who followed population dynamic using semiochemical traps baited with three floral volatile blends (from Bruce *et al.* (2011)). They could not significantly catch *B. rufimanus* before crops bloomed, and mean number of catches only increased after blooming finished while insects were present in crops. This suggests that diapausing adults emerging from overwintering sites in spring are attracted by large amount of floral crop scent that strongly competes with synthetic lures. This massive odors emission triggers population movement over great distances to colonize fields. Whether these floral scents attract *B. rufimanus* emerging form seeds in late-summer needs clarifying (*cf.* early emergence strategy).

4.3.2 Pheromonal communication

After landing on *V. faba*, *B. rufimanus* feed on pollen and nectar to terminate its reproductive diapause, and mate on flowers **(Figure 8 – II)**. Bruce *et al.* (2011) investigated which volatiles were involved in the mating behavior by testing the response of mature field-collected adults to volatiles from their mating partner. They found that only one male emitted VOC, 1-undecene, was attractive to female. Subsequent bioassay in laboratory validated this single component VOC as a sexual pheromone. Yet, a field test of traps containing just 1-undecene failed to catch females, but associating it with host plant kairomones was more effective. Thus, host plant kairomones synergistically act with male emitted sexual pheromones in *B. rufimanus* communication. This was later confirmed using flight tunnels, with females being more attracted to combination of male and flowering plants than just flowering plants (Frerot *et al.* 2015).

A male emission of sexual or aggregation pheromone is common to several Coleoptera, especially within Curculionidae and Chrysomelidae families (Smyth and Hoffmann 2003; Witzgall *et al.* 2010). Such pheromones are therefore difficult to distinguish as they attract both sexes to the same site and induce mating after supposed tactile recognition of sexual partner (Qi

and Burkholder 1982; Nojima *et al.* 2007; Sauvion *et al.* 2013). The synergetic effect of host plant kairomones is also frequent concerning Chrysomelidae species. Many examples of male emitted pheromones whose attractiveness was boosted by association with host plant VOCs could be highlighted in the literature (Dickens *et al.* 2002; Cossé *et al.* 2002; Rao *et al.* 2003; Bartelt *et al.* 2006, 2008). This suggest the colocation of olfactive receptors neurons (ORN(s)) in the same sensillae and an eventual dimorphism in expression of olfactory neurons when male/female are more sensitive to certain chemical signatures (Unelius *et al.* 2013; Park *et al.* 2013).

The 1-undecene lack of attractiveness to females during field test is thought to be due to a weak attractive range of this VOC (Bruce *et al.* 2011). Alternatively, the experimental design might have been unsuitable for detecting all blended components in the pheromone. Single component pheromones being rare (Blomquist *et al.* 2005); other VOC(s) could probably be blended with 1-undecene and exert a better attraction to females and/or constitute aggregation pheromones. Further investigations are necessary for a better understanding of pheromonal communication.

4.3.3 Kairomones involved in locating Vicia faba pods and oviposition

After mating on flowers, gravid females oviposit on forming pods at the base of *V. faba* **(Figure 8–III)**. This behavior is also regulated by VOCs, recently identified by (Leppik *et al.* 2016). The authors tested the EAG response of gravid females to host plant odor during the fructification stage, during which green odors mixed with monoterpenes were emitted. Gravid females clearly responded to pod odor. Although no precise data was published, an INRA patent describes the composition of this odor as containing cis-3-hexenyl acetate (30–40%), plus five minor compounds similar to those identified at the flowering stage by Bruce *et al.* (2011): ocimene (15–20%), linalool (10–20%), α and β caryophyllene (10–20%), and limonene (15–20%) (Frérot and Leppik 2018).

4.4 Semiochemical-based control method of *B. rufimanus* in IPM programs

Semiochemicals offer several opportunities for manipulating pest behavior, and could hinder one or more of the three crucial stages preventing the completion of their life cycle (Evenden 2018). Four semiochemical-based methods have been developed for pest management: (i) monitoring, (ii) mass annihilation (including "mass trapping" and "lure and kill"), (iii) mating disruption, and (iv) push-pull (Smart *et al.* 2014). Based on current knowledge of the chemical ecology of *B. rufimanus*, mating disruption could not be implemented, because it requires efficient sexual pheromones. However, the other methods have potential, with monitoring and mass trapping already being implemented/under implementation.

Monitoring methods based on the use of semiochemical baited traps consists in detecting the presence of pests, and keeping track of population dynamics along crop development in order to determine when to apply pesticides (Witzgall *et al.* 2010). Integrated pest management typically uses this method of control as this provides a way to rationalize the use of pesticides at the most sensitive times for growers named "economic thresholds" (Smart *et al.* 2014). In the case of *B. rufimanus* infestations, the determination of economic threshold is currently based on the surveillance of temperatures rather than on the pest population density due to the great influence of this environmental factor on adult mating and ovipositing activities (*cf.* section 2.2). Economic threshold is defined as two consecutive days of sunny weather manifesting maximum temperatures above 20°C at the time of first pod setting (Ward and Smart 2011; Ramsden *et al.* 2017). When these meteorological conditions are met, semiochemical traps will enable growers to detect and monitor *B. rufimanus* populations to assess whether an intervention or second treatment is necessary depending on the pest persistence.

Practically, the monitoring method consists in displaying semiochemical traps on the edges or inside the crop, and lures them with three components floral attractants elaborated by Bruce *et al.* (2011). Two trap

design are commonly used, (i) green 'cone' traps (*cf.* traps for monitoring boll weevil) mounted on one meter poles; and (ii) green funnel traps with barrier cross which are suspended at the same height as *V. faba* flowers (Dickerson 2001; Ward 2018). The monitoring period runs throughout the entire *V. faba* flowering and pod setting period.

However, the efficiency of this method is limited by numerous factors. First of all is the lack of pesticide efficiency and bad side effects on beneficials (*cf.* introduction). Then, *V. faba* strong floral odors are observed to reduce the lure attractiveness (*cf.* section 3.2). This uncompetitive lure issue was therefore put under further observation at the Institute of National Research in Agronomy and Environment (INRAE); which led to the development of two efficient kairomonal attractants: one reproducing a more competitive flower scents (under unavailable patent – license number 20 02150), and the other reproducing the odor of pod scent, as presented at section 3.4 (Frérot and Leppik 2018). These lures are currently under study for a potential implementation as mass trapping control method.

Mass trapping is a similar method to monitoring, except that it does not relies on pesticides, but rather aims to catch as many individuals as possible to reduce or eradicate pest population (Smart *et al.* 2014). Kairomonal attractants developed by INRAE are under investigation by AgriOdor company for mass trapping of *B. rufimanus*. A prototype of pan trap (patent pending), specific to the capture of *B. rufimanus,* was also developed. Mass trapping of Coleoptera species could already be successful proceeded with other pests (Faleiro *et al.* 2003; Dufour and Frérot 2008). Concerning *B. rufimanus*, pods kairomones have the particular advantage of attracting females offering better chances for avoiding oviposition (Smart *et al.* 2014). However, this method still faces one principal constraint to reach economical and logistical viability: determining the trap density per unit of area to catch sufficient proportions of the *B. rufimanus* population and avoid oviposition (Montagné *et al.* 2018).

4.5 Future research Areas to control *B. rufimanus*

All the information provided so far concerning the biology and chemical ecology of *B. rufimanus*, as well as semiochemical-based control methods raised four main gaps of knowledge having potential application in IPM strategy : (i) determination of temperature influence on developmental rate and fecundity of *B. rufimanus*, (ii) methodological improvement of pheromonal studies and other pheromonal communication, (iii) implementation of monitoring methods providing quantitative description of population size and movements, and (iv) implementation of other semiochemical-based control methods.

4.5.1 Influences of temperature on the developmental rate and fecundity of B. rufimanus

Temperature dependent development models are often used to anticipate pest activity and seasonality (Lamb 1992). There is extensive literature emphasizing the influence of temperatures on the developmental rates and fecundity of multivoltine bruchid species (Howe and Currie 1964; Kistler 1982; Wu *et al.* 2013; Soares *et al.* 2015; Maharjan *et al.* 2017). But none laboratory study were carried out on univoltine species such as *B. rufimanus*, probably because of economical differences in pest importance, and also due to the difficulty of establishing fairly productive rearing of these insects with complex biology. Only one field study has emphasized the influence of temperature on *B. rufimanus*, and shown that temperature could be a key factor influencing overwintering emergence and oviposition, although data could not statistically prove it (Ward 2018). Defining temperature thresholds and thermal constants for each development stages of *B. rufimanus* (*cf.* overwintering and ovipositing adults, eggs incubation and post embryonic development - **Figure 5**), could provide a better understanding of interannual fluctuations of damages as well as an accurate determination of the duration of each life cycle stages. This could overall provide growers with useful indications to anticipate the emergence of insects either from overwintering site in spring, or from matures seeds in late summer; and allow them to adopt adequate management measures to

hinder pest population. Defining the appropriate time to deploy traps for mass trapping, or proceeding to harvest before the emergence of adults would probably represent a good opportunities minimize short-term damage, and progressively decrease *B. rufimanus* population in the long run, as suggested with *Bruchus pisorum* L. (Mihiretu and Wale 2013).

4.5.2 Methodological improvement and other pheromonal communication

Further knowledge on pheromonal communication of *B. rufimanus* is necessary to provide new insights for efficient semiochemical-based control methods. This could be provided on the one hand by adapting experimental odor sampling designs, and on the other hand by investigating whether other type of pheromonal communication such as the oviposition pheromone could have a deterrent effect on egg laying behavior.

Unsuccessful application of sexual pheromone in field trials (*cf.*, section 3.3) suggested that other VOC(s) may be blended with 1-undecene in the pheromone composition, which may therefore constitute an aggregation pheromone. This important issue should be investigated through some improvements in the sampling odor design. Firstly, an increasing pheromone production could increase chances to detect other volatiles. This could be done either by antenectomy, which reduces antennal input of chemical communication and induces a response of increasing pheromonal production, or by application of juvenile hormone JHIII that stimulates pheromone production as long as JHIII is involved in the biosynthetic pathway of the pheromone production (Dickens *et al.* 2002; Seybold and Vanderwel 2003). Secondly, the odor sampling method should be performed with adults feeding on host plant instead of insulating them in a vial. Indeed, most beetles need to consume host plants in order to emit volatiles as host plant feeding contains necessary precursors (Tillman *et al.* 1999; Sauvion *et al.* 2013).

"Host marking pheromones" (HMP) or "Oviposition deterring pheromones" (ODP) are semiochemicals regulating or suppressing the egg-laying behavior of insects. Their role is to signal the presence of egg/larva to another conspecific female to avoid supplementary oviposition on the same substrate. This intraspecific regulation of egg density aims to increase fitness of emerging larvae by reducing the risk of competition in a limited food resource, which may lead to cannibalism (Prokopy 1972; Thiéry 1991). Several studies have highlighted the deposition of HMP by many species, but none has yet tried to study such pheromonal communication with *B. rufimanus,* while numerous biological indications support the presence of HMP. Indeed, their heir larvae develop inside limited food resources, *i.e.*, one single seed (Hoffman *et al.* 1962). High larval density induces a risk of cannibalism where the largest larvae can damage/kill the smallest (Pölitz and Reike 2019), and the number of eggs laid per green pods in the field rarely exceed 10 (Huignard *et al.* 2011).

4.5.3 Standardization of semiochemical-based monitoring methods for quantitative demographical description

Lacks of knowledge concerning seasonal population dynamics and long distance dispersal of *B. rufimanus* are related to the absence of standard monitoring methods. Semiochemical-based monitoring methods taking into account the landscape influence (*cf.* presence of storage facilities and woods as overwintering sites) offers good opportunity to fillfull this methodological gap and would allow to anticipate areas presenting high risks of infestation for growers.

Establishment of semiochemical monitoring of *B. rufimanus* was already performed by Ward (2018) and could thus be considered as a basis of recommendation for standardization. They displayed floral scent lured traps at the edge of faba bean crops at a height of 0.8-1 meter, spaced of about 20 meters, and registered catches at least once a week. This method is thought to find potential improvements in the use of more efficient semiochemicals and trap designs for catching *B. rufimanus* (*cf.* AgriOdor researches), or in technological advances such as connected detection

devices providing real time information of pest population to growers, which reduces the logistical burden of monitoring (Bordes 2017).

Information obtained on spring population densities (number of adults per ha), and on how crops are subsequently damaged (number of damaged seeds per ha) will provide all the necessary data to determine a robust quantitative economic threshold (ET), as well as an eventual decision support model like that developed to manage *Sitodiplosis mosellana* (Gehin, 1857) (Ellis *et al.* 2009; Ramsden *et al.* 2017; Gahukar and Reddy 2018).

4.5.4 Implementation of other semiochemical control methods: "Lure and kill" and "push-pull"

"Lure and kill", also called "attract and kill", "male annihilation", "bait sprays" or "attracticide", consists in combining a pesticide/sterilant/pathogenic agent with an attractant in order to kill/sterilize/infect the insect (El-Sayed *et al.* 2009). Many implementation modalities are possible: lures can be pheromonal and/or kairomonal attractants, while the co-formulation of killing agent (insecticide, sterilant or pathogen) may be arranged in a trap or immediately dropped in the crop (El-Sayed *et al.* 2009; Smart *et al.* 2014). To insure a successful implementation, the attractiveness on the insect should not be hampered by the odor of the killing agent (Cayley *et al.* 1984), and non-chemical killing agents (*e.g.*, entomopathogenic fungi) should be suggested to increase the specificity of the control method and avoid the appearance of resistance mechanism (Roush *et al.* 1990). Concerning *B. rufimanus*, an interesting implementation of attract and kill strategy would consist in attracting males and females in devices lured with host plant kairomones (*cf.*, section 3) containing entomopathogenic fungi so as to infect insects by contact with conidia. The infected insects could then disseminate the pathogen to other members of their population (Klein and Lacey 1999). This approach named "autodissemination" was already performed with other insects and is particularly promiscuous for *B. rufimanus* as attractive kairomones are already developed and two efficient pathogenic fungi species were identified, namely Beauveria *bassiana* (Bals.-Criv.) Vuill. 1912 and

Metarhizium anisopliae (Metschn.) Sorokīn 1883 (Sabbour *et al.* 2007). Proceeding with this method during field colonization of *B. rufimanus* (*cf.,* **Figure 5-I**) could furthermore benefit from a natural infection between sexual partners inducing sublethal effects like the inhibition of oviposition, as highlighted with sap beetles (Vega *et al.* 1995). Proceeding with this method during the emergence of adults (*cf.*, **Figure 5-V**) could also favor the transmission of pathogen between overwintering adults.

"Push Pull" is another implementable semiochemical control method of *B. rufimanus,* consisting in regulating the insect behavior by "pushing" it from protected crops, using repellent or deterrent from associated plants or synthetic compounds, and "pulling" it to the perimeter of the crop where it will develop on other plants. Ideally, the pushing effect also attracts natural enemies such as predators or parasitoids, and pulling plants reduce population with innate defense or with incorporated pesticide (Pickett *et al.* 2014). In the case of *B. rufimanus*, botanical oils of *Artemisia campestris* L., nigella and mustard were identified as having efficient repellence / oviposition deterring / insecticidal effects on *B. rufimanus* (Sabbour *et al.* 2007; Titouhi *et al.* 2017). These could then constitute potential repellent agents, as far as their harmlessness effects against beneficial organisms of faba bean crops, and their stability against oxidation and photo-deterioration can be insured (Smart *et al.* 1994; Ketoh *et al.* 2005). Application of these essential oils could be performed on the protected crop using standard flat fan hydraulic nozzle for pushing insects to perimeter trap crops where synthetic kairomones or eventual aggregation pheromones would serve as attractant agents. No information can currently provide input on the concurrent attraction of natural enemies (*i.e.* predators and parasitoids) as well as on the practices favoring their presence. However, it can be pointed out that two types of parasitoids were identified to date: larvaphageous parasitoids including species from *Braconidae* (Nees, 1811) family such as *Sigalphus pallipes* Nees von Esenbeck, 1816, *S. gibberosus* Szépligeti, 1901, *Triaspis thoracica* (Curtis, 1860), *T. similis* (Szepligeti, 1901), *T. luteipes* (Thomson, 1874) and *Chremylus rubiginosus* (Nees, 1834), and

oophageous parasitoids from *Trichogrammatidae* family such as *Uscana senex* (Grese, 1923) (Boughdad 1994; Medjdoub-Bensaad *et al.* 2007; Titouhi *et al.* 2017).

4.6 Conclusions

Considering the expected increase in *Vicia faba* cultivation throughout Europe (*cf.* multiple agricultural benefits and promotion by European policies), and the multiple impacts of *Bruchus rufimanus* on seeds, confronted to restricted and low efficiency uses of pesticides, new eco-friendly control strategies must be developed in the coming years. Among the alternatives highlighted to date, this review emphasized on the semiochemical-based methods of control as well as on biological missing information. Indeed, despite the extensive literature describing the biology and ecology of *B. rufimanus*, some key elements are still missing such as the influence of temperature on the pest developmental rate and fecundity, or the seasonal population dynamic and long distances dispersal capacity. Such aspects should now be investigated as they would provide growers with precious tools to proceed with adequate measures to hamper the pest life cycle.

Numerous existing chemical ecology studies provide a comprehensive overview of the semiochemical processes regulating the specific interactions between *B. rufimanus* and *V. faba*. Further studies on pheromonal communication should be undertaken by adapting the sampling odor designs and assessing the presence of HMP, so as to improve both current monitoring and mass trapping control methods. Attract and kill or push-pull methods meet each of the required elements for their implementation, and could constitute a promiscuous alternative method to investigate. All these elements, completed with other control levers, are essential to provide growers with efficient management strategies, without resorting to chemical pesticides.

5 Baseline information about biocontrol strategie and biopesticides

Some undefined concepts of biocontrol or biopesticides were evocated in the previous section. Before broaching the thesis objective that is entierly dedicated to the biocontrol strategies and alternative to the use of insecticide against *B. rufimanus*, a quick reminding of baseline definitions sounds adequate at this stage to avoid potential confusions in the different concepts being treated in next chapters.

Biocontrol management against pest is defined as a group of strategies involving self-replicating biotic entities such as natural enemies (predators and parasitoids) or pathogens that will exert an antagonistic effect on pest populations with the objective to decrease their level below the economic threshold (Woodring and Davidson 1996). This concept should not be confused with that of biopesticides, which is a generic term referring to substances derived from the nature that may be formulated and applied in a similar manner to a conventional insecticide (FAO and WHO 2017). In this sense, insect pathogens are included in these two concepts, unlike semiochemicals, vegetable oils or double stranded RNA (dsRNA) suspensions, which do not benefit from the self-replicating characteristic.

Biocontrol may be operated following three principal strategies named the classical approach, the augmentative approach and the conservative approach. Classical approach consists in the introduction of a natural enemy which is, in most case, an exotic parasitoid or predator (present in the geographical center of pest origin) that will settle in the new environment where pest are rife and regulate their population on a long term (Becker *et al.* 2003). Other approach such as inoculation, augmentation or inundation consist in the release of laboratory-bred natural enemies or pathogens (Huis 1991). Finally, conservation strategies consist in the modification of the pest environment to favor the communities of natural enemies (Venzon and Sujii 2009). In this thesis, specific biocontrol tools

based on insect pathogens or dsRNA based biopesticide are gathered under the denomination "biotechnological control".

6 Thesis objectives and structure

The sustainable promotion of faba bean crops in Wallonia must provide producers with sufficient remuneration for their production. In this sense, the development of effective control methods against *B. rufimanus* is an essential step. This thesis addresses the general objective of developing biological control methods and alternatives to the use of insecticides against *B. rufimanus* in faba bean crops. This general objective is based on four control levers, *i.e.*, semiochemical control, varietal control, improvement of *B. rufimanus* bioecological knowledge and biotechnological control (microbial biopesticides and RNAi). Five main specific objectives (SO) and associated hypotheses were followed:

SO1: Improvement in the bioecological knowledge of *B. rufimanus* in the Walloon Region (Chapter II)

- **Sub-objective 1.1:** Determine the infestation dynamics of adults in faba bean crops
    - **Hypothesis**: Infestation patterns of adults in Belgium are similar to neighboring countries (Germany, France, and England)
- **Sub-objective 1.2:** Determine the diversity of bruchid species and associated natural enemies on faba bean crops in the Walloon Region
    - **Hypothesis 1:** Presence of five bruchid species on *V. faba* cropsincluding *B. rufimanus, B. affinis, B. atomarius, B. pisorum, B. brachialis*.
    - **Hypothesis 2:** Presence of one species of oophageous parasitoid (*U. semifumipennis*) and 13 species of larvaphageous parasitoids (T. *forbesii, T. luteipes, T. obscurella, T. pallipes, T. stictostiba, T. thoracica, A. calandrae, D. acutus, D. basalis, D. magnus, E. swezeyi, E. wachtli, L. distingendus*)

SO2: Breeding and evaluation of the thermal development of *B. rufimanus* (Chapter III)

- **Sub-objective 2.1:** Establishment of functional breeding
- **Sub-objective 2.2**: Determination of the thermal balance relative to embryonic development and total development of *B. rufimanus*
    - **Hypothesis:** The development of *B. rufimanus* based on temperature follows a relationship similar to those proposed by *Briere-1* or *Analytis* models, which fit to other bruchid species

SO3: Identification of the most interesting varieties to cultivate in the Walloon Region for human food valorization (Chapter IV)

- **Hypothesis 1:** Infestation rates and protein production are influenced by varietal effects
- **Hypothesis 2:** Infestation rates and protein production are influenced by climatic variables
- **Hypothesis 3:** Varieties can be classified using a two principal component space (PCA) resulting from a linear combination of multiple variable including agronomic, varietal, and bruchid sensitivity factors.

SO4: Identification of the most effective kairomone-based semiochemical traps for bruchid capture in faba bean crops (Chapter V)

- **Hypothesis 1:** There is competition between the smell of the crop and the capture of semiochemical traps reproducing the same smell
- **Hypothesis 2:** There is an optimal combination of different semiochemical lures and trap designs for capturing *B. rufimanus*
- **Hypothesis 3:** Semiochemical traps specifically target *B. rufimanus* and have no impact on crop auxiliaries

SO5: New biotechnological tool assessment against *B. rufimanus*: Hypocrealean Entomopathogenic Fungi (HEF) and gene silencing via RNA interference (Chapter VI)

- **Sub-objective 5.1:** Assessment of lethality of five strains of entomopathogenic fungi (*B. bassiana* (GHA), *A. flavus*, *M. acridum*, *M. brunneum* (V275), *M. brunneum* (USDA 4556))
    - **Hypothesis:** All entomopathogenic fungi strains tested *in vitro* present LT50 inferior to five days and are effective as foliar sprayed conidial suspension in field condition
- **Sub objective 5.2:** Assessment of RNAi as potential new biotechnological tool for bruchid control with gene silencing of *laccase 1* experiments on *C. maculatus*
    - **Hypothesis 1:** RNAi core machinery components are present in the genome of *C. maculatus*
    - **Hypothesis 2:** *Alpha tubuline* 1, *Arginine kinase* and *beta-actin* constitute ideal reference gene for RT-qPCR analyses in *C. maculatus*
    - **Hypothesis 3:** Gene expression patterns show a decrease of *laccase 1* expression after the double stranded RNA administration that are simultaneously combined with lethal phenotypes of *C. maculatus* adults.

Chapter II

Bioecology of *B. rufimanus* in Wallonia

1 Dynamic of *B. rufimanus* field infestation

1.1 Introduction

Several studies have followed the *B. rufimanus* infestation, commonly named the broad bean weevil (BBW), in faba bean crops across European and Mediteranean courntries like Maroco, Algeria, Germany, United Kingdom, France and Latvia (Boughdad and Lauge 1997; Medjdoub-Bensaad *et al.* 2007; Ward 2018; Pölitz and Reike 2019; Hamidi *et al.* 2021; Gailis *et al.* 2022). Methodologies used for the description of *B. rufimanus* population dynamic included active countings of bruchids or semiochemical monitoring.

In Belgium, no information is available for the description of BBW's population dynamic. In this chapter, first description of BBW infestations is provided using manual and semiochemical monitoring in faba bean crops. The focus is done on adults infesting flowering crops, aiming to identify specific external phases that could be targeted by crop control strategies.

1.2 Monitoring procedure

Four field bean crops were followed during the cropping seasons 2018-2019, 2019-2020, 2020-2021 and 2021-2022. All these crops were located in the same area in the vincinity of Gembloux (Belgium) at the locality called "Isnes". A maximal distance of 1,122.4 m separed crops (**Figure 9**). In all of the study sites, winter faba bean (variety Nebraska) and spring faba bean (variety Fanfare) were grown at respective densities of 35 plants/m² and 50 plants/m².

Bruchid populations were monitored with manual catches during the two first seasons and with semiochemical traps during the other seasons. Manual catches were performed using similar methodology to other studies (Medjdoub-Bensaad *et al.* 2015; Titouhi *et al.* 2015; Pölitz and Reike 2019). It consisted in weekly prospections on a fixed pathway of 100 m length x 1 m width at the same daily period from 15:30 to 16:30. Adults of

BBWs detected by operators in the flowers or apical leaves were captured using a truncated cone reversed over a pill box or counted if adults flew away. Semiochemical trappings (monitoring of 2021 and 2022) were performed using white pan trap with a transparent cylinder (AgriOdor, Rennes, France) placed in the center of 27m wide parcels. These traps were lured with kairomonal attractant reproducing the odors of flowers commercialized by International Pheromone System Ltd. (Neston, UK) and filled with water containing an odorless surfactant (TRITON X-100, 0.1% v/v). Phenological stages of faba bean crops were also followed in parallel of bruchids populations, *i.e.*, the flowering period of faba bean and pods setting periods. Informations about study sites as well as the monitoring methodology of each year are presented in **Table 3**.

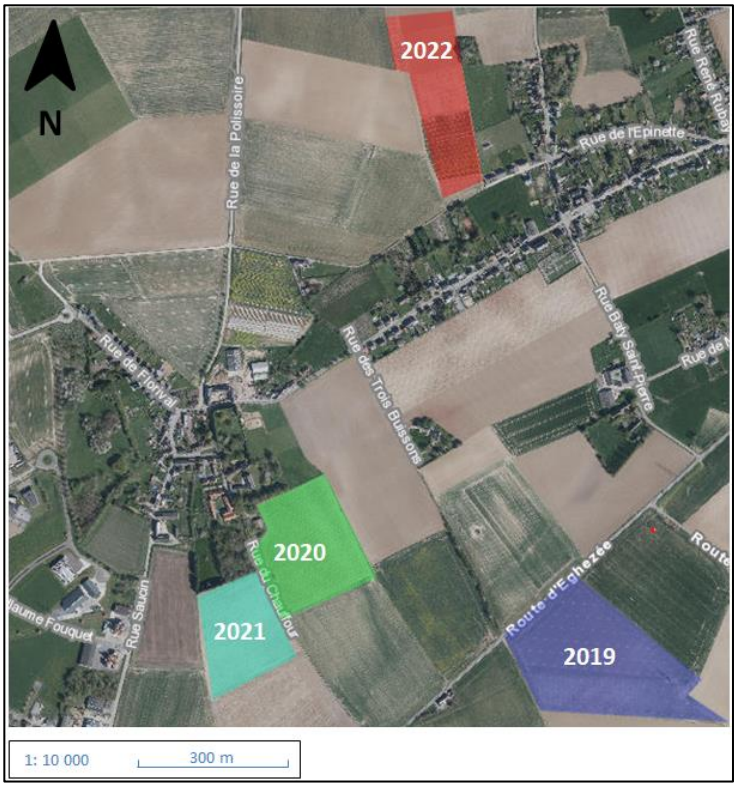


Figure 9: Localization of crops where *B. rufimanus* infestation were monitored in 2019, 2020, 2021 and 2022

Table 3: Monitoring modalities during the four year of study (W = winter field bean; S = spring field bean). The number of repetitions of semiochemical traps is also presented in the table

Growing season	Geographical coordinates and altitude	Surface	Crop monitored (W/S)	Monitoring method
2018-2019	(50◦49'91''N, 4◦74'34''E) 167.12 m	7.84 ha	W+S	Manual catches
2019-2020	(50◦50'34''N, 4◦73'19''E) 174.75 m	4.75 ha	W+S	Manual catches
2020-2021	(50°50'34''N, 4°73'19''E) 174.75 m	3.46 ha	W+S	White traps + IPS lures (4)
2021-2022	(50°51'18''N, 4°73'61''E) 177.20 m	4.99ha	W	White traps + IPS lures (6)

1.3 Results and discussion

Phenologcal stages of flowering and pods formation and BBW populations effectives are respectively presented in **Table 4** and **Figure 10**. This data provide first insight on *B. rufimanus* infestation of faba bean in the locality of Isnes over four years.

Table 4: Flowering and pod-setting periods in faba bean monitored crops

	Flowering period	Pods-setting period
2019 (W)	06/05 - 17/06	21/05 - 12/07
2019 (S)	31/05 - 30/06	20/06 - 23/08
2020 (W)	19/04 - 02/06	05/05 - 24/06
2020 (S)	30/05 - 28/06	10/06 - 26/07
2021 (W)	29/04 - 20/06	10/06 - 02/08
2021 (S)	03/06 - 22/07	15/06 - 02/08
2022 (W)	02/05 – 07/06	13/05-29/06

As it could be observed in other studies, the dynamic of populations is greatly correlated to the host plant phenology which is itself depending

on climatic conditions. Faba bean responds to thermal development for floral induction, with an accumulation of 830 to 1000 degree-days (Patrick and Stoddard 2010). Most infestations were observed in crops during the flowering stages as the first pods began to form (*i.e.*, at the stage "young pods of 2 cm length - YP2"). The population movement patterns closely resembled those witnessed in Germany, with the highest infestation peak occurring on May 22 and a subsequent one on June 16 (Pölitz and Reike 2019). However, observations of males colonizing crops before the flowering phase and sustaining by feeding on extrafloral nectaries could not be observed. A migration of the bruchid population was noted, shifting from winter to spring field bean crops following the flowering of the winter faba bean. This likely occurred due to the proximity of these delayed crops in neighboring plots. The bruchid population appears to move towards the adjacent spring faba bean, which remains in bloom, thereby offering a continued food source for the insects.

Interannual phenology of faba bean crops greatly differ interranually because contrasted climate were observed. Particularly, the temperatures greatly influenced the timing of flowering. In 2021, a cold period observed after the flower induction prolungated the flowering period compared to other seasons. This likely caused variations in the interannual bruchids population movement patterns.

The population movement patterns also vary according to the method of monitoring. Bruchids are earlier detected during the flowering period using manual catches rather than semiochemical traps reproducing the flower scent. This aspect of competition between the crop flowering and the attractiveness of semiochemical lures is discussed in Chapter V. This competition is illustrated with the peak of captures at the end of the season when the spring faba bean ceases to flower (*cf.*, 252 captures in 2021 and 96 captures in 2022). The adults are presumed to leave the crops that no longer provide a food source and are more easily attracted to traps replicating the flower scent. These observations were made during two consecutive years of semiochemical monitoring.

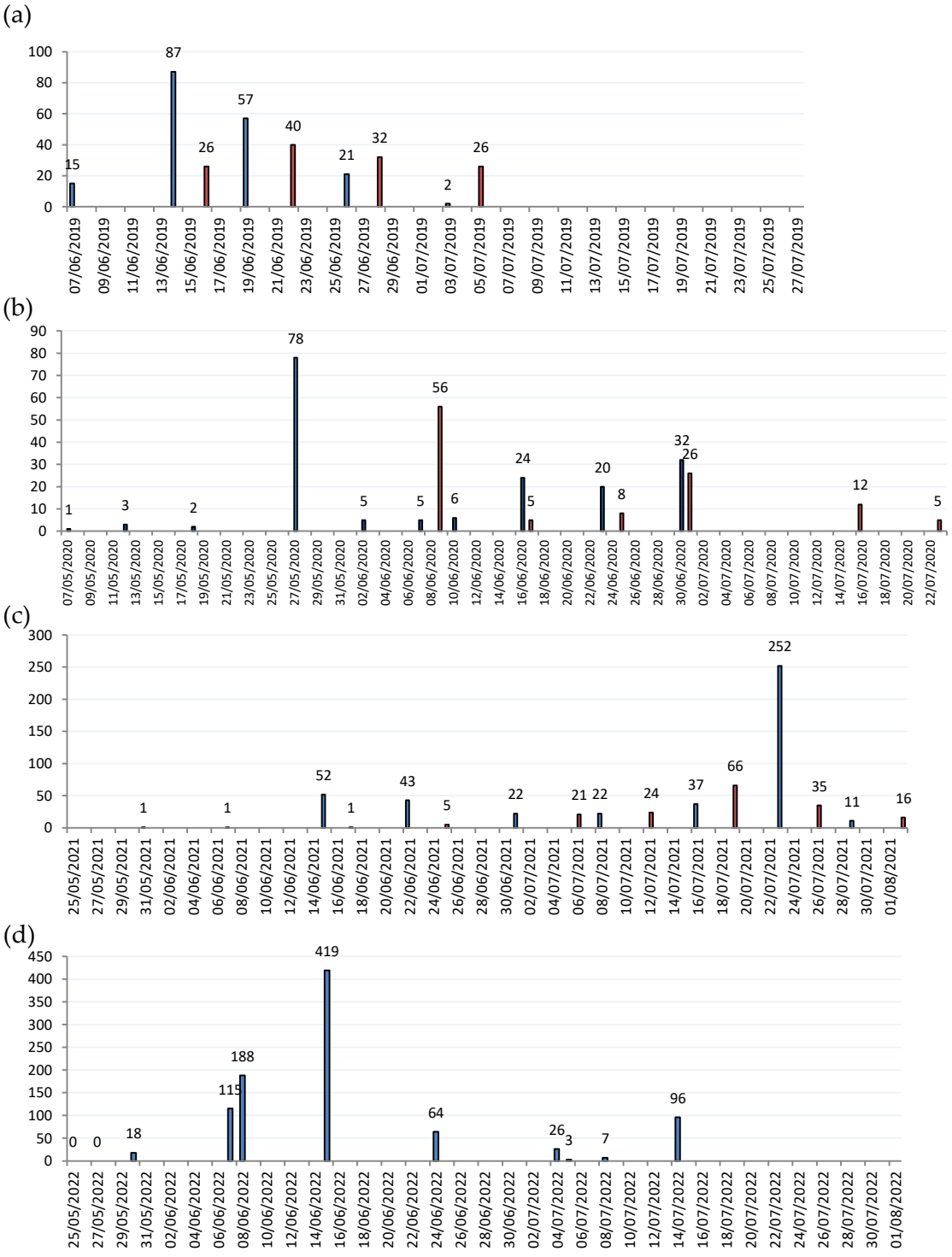


Figure 10: Dynamic of *B. rufimanus* field infestation in 2019 (a), 2020 (b), 2021 (c) and 2022 (d) in winter field bean (blue) and spring field bean (red)

These local findings provide insights into the periods when control measures should be implemented against adults and subsequent egg-laying. Manual captures indicate that bruchids were present in the crops starting from late May and typically peaked around mid-June in winter crops. Regarding spring crops, drawing conclusions is more challenging as trends varied considerably over the four years of monitoring. Nevertheless, bruchids most commonly appeared when young pods emerged, aligning with the economic threshold against faba bean bruchid (*i.e.*, the application of phytosanitary products), defined as two consecutive days with a daily temperature exceeding 20°C during young pod formation. These indications are valid for the study region but may not be applicable to the entire Walloon territory, which encompasses various bioclimatic regions where time shifts may occur for the crop developments and field infestations. As regional temperatures tend to decrease towards the southeast of the country, the development of crops should also be expected to be shifted as well as the pest occurrences and the related management measures to avoid ovipoistion.

2 Spatial incidence and richness of *Bruchus* species and associated parasitoids in Wallonian faba bean crops

Taken from a project of paper in preparation for submission:

Segers A., Lugendo R., Fagot J., Rasplus J-Y, Caparros Megido R., Francis F. **(2023)** *Spatial incidence and species richness of Bruchus species and associated parasitoids in Wallonian faba bean crops* Faunistic Entomology

Abstract: No study describes the Belgian diversity of bruchids and their parasitoids in faba bean crops. In this context, twelve sites were inventoried in different Wallonian bioclimatic zones over three years (2020, 2021 and 2022) to asses the spatial distribution of bruchids and their parasitoids as well as the damage caused to seeds. A total of 1745 insects were collected from harvested seeds placed in emergence traps. Only one bruchid species was identified, *i.e.*, *Bruchus rufimanus* Boheman 1833 and four larval parasitoids, *i.e.*, *Triaspis luteipes* (Thomson, 1874), *T. thoracica* (Curtis, 1860), *Pteromalus sequester* Walker, 1835 and *P. fasciatus* (Thomson, 1878). This is the first report of parasitation of *B. rufimanus* larvae by *Pteromalus* species to our knowledge. Differences were observed in terms of *B. rufimanus* infestation rates and emergence timings within different bioclimatic areas. All these observations are discussed as baseline information in the development of integrated pest management of *B. rufimanus* in Wallonia.

Key words: Bruchids; Chalcidoid; Parasitoids; Faba bean; Triaspis; Pteromalus; Bruchus; natural enemies

2.1 Introduction

In Belgium, a limited number of works have compiled the list of bruchids. The initial work by Decelle (1989) identified sixteen species of Bruchinae. The same author provided an updated review five years later, but no further lists were published for the next two decades. Recently, Baugnée *et al.* (2021) added three new species to the Belgian list, namely *Bruchidius imbricornis* (Panzer, 1795), *Bruchus occidentalis* Lukjanovitch & Ter-Minassian, 1957, and *Bruchus brachialis* Fåhraeus, 1839. This study also summarized the different status of the Bruchinae species in Belgium, noting that fifteen species are indigenous to Belgium, while four species (including *B. rufimanus*) have become naturalized due to the introduction of their host plants.

Considering the works of Zampetti and Ricci (2012) that provide information on the larval host plants of various bruchid species, and when considering the list of recorded bruchids in Belgium, it can be inferred that larvae of five *Bruchus* species, namely *Bruchus atomarius* (L. 1761), *B. pisorum* (L. 1758), *B. brachialis* Fåhraeus 1839, *B. affinis* Frölich 1799, and *B. rufimanus* Boheman 1833, are capable of developing in *Vicia faba* seeds. While *Bruchus rufimanus* is reported as the main species infesting *V. faba* in Tunisia (Titouhi *et al.* 2015), no comprehensive inventory has been conducted to assess the diversity of bruchids within faba bean crops.

A complete knowledge of the bruchid species richness infesting faba bean is essential for the developmet of the most effective control strategies that would take into consideration all the potential pests. Moreover, this would allow the identification of potential natural enemies that should be present in Wallonia and provide baseline information for the development of biological control strategies (Roubinet 2016; Segers *et al.* 2021). Natural enemies identified for the genus *Bruchus* in Europe was recently reviewed by Bellifa and Chapelin-Viscardi (2021). They reported that three type of natural enemies against bruchids were present in Europe, including Hymenoptera parasitoids developing in eggs or larvae, and a generalist

bug predator named *Zelus renardii* Kolenati, 1856 (Heteroptera: Reduviidae), which is not really specific to *Bruchus* spp (Cox 2007). Oophagous parasitoids appear to be the most promising organisms for control purposes preventing the eggs hatching and seeds infestations. Larval parasitoids could be interesting for the long term regulation of bruchids infestations in the same area, depending on the prevalence of their abundance compared to bruchids populations (Segers *et al.* 2021). Larval parasitoids encompasses 10 species of from the genus *Triaspis* (Ichneumonoidea) and 33 species from the genera *Anisopteromalus, Baryscapus, Dibrachys, Dinarmus, Eupelmus, Eurytoma, Gastrancistrus, Lamennaisia, Lariophagus, Microdontomerus, Pnigalio, Pteromalus, Tetrastichus, Torymus, Trichomalopsis, Trichomalus, and Trimeromicrus* (Chalcidoidea). Egg parasitoids include only two species, *Uscana semifumipennis* Girault, 1911 and *U. senex* (Grese, 1923) from the family Trichogrammatidae (Chalcidoidea).

To date, the knowledge about these parasitoids and the *Bruchus* species richness in faba bean crops is scarce. In this context, twelve sites were identified in different bioclimatic areas of Wallonia and sampled over two years to evaluate the diversity of pests and their natural enemies. Comparaison of infestation rates were also performed to assess the occurrence and the damage caused to seeds in each bioclimatic zone.

2.2 Material and methods

2.2.1 Sampling site of infested seeds in different bioclimatic areas

Samples of faba bean seeds were collected for the detection of larval parasitoids, mostly in organic farms. Eight sites were sampled in 2021 and six sites in 2022 (**Figure 10**). All sites covered the different bioclimatic regions of Wallonia. Geographic coordinates and altitudes of sampling sites as well as the mode of cultivation (pure/association) and the harvesting dates are presented in **Table 5**. The seeds were collected immediately after harvest in canvas bags and displayed in emergence

cages in the Functional and Evolutionary Entomology Laboratory at Gembloux Agro-Bio Tech. Concerning the oophageous parasitoids, only one site *(i.e.,* Isnes) was monitored using vacuum traps placed in flowering crops during 2020 and 2021 seasons.

Table 5 : Geographical and cultural indications about all sampled sites

Site	**Year**	**Geographical coordinates and altitude**	**Ref. Figure 11**	***V. faba* crop**	**harvesting date**
Isnes	2021	(50°50'34'' ; 4°73'19'') 174.75 m	2	Pure (conventionnal)	03/09/2021
Beho	2021	(50°18'86'' ; 5°98'41'') 455.11m	8	Association (Organic)	07/09/2021
Verdenne	2021	(50°23'52'' ; 5°38'85'') 290m	6	Association (Organic)	30/08/2021
Battice	2021	(50°64'59'' ; 5°78'84'') 300m	7	Pure (Organic)	27/08/2021
Assesse	2021	(50°38'09'' ; 4°99'73'') 280 m	4	Pure (Organic)	08/09/2021
Bierwart	2021	(50°54'78'' ; 5°01'35'') 190.7m	3	Association (Organic)	07/09/2021
Ciney	2021	(50°29'77'' ; 5°10'45'') 275 m	5	Pure (Organic)	13/08/2021
Rebecq	2021	(50°41'14'' ; 4°07'22'') 71.14 m	1	Pure (Organic)	18/08/2021
Assesse	2022	(50°38'09'' ; 4°99'73'') 280 m	4	Pure (Organic)	09/08/2022
Erezée	2022	(50°28'04'';5,54'34''8) 300m	9	Pure (Organic)	01/08/2022
Ferrière	2022	(50°39'54''; 5,62'48'') 302.5m	10	Association (Organic)	09/08/2022
Isnes	2022	(50°51’18’’, 4°73’61’’) 177.20 m	2	Pure (conventionnal)	08/08/2022
Itre	2022	(50°65'78'';4°26'02'') 138.13m	11	Association (Organic)	31/07/2022
Rotheux	2022	(50°53'24'' ; 5°48'90'') 233.7m	12	Association (Organic)	12/08/2022

2.2.2 *Laboratory monitoring of insect emergence from seeds*

Seed samples were placed in emergence traps at 20 ± 2°C for a period of two months to capture post-harvest emerged bruchids and their potential parasitoids. The emerged insects were collected twice a week with a mouth aspirator and preserved in ethanol 70% (v/v) for preparation and identification.

2.2.3 *Preparation of bruchid and parasitoid specimens and identifications*

The method used for the preparation of insects followed the guidelines of Fagot *et al.*, (2022). The specimens were then morphologically identified to the species level by Chrysomelid specialist Jean Fagot, on the base of identification keys of Decelle (1989), Kingsolver (2004) and Zampetti and Ricci (2012). Hymenopteran parasitoids were identified up to the family level using morphological key of Noyes (2019), and then identified at the species level from ethanol stored insects by the specialist Jean-Yves Rasplus (National Institute for Agriculture, Food, and Environment Research, INRAE, and the Biology Centre for Population Management - Montferrier-sur-Lez, France).

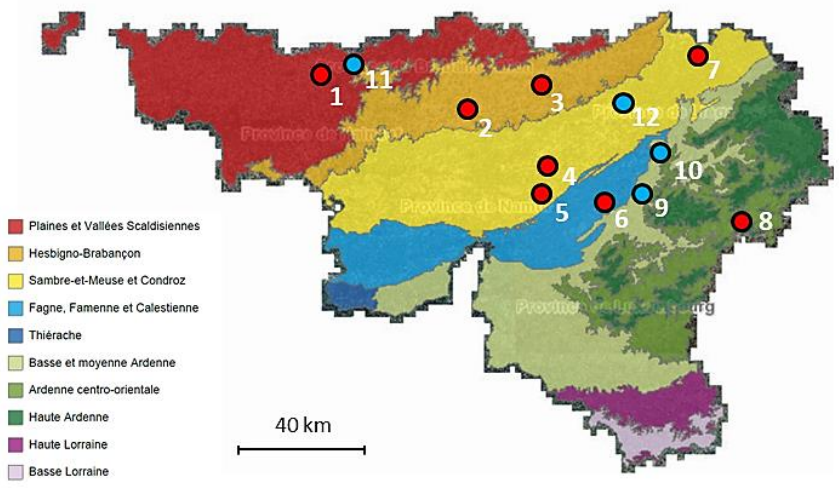


Figure 11: Sampling sites in 2021 (red) and 2022 (blue) in different Wallonian bioclimatic regions: 1=Rebecq, 2=Isnes, 3=Bierwart, 4=Assesse, 5=Ciney, 6=Verdenne, 7=Battice, 8=Beho, 9=Erezée, 10=Ferrière, 11=Itre and 12=Rotheux. Source: Wallonmap (©A. Segers)

2.2.4 *Comparison of infestation rates of faba bean seeds collected in different bioclimatic regions*

To analyze the extent of damage caused by Bruchus spp. on seeds sourced from diverse farms, we employed the direct method outlined by Roubinet (2016) and El Miziani *et al.* (2017). For each sample, 50 faba bean seeds were randomly collected by manual counting in 4 replicates (*i.e.*, 200 seeds from each site). Healthy seeds were counted as well as seeds infested (*i.e.*, presenting larval development traces or emergence whole), and the infestation rates of seeds from each sampling site were determined.

2.3 Results

1745 insects were collected from seeds of *V. faba* in 2020, 2021 and 2022, with respectively 82, 612 and 1051 insects collected annually. All these collection included one bruchid species, four species of larval parasitoid but none egg parasitoids from genus *Uscana* (**Table 6).** The complete database including identifications with all emergence dates and sampling sites are presented in **Appendix A**. In 2020, a total of 82 parasitoids were collected in the location of Isnes, including three different species, *Trisapis thoracica* (Curtis, 1860) - 43 specimens, *Triaspis luteipes* (Thomson, 1874) – 38 specimens and *Pteromalus fasciatus* (Thomson, 1878) - 1 specimen. In 2021, a total of 534 bruchids and 81 parasitoids were collected in 8 sites. All the *Bruchus* species corresponded to *B. rufimanus* and parasitoids included three different species. *Triaspis luteipes* was the most abundant species – 61 specimens, followed by *T. thoracica* - 13 specimens and *Pteromalus sequester* Walker, 1835 - 4 specimens. In 2022, a total of 992 bruchid species were collected from six sites, all corresponding to the species *B. rufimanus*. Parasitoids were collected and classified in *Triaspis* and *Pteromalus* genera according to morphotypes determined by the identifications of 2019 and 2020. Fourty eight specimens corresponded to the genera *Triaspis* and 11 specimens to the genera *Pteromalus*. The four species of parasitoids are illustrated in **Figures 13, 14 and 15**. Some specimens of *Triaspis sp.* could also be identified within dissected seeds

and revealed pre-imaginal stage that were probably about to emerge (**Figure 16).**

All sampled sites over the three study years were infested by *B. rufimanus*, none of other potential bruchids species were recorded. The infestation rates (**Figure 12**) greatly varied according to the sampling site, the less infested crops were Rebecq and Battice in 2021 with mean infestation rates of 5.5 ± 0.95% and 4.0 ± 1.82% respectively. The most infested crops were Rotheux and Assesse in 2022 with 46 ± 2.55% and 46 ± 11% respectively.

In 2021, climatic conditions were exceptionnaly rainy during the months of July and August which delayed the harvesting period of seeds at September. As indicated in **Table 5** harvesting times ranged from the 18th of August to the 8th of September in 2021 while in 2022, harvests were performed from the 30th of July until the 12th of August. The climate conditions during 2022 were more dry and warm, which was more favorable for bruchids activity.

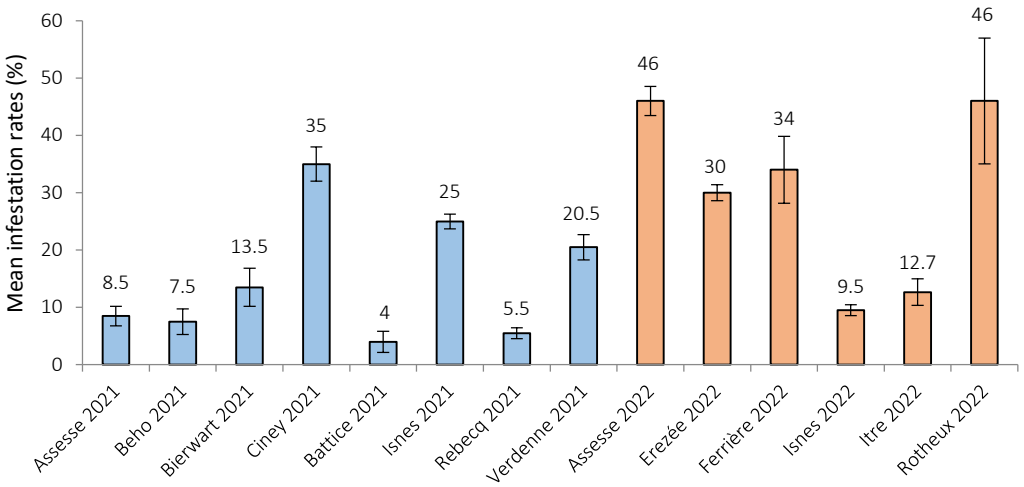


Figure 12: Average of *B. rufimanus* infestation rates in samples collected in 2021 (blue), and 2022 (orange)

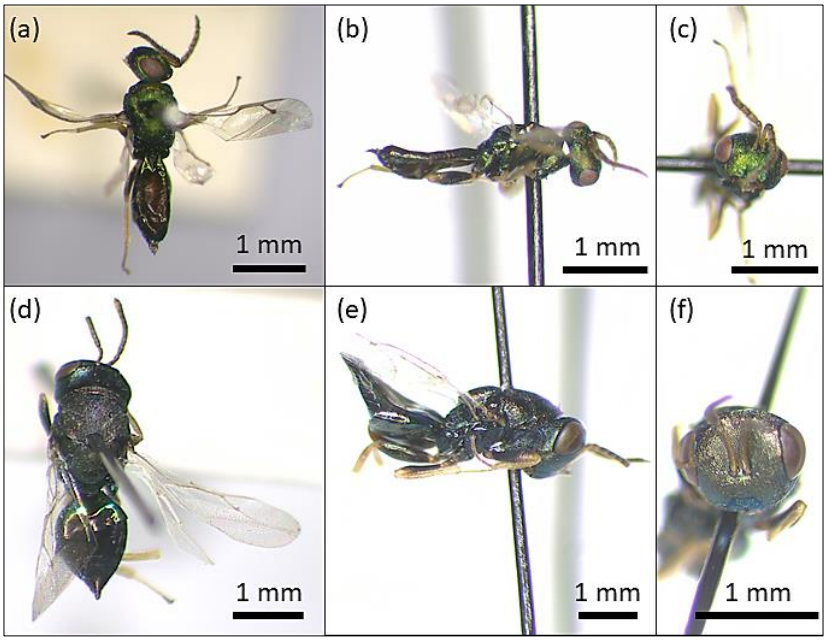


Figure 13: Illustrations of *Pteromalus fasciatus* (a,b,c) and *Pteromalus sequester* (d,e,f)

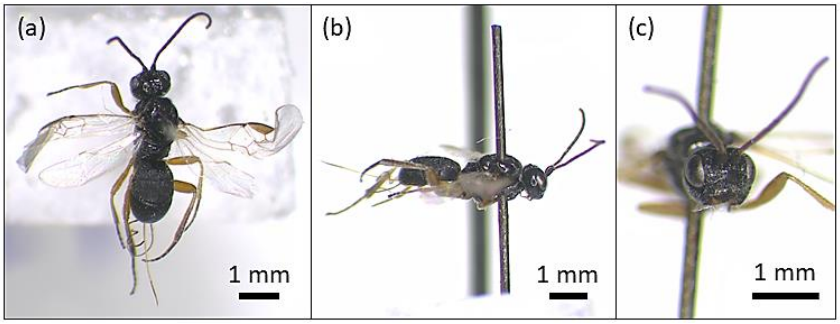


Figure 14: Illustration of *Triaspis luteipes* (female)

Table 6: Summarized identifications and abundances of all collected insects

Year	Site	Species	Abundance
2020	Isnes	*Pteromalus fasciatus* (Thomson, 1878)	**1**
		Triaspis luteipes (Thomson, 1874)	**28**
		Triaspis thoracica (Curtis, 1860)	**43**
2021	Assesse	*Bruchus rufimanus* Boheman 1833	**120**
		Triaspis luteipes (Thomson, 1874)	**2**
	Battice	*Bruchus rufimanus* Boheman 1833	**5**
	Beho	*Bruchus rufimanus* Boheman 1833	**33**
	Bierwart	*Pteromalus sequester* Walker, 1835	**1**
		Triaspis luteipes (Thomson, 1874)	**2**
		Bruchus rufimanus Boheman 1833	**83**
	Ciney	*Triaspis luteipes* (Thomson, 1874)	**28**
		Triaspis thoracica (Curtis, 1860)	**12**
		Bruchus rufimanus Boheman 1833	**85**
	Isnes	*Pteromalus sequester* Walker, 1835	**3**
		Triaspis luteipes (Thomson, 1874)	**28**
		Bruchus rufimanus Boheman 1833	**193**
	Rebecq	*Triaspis luteipes* (Thomson, 1874)	**1**
		Triaspis thoracica (Curtis, 1860)	**1**
		Bruchus rufimanus Boheman 1833	**12**
	Verdenne	*Bruchus rufimanus* Boheman 1833	**3**
2022	Assesse	*Bruchus rufimanus* Boheman 1833	**389**
		Pteromalus sp.	**9**
		Triaspis sp.	**17**
	Erezee	*Pteromalus sp.*	**1**
		Triaspis sp.	**7**
		Bruchus rufimanus Boheman 1833	**38**
	Ferrière	*Bruchus rufimanus* Boheman 1833	**54**
		Triaspis sp.	**4**
	Isnes	*Triaspis sp.*	**10**
	Itre	*Bruchus rufimanus* Boheman 1833	**20**
		Pteromalus sp.	**1**
		Triaspis sp.	**1**
	Rotheux	*Triaspis sp.*	**9**
		Bruchus rufimanus Boheman 1833	**491**

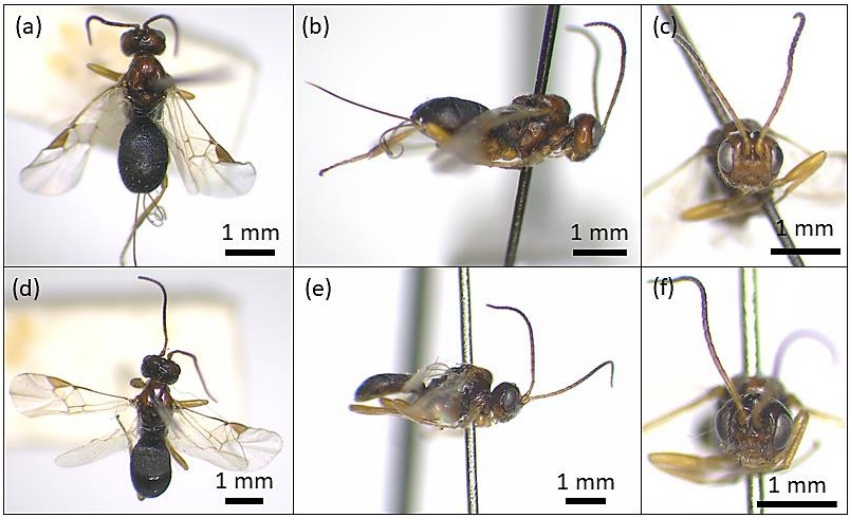


Figure 15: Illustration of *Triaspis thoracica* female (a,b,c) and male (d,e,f)

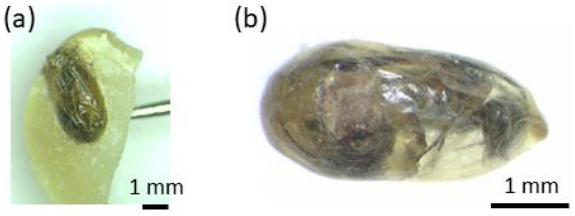


Figure 16: Illustration of *Triaspis sp.* nymphosis located in the nymphal loge of *B. rufimanus* in dissected seed of *V. faba*

2.4 Discussion

This work is the first study carried out in Wallonia assessing the diversity of bruchids and their parasitoids in faba bean crops. All bruchids species corresponded to *B. rufimanus*, which join observations of Titouhi *et al.* (2015). This indication would facilitate future management methods that can consider only one target species, which is usefull for the adjustment of selective method of control like semiochemical based management. Semiochemical attractant would present the same effectiveness against a single pest species ratrer than multiple potential species that do not share the same sensitivities to volatile cues.

All bioclimatic areas in Wallonia were infested by *B. rufimanus* but differences were observed in terms of levels of infestation rates (**Figure 12),** and also in terms of the emergence timings. The dynamic of emergences were time shifted according to bioclimatic areas (**Appendix A**). For example, seeds collected in Beho did not presented any emergence hole at the harvesting time and the first bruchids emergences occurred ten days after the harvest. This suggests that climatic conditions in this area would be favorable for a faster development of the host plant rather than bruchids. In consequence the treatment of harvested seeds in storage facilities could prevent the insect emergence, *i.e.*, the recruitment of the future pest population (Mihiretu and Wale 2013; Bachmann *et al.* 2020).

The parasitoid species richness highlighted by this study was poor compared to the potential richness indicated in the litterature. No oophageous parasitoids could be detected and only four species were identified while up to 12 species could parasite *B. rufimanus* during embryonic or post-embryonic development (Bellifa and Chapelin-Viscardi 2021a) including six species from the Braconidae family belonging to the genus *Triaspis*, named *T. forbesii* (Dalla Torre), *T. luteipes* (Thomson, 1874), *T. obscurella* (Nees, 1816), *T. pallipes* Nees (1816), *T. stictostiba* Martin, 1956 and *T. thoracica* (Curtis 1960), five species of Pteromalidae named *Anisopteromalus calandrae* (Howard, 1881), *Dinarmus acutus* (Thomson,1878), *Dinarmus basalis* (Rondani, 1877), *Dinarmus magnus* (Rohwer, 1934), *Lariophagus distinguendus* (Förster, 1841), and *Stenomalina micans* (Olivier, 1813) and one species of egg parasitoid named *U. semifumipennis*. None Dinarmus species were recorded while *D. basalis* could be identified in the UK (de Luca 1965). The recordings of *P. sequester* and *P. fasciatus* emerging from *B. rufimanus* larvae was not yet mentioned in the literature. *Pteromalus sequester* is known to be related to *B. affinis*, *B. atomarius* or *B. pisorum* according to Pérez-Benavides *et al.* (2019). To our knowledge, no study recorded any *Pteromalus* species developing within *B. rufimanus* larvae to date. The taxonomic identification must be

confirmed by the specialist J-Y Rasplus from prepared insects for further conclusions.

Regarding the braconid species, the two most occurent parasitoid species, *i.e.*, *T. luteipes* and *T. thoracica*, were already reported in other studies as reducing *B. rufimanus* infestation rates of 9 and 10% respectively (Boughdad 1994; Medjdoub-Bensaad *et al.* 2015). This level of control is too low to consider these parasitoids as effective biocontrol agent (Ward 1999). However, these organism represents a regulation lever of *B. rufimanus* population that should not be neglected as far as no conservative biocontrol strategies could be assessed for the enhancement of their presence in semi-natural habitat of faba bean crops (Williams 2010). The lack of such information is a direct consequence of the scarce biological or ecological information concerning these organisms, especially concerning the *Triaspis* species (Wharton and Lopez-Martinez 2000; Koldas *et al.* 2018). In consequence, the recomandation of conservative practices remain challinging.

Chalcidoïdea parasitoids (*i.e.*, *P. sequester* and *P. fasciatus*) are ectoparasitoids while Ichneumonoïdeae (*i.e.*, *T. thoracica* and *T. luteipes*) are endoparasitoids. *Triapsis* females are reported to oviposit in spring on eggs or early larval stages of several species of bruchids (Parker 1957). The larva will develop without killing their host that will continue to grow after parasitation (Wharton and Lopez-Martinez 2000). The host is supposed to be killed as a pre-pupa. Ward (2018) reported that the parasitoids larva exit after the host death and feed externally before making a cocoon inside the host's nymphosis chamber. Our data supported this observation by providing illustration of a *Triaspis sp.* cocoon (**Figure 16**). In consequence, the life cycle of *Triaspis* parasitoids is longer than *Pteromalus* species that oviposit on paralysed larvae of bruchids inside the seed (Sanon *et al.* 1998). Hatching of *Pteromalus* larvae occurs in average 30 h later and is followed by a rapid larval development to avoid bacterial contamination. The total development surround fourteen days until next generation emergence (Mondedji *et al.* 2002). In

overall, the emergence of *Pteromalus* species occurred earlier than the emergence of *Triaspis* species, which could be the result of the different life cycle duration (J-Y Rasplus personal communication, 2022). Further field monitoring studies for the detection of parasitoids field colonization and the timing of *B. rufimanus* parasitation are required for more comprehensive information on the bioecology of these beneficials.

2.5 Conclusions

Bruchus rufimanus was the only species observed in all faba bean crops within different Wallonian bioclimatic regions. Infestation rates vary widely according to bioclimatic areas, with certain areas that seem more favorable to the growth of *V. faba* than *B. rufimanus*. This suggests that seed treatment could constitute a local preventive tool for future infestations. Four species of parasitoids were identified, including *T. luteipes*, *T. thoracica, P. sequester* and *P. fasciatus*. This is the first report of parasitation of *B. rufimanus* larvae by *Pteromalus* species to our knowledge, but the confirmation based on prepared insect rather than ethanol conserved insects is still needed by the specialist. These parasitoids are considered as innefective biocontrol agents. However, they may represent a population control lever via conservative biocontrol strategy that should be more studied. Meanwhile, the knowledge on these insects is too poor to provide any recommendation for IPM strategies against *B. rufimanus*.

Chapter III

Embryonic and total development of *Bruchus rufimanus* Boheman 1833 (Coleoptera: Chrysomelidae) over temperature: Rearing protocol and thermal modelling approach

Embryonic and total development of *Bruchus rufimanus* Boheman 1833 (Coleoptera: Chrysomelidae) over temperature: Rearing protocol and thermal modelling approach

Taken from a project of paper in preparation for submission:

Segers A., Rossini L., Caparros Megido R., Francis F. **(2023)** *Embryonic and total development of Bruchus rufimanus Boheman 1833 (Coleoptera: Chrysomelidae) over temperature: Rearing protocol and thermal modelling approach*

Abstract: Thermal development modelling of insect pests offers a valuable tool for predicting population movements throughout their life cycle and enable timely interventions to disrupt subsequent generations. This study focuses on *Bruchus rufimanus*, a specific pest of *Vicia faba* L., which causes unavoidable damage through annual larval development inside seeds. Given the lack of existing data on the cryptic annual development process in terms of degree-days, this study tackled the thermal modeling of this pest in a two-fold approach. First, baseline data were established for rearing *B. rufimanus* under laboratory conditions. Subsequently, various models were evaluated to assess the thermal influence on the embryonic and total developments. The effect of temperature on the sex ratio of the next generation was also investigated. The most accurate model for the description of both embryonic and total development was the *Briere-2* model. Developmental threshold temperatures were 8.2 ± 0.5 °C for eggs and 12°C for total pest development. Cumulative degree-day requirements were found to be 83 degree-days for eggs and ranged from 550 degree-days for total development. Temperature had no effect on sex ratio. These findings hold implications for IPM strategies against *B. rufimanus* that are discussed in this study.

Key words: Bruchids, Broad bean weevil, thermal development, faba bean

1 Introduction

Bruchid beetles (Coleoptera : Chrysomelidae) are pests of dry or immature leguminous seeds (Fabaceae), responsible for important damages all over the world. In the last few years, the species *Bruchus rufimanus* Boheman 1833 (Coleoptera: Chrysomelidae), commonly named the broad bean weevil (BBW), gained particular attention in Europe, given the significant and increasing economic concerns on seeds of faba bean crops (*Vicia faba* L. – Fabaceae).

This species has a univoltine life cycle closely depending on the climatic conditions and on the phenology of the host plant (Hamidi *et al.* 2021). Adults overwinter in wooded sites in reproductive diapause and colonize flowering fields in spring when temperatures overreach 15 °C (Hoffman *et al.* 1962) Above this temperature threshold, the adults start to feed pollen and nectar to complete the sexual maturation, and, after the coupling, females lay their eggs on young forming pods. According to the current literature, the oviposition starts when temperatures reach 20 °C (Tran and Huignard 1992). Emerging larvae penetrate the pod tissues and settle inside a seed to undergo larval (four instars) and nymphal development throughout seed formation and maturation (Boughdad 1994). Adults emerge at the end of the season, either before or after harvest, and spend a period of reproductive diapause in forested areas waiting for favourable conditions for the development (Medjdoub-Bensaad *et al.* 2007; Huignard *et al.* 2011).

The feeding larvae inside forming pods leads to a series of harmful consequences for seed quality, such as a decline in nutritional values or flavour quality, a diminished aesthetic value due to necrotic areas and emergence holes on the seed coats, a reduced germination capacity of the seeds, and an increased risk of fungal contamination during storage or sowing (Boughdad and Lauge 1995; Kaniuczak 2004; Huignard *et al.* 2011; Khelfane-Goucem and Medjdoub-Bensaad 2016).

The management of BBWs field infestations consist of repeated insecticide treatments (*i.e.*, pyrethroïds) throughout the field colonization and oviposition period, which can last up to five weeks. The effectiveness of this management approach has become obsolete due to climatic, technical and ecological reasons (Mansoor *et al.* 2015 ; Segers *et al.* 2020). The resulting increase infestation levels observed in Europe has led to the collapse of the export market to Egypt, which is one of the most lucrative markets for growers (Lacampagne 2021). This issue is expected to worsen in the coming years, as the European Union promotes the cultivation of field bean crops for their multiple benefits that improve the sustainability of cropping systems (Köpke and Nemecek 2010) while adopting a more restrictive regulations on pesticide use at the same time (Ward 2018). This endorses even more the formulation of alternative control strategies against *B. rufimanus*.

In this sense, some authors suggested that harvesting seeds before the emergence of bruchid adults, followed by seed treatment, would lead to a reduction in pest populations in subsequent years (Mihiretu and Wale 2013). However, the necessary information to anticipate the emergence of BBWs at the end of seed maturation (*i.e.*, thermal development modeling that provides the necessary degree-days) is still lacking. The first field study highlighting the influence of temperature on *B. rufimanus* was conducted by Ward (2018) and showed that temperature could affect overwintering emergence and oviposition, although the data did not statistically prove it. The thermal development of *B. rufimanus* was better investigated by Gailis *et al.* (2022), but the experiment was conducted under field conditions, where the ability to control environmental factors is very limited.

The establishment of a thermal model for the development of BBWs would require the exposure of *B. rufimanus* to numerous cycles within a range of temperature conditions. To date, there are no studies that provide fundamental insights into the establishment of BBW rearings. This might be due to varying economic priorities regarding the pest's significance, or the

challenges inherent in establishing a productive rearing system for insects with intricate biological requirements.

In this context, the aim of this study was to provide a first thermal modeling of *B. rufimanus* development under controlled laboratory conditions.This goal was achieved in two steps.The first step focused on the definition of methodological guidelines to establish *B. rufimanus* rearings. The second step aimed at repeating the rearings under different constant temperatures and focusing on the embryonic and total development (*i.e.*, egg to adult). The observed differences in developmental times were used for the mathematical description of the developmental rate variations with temperature and for the comparison of several non-linear models. Finally, the potential influence of temperature on the sex ratio of emerging adults was investigated.

2 Material and methods

2.1 Cultivation of host plants

Tiffany cultivars (spring faba bean) were sown in 45 x 50 x 20 cm trays (16 seeds/tray) containing composted soil on a bed of clay balls. Four cylindrical wire mesh were placed in each tray to support plants (**Figure 17**). Plants were grown in climatic chamber (model/equipment of the chamber) under conditions of 16h/8h photoperiod using 23 watts bi-phosphorous white vegeleds ® (Seraing, Belgium) ensuring a minimal light intensity of 150 µmol/m²s. for floral induction. The environmental conditions (*i.e.*, temperature and relative humidity) were automatically regulated by a thermostat to ensure full control of the environment. To increase precision, the temperatures inside the rearing cages were recorded with a data logger (EasyLog EL-USB-2, LASCAR electronics, Wiltshire, United Kingdom), which acquired data every 30 minutes. The final temperature used in the data analysis was therefore the average of all the values recorded. This choice ensured a constant double check of the conditions inside the growth chambers and a more accurate estimation of the uncertainties associated

with temperature. The rearing system provided a light spectrum with a Photosynthetic Active Radiation (PAR) of 98.37%, including 16.16% of blue light, 37.30% of green light, and 44.91% of red light. No fertilizer was provided to plants, and they were regularly watered at their base rather than at the canopy level, avoiding the occurrence of fungal diseases. Three types of phytosanitary interventions were carried out to maintain the plants in good sanitary condition throughout the whole cycle in the confined environment: (i) fungicide application with Fungaflor® (imazalil 100g/l) at the stage GS105 of faba bean (Knott 1990) to avoid Oidium sp. infections, (ii) acaricide application at the stage GS201with Floramite® (bifezanat 240 g/l) to avoid Tetranichus sp. proliferation, and (iii) insecticide application at the stage GS201 with Karate Zeon® (λ-cyhalothrin 100 g/l) to avoid thrips and whitefly infestations. It is worth saying that insecticide applications were carried out at least three weeks before the release of *B. rufimanus* adults in the growth chamber, widely respecting the shortage period.

According to the literature, faba bean requires a thermal time of 830 – 1000 degree-days computed from a base temperature of 0°C (Patrick and Stoddard 2010). Under climatic chamber conditions of 21°C ± 1.5°C flowering induction (*i.e.*, flower bud apparition) occurred 40 days after sowing and lasted up to 60 days circa. Given that faba bean has a mixed breeding system requiring insect pollination (Stoddard 1986; Stoddard and Bond 1987), manual pollination was carried out to favour the pods formation. The first pods were observed on plants measuring 60 cm in height around 60 days after sowing. Pods maturation lasted until the 106th days after sowing, when pods were ripened.

2.2 Insects collection and rearing procedure

The continuous rearing of insects ensuring the specimens for the constant temperature trials was carried out as follows. Broad bean weevils in reproductive diapause were collected from harvested faba bean seeds in August and September 2020 at the Gembloux Agro-Bio Tech experimental farm and kept in boxes of 10 cm x 10 cm x 7 cm (100 adults/box) containing

corrugated cardboard with a filter paper soaked in 10% sucrose solution at a temperature of (10 ± 1) °C and a constant darkness (hereafter denoted by "overwintering conditions"). Sucrose solution was autoclaved to avoid the risk of moulding and renewed twice a month for each box. Under these overwintering conditions, adults can survive up to eight months (Tran and Huignard 1992). For the sake of completeness, we reported the survivals observed under the above-mentioned conditions in **Table 7**.

Table 7: Survival of adults under overwintering conditions

	Sept. 2020	**Oct. 2020**	**Nov. 2020**	**Dec. 2020**	**Janv. 2021**	**Feb. 2021**	**Mar. 2021**	**Apr. 2021**	**May 2021**	**Jun. 2021**	**Jul. 2021**
Box 1	**100**	88	86	85	80	79	69	49	38	35	15
Box 2	**100**	76	76	74	73	70	68	54	42	33	23
Box 3	**100**	85	81	78	75	74	74	63	52	49	34
Box 4	**100**	91	88	88	85	82	72	66	59	49	30

Previous studies showed that at least 80% of adults (both male and female) can overcome their reproductive diapause after a period of seven month under overwintering conditions when they are in presence of their host plant and a photoperiod of 18h light – 6h darkness (Tran *et al.* 1993). Batches of 25 bruchids (15 females and 10 males) were therefore placed in cages (50x50x120cm) containing 16 flowering faba bean plants supplied by a petri dish containing pollen stains stuck on a honey imbibed filter paper as supplementary food supply (**Figure 17**). The rearing conditions in these cages were (22.02°C ± 2) °C, (62 ± 8) % RH and a 16:8 (LD ratio) photoperiod with a light intensity of 185µmol/m²s at the mid-cage height.

Insects' activity (mobility and feeding or mating behaviour) was daily monitored in each cage. Adults stayed immobile for three days in the top of cages before they begin to prospect for food resources by walking on stem or leaves of their host plants. First feeding behaviours were observed four days after the placement of adults in rearing cages. Adults fed first on the extrafloral nectaries before they went into flowers (**Figure 18 a - b**). First

couplings were observed (**Figure 18 c**) eleven days after the placement of adults in rearing cages and was correlated with first flying activities. All greening pods were prospected for oviposition after 18 days (**Figure 18 d**). As the number of gravid females was excessive compared to the number of produced pods, a high number of eggs was continuously laid over a period of 40 days. The total number of eggs laid on pods greatly differed between cages and ranged from 627 to 2308, reporting an average fecundity of 97.8 eggs under the assumption that all females presented the same survival rate of 100% over the oviposition period.

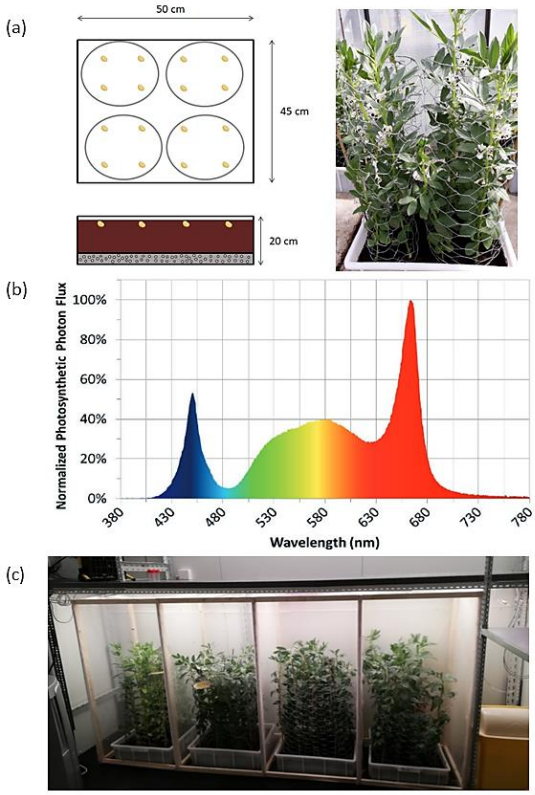


Figure 17: Sowing modalities of faba bean in trays (a) ; Light spectrum of climatic chambers (b) and cages displayed in climatic rooms (70 days after sowing) for *B. rufimanus* rearings (c)

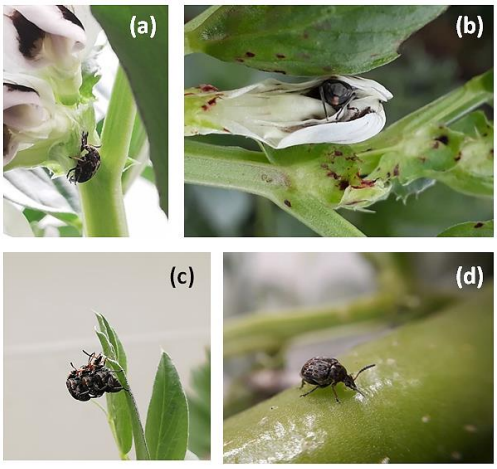


Figure 18: Behavioral observations in *B. rufimanus* rearings including feeding on flowers or extrafloral nectaries (a) and (b), mating (c), and oviposition (d)

The transparent nature of eggs shell allowed the distinction of four morphological stages during the embryonic development of *B. rufimanus* (Huignard *et al.* 2011; Bellifa and Chapelin-Viscardi 2021b): *i)* the freshly laid egg with homogenous whitish opaque appearance, *ii)* the mid-grey stage corresponding to the first tissue formation of the larvae, *iii)* the black-head stage corresponding to cephalic capsule of the larvae, and *iv)* the hatched egg presenting the perforation larval whole on the pod surface.

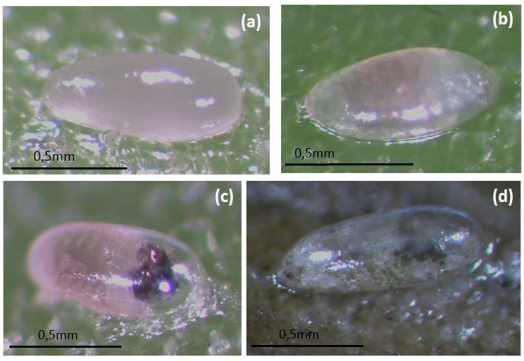


Figure 19: Different stages of *B. rudimanus* embryonic development (a) Freshly oviposited egg, (b) mid-grey stage, (c) black head stage, (d) hatched egg

The cryptic development of larvae limited the observation to oviposition and emergence of adults of the next generation in order to avoid destructive samplings. First emergences were observed from the 60th day to the 80th day after oviposition (i.e., day where seeds were dissected to check for adults that stayed inside seeds).

2.3 Egg incubations and total developments under different temperatures

2.3.1 Egg-to-adultdevelopment (total development)

Following the aforementioned rearing procedure, eight different constant temperatures were tested to explore the egg-adult stage development, namely: (14 ± 2), (17 ± 1), (21.0 ± 0.6), (21.1 ± 0.7), (21.1 ± 0.7), (22 ± 2), (24 ± 1), (27 ± 2), (28.1 ± 0.8) °C. In this phase, we have considered as a day zero the day the egg was laid by the female and as last day the emergence of the new generation adult from the faba seed. In each thermal condition, we recorded the delays from first oviposition to adult emergence as well as the sex ratio of next emergence and the percentage of adults that emerged from seeds. For each rearing cage, the emerging adults were sexed by observation of sexual dimorphism, i.e., the presence of spurs at the extremity of mesotibias and the intended pygydium of male.

It is known from the literature that generally, faba bean do not produce pods above 28 °C (Ellis *et al.* 1988), accordingly, the total development cycle at 28 °C was carried out from gravid females displayed in contact of plants during five days for oviposition at 21°C, the adults were then removed from the plants before raising the temperature to 28 °C.

2.3.2 Egg-to-larva development (post-embryonic development)

Eleven batches of pods carrying freshly laid eggs were placed in growth chambers and/or incubators to assess the thermal influence on egg incubation. Each pod bearing eggs were daily checked to assess the embryonic development. Incubations were carried out at eleven constant

temperatures, namely: (12.4 ± 0.4), (14.9 ± 0.9), (17.7 ± 0.7), (20.5 ± 0.6), (22 ± 1), (23 ± 2), (23.4 ± 0.6), (28.0 ± 0.6), (31.1 ± 0.4), (35.0 ± 0.4), and (40.0 ± 0.4) °C.

2.4 Thermal development and data analysis

2.4.1 Egg-to-larva and egg-to-adult development: life tables values calculation

The life table values reported as a result of this study were calculated as follows. For each experimental dataset, synthetic values were calculated from the developmental times of each reared individual, including the mean, the corresponding standard error of the mean, the median (the middle value separating the upper and lower halves of the dataset), the mode (the most frequent value), the kurtosis (thickness of the tails of the distribution), and the skewness (measure of the asymmetry of the distribution).

For the sake of completeness, the thermal accumulation in terms of degree day was calculated for each constant temperature T_i as well according to:

$$DD(T_i) = \sum_{n=0}^{N} (T_n - T_L) \quad (1)$$

where T_L is the baseline temperature calculated through the equation (4). Analogously to the development times, we calculated the mean value and the standard error of the mean, as well as the median, mode, kurtosis, and skewness in terms of degree days.

The dataset corresponding to each constant temperature was graphically represented by plotting the raw data, given the greater amount of information deriving from this kind of plot (*e.g.*, minimum amount of day for egg hatching or adult emergence). The calculations were carried out using Matlab (version 2023a).

Sex ratios (F/M) were also computed in each experimental temperature. The emerging adults were sexed by observation of sexual dimorphism, *i.e.*, the presence of spurs at the extremity of mesotibias and the intended pygydium

of male. Comparisons were performed using Kruksall-Wallis test with Rstudio software (version 2022.07.1+554) and R (version 4.1.2).

2.4.2 Egg-to-larva and egg-to-adult development: mathematical interpretation of development over temperature

The development times, $D_i(T)$, of each individual i observed at the different constant temperatures were first converted in development rates, $G_i(T)$, following the standard procedure, according to the formula:

$$G_i(T) = \frac{1}{D_i(T)} \tag{2}$$

The development rates, $G(T)$, over temperature of both egg-to-larva and egg-to-adult provided the typical increasing-decreasing profile that can be mathematically interpolated using the so-called development rate function equations. In particular, the literature offers several options (Quinn 2017), but the empirical nature of these functions let it difficult to choose one instead of the others. For this purpose a preliminary fitting was carried out considering the non-linear models of Logan (Logan *et al.* 1976), Lactin (Lactin *et al.* 1995), Briere (Briere *et al.* 1999), and Sharpe and De Michele (Sharpe *et al.* 1977). After the preliminary evaluation, the focus was narrowed down to the functions that yielded meaningful results during the fitting process. These functions included:

– The Logan development rate function (Logan *et al.* 1976):

$$G(T) = \psi\left[\exp(\rho T) - \exp\left(\rho T_M - \frac{T_M - T}{\Delta T}\right)\right] \tag{3}$$

where ψ and ρ are empirical parameters, T_M is the maximum temperature above which the development is theoretically not possible, and ΔT is the temperature range between the maximum of the function $G(T)$ and T_M.

– The Briére development rate function (Briére *et al.* 1999):

$$G(T) = aT(T - T_L)(T_M - T)^{1/m} \quad (4)$$

where a and m are empirical parameters, and T_L and T_M are the lower and maximum temperatures below and above which the development is theoretically not possible, respectively.

– The Sharpe and De Michele rate function (Sharpe and De Michele 1977):

$$G(T) = \frac{T \exp\left(A - \frac{B}{T}\right)}{1 + \exp\left(C - \frac{D}{T}\right) + \exp\left(E - \frac{F}{T}\right)} \quad (5)$$

where A, B, C, D, E, and F are parameters related to the enzyme kinetics (Rossini *et al.* 2019)

- The Lactin rate function (Lactin *et al.* 1995):

$$G(T) = \exp(a \cdot T) - \exp\left(a \cdot T_M \cdot \left(\frac{T_M - T}{\Delta T}\right)\right) \quad (6)$$

Where T_M is the maximum temperature above which the development is theoretically not possible, and ΔT is the temperature range between the maximum of the function $G(T)$ and T_M, a and b are empirical parameters

The non-linear fits were carried out using the fitting library included in the Matlab software (vers. 2023a), and the goodness of fit was evaluated considering the sum of squares error (SSE), the coefficient of determination (R^2) and the root mean of squared errors (RMSE).

3 Results

3.1 Egg-to-larva and egg-to-adult development: life tables results

The response of *B. rufimanus* under different environmental temperatures were observed, more precisely the egg-to-larva development under eleven temperatures and the egg-to-adults development under six temperatures. A synthesis of rearing initial effectives for egg-to-adults development observations including observations of oviposition delays as well, sex ratios of the next generation and percentage of emerging bruchids is presented in **Appendix B**.

Egg developments showed different trends in terms of incubation time distributions over temperature (**Figure 20**). In general, eggs incubation time decreased with increasing temperatures from 12°C until 40°C with respective survival rate of 44.9% and 0% **(Table 8)**. According to the dataset, the shortest delay in egg hatching occurred at higher temperature, where the minimum amount of days to observe hatching is below 10 days (*cf.*, 3 days at 35 ± 0.4°C). On the other hand, at low temperatures the hatching time is strongly delayed, requiring until 28 days at 12 ± 0.4°C °C.

Even with differences among the temperatures, all the eggs hatch in a relatively short time, as shown by the standard errors of the mean listed in **Table 8**. The parameters of the distribution listed confirm this observation, given that the median and mode values were comparable to the mean development times.

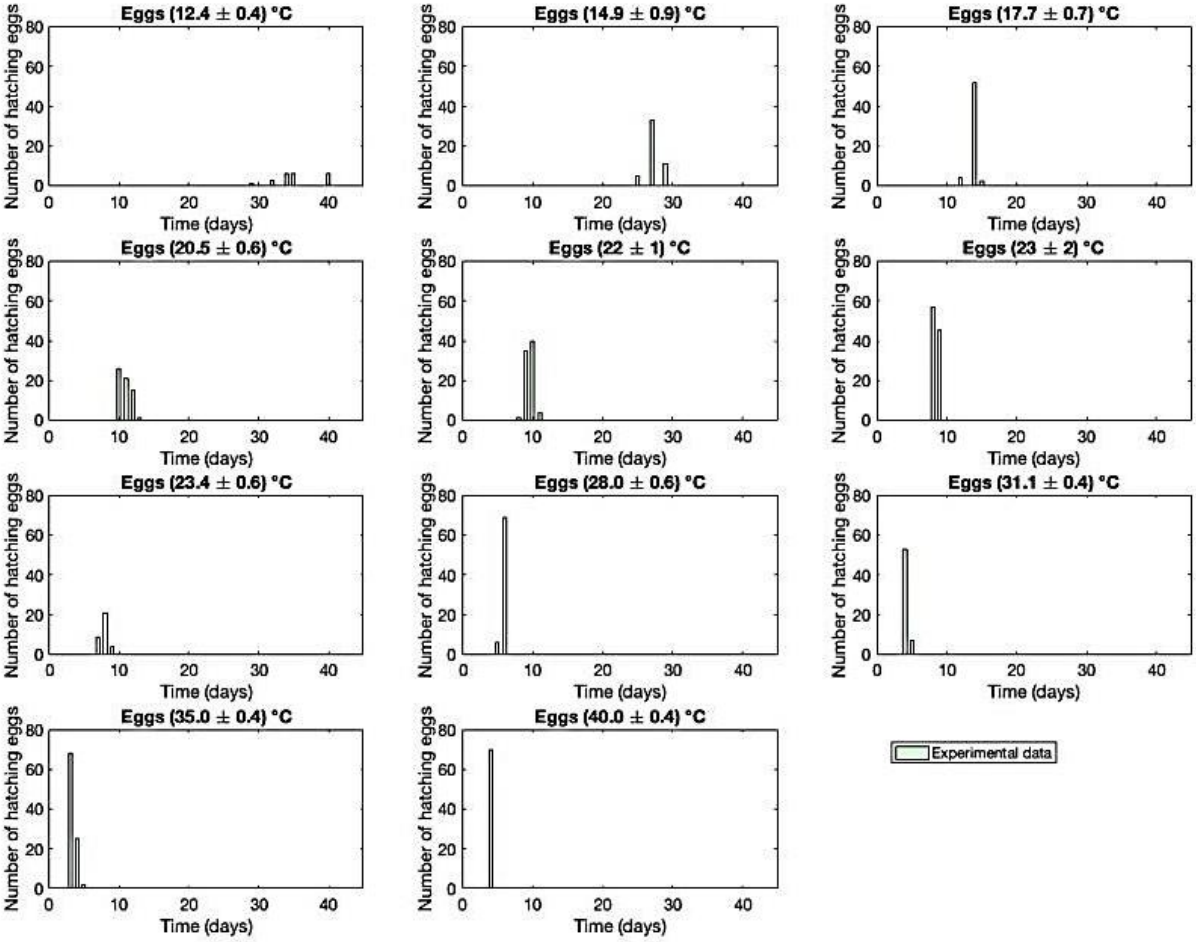


Figure 20: General plot of the distributions of egg incubations time under different constant temperatures

A different scenario, compared to the egg-to-larva development, was observed for the egg-to-adult development. The shortest time at which adults started to emerge was assessed at 28 °C (**Figure 21**), that can be considered as the optimum temperature for the development according to the synthetic values listed in **Table 8**.

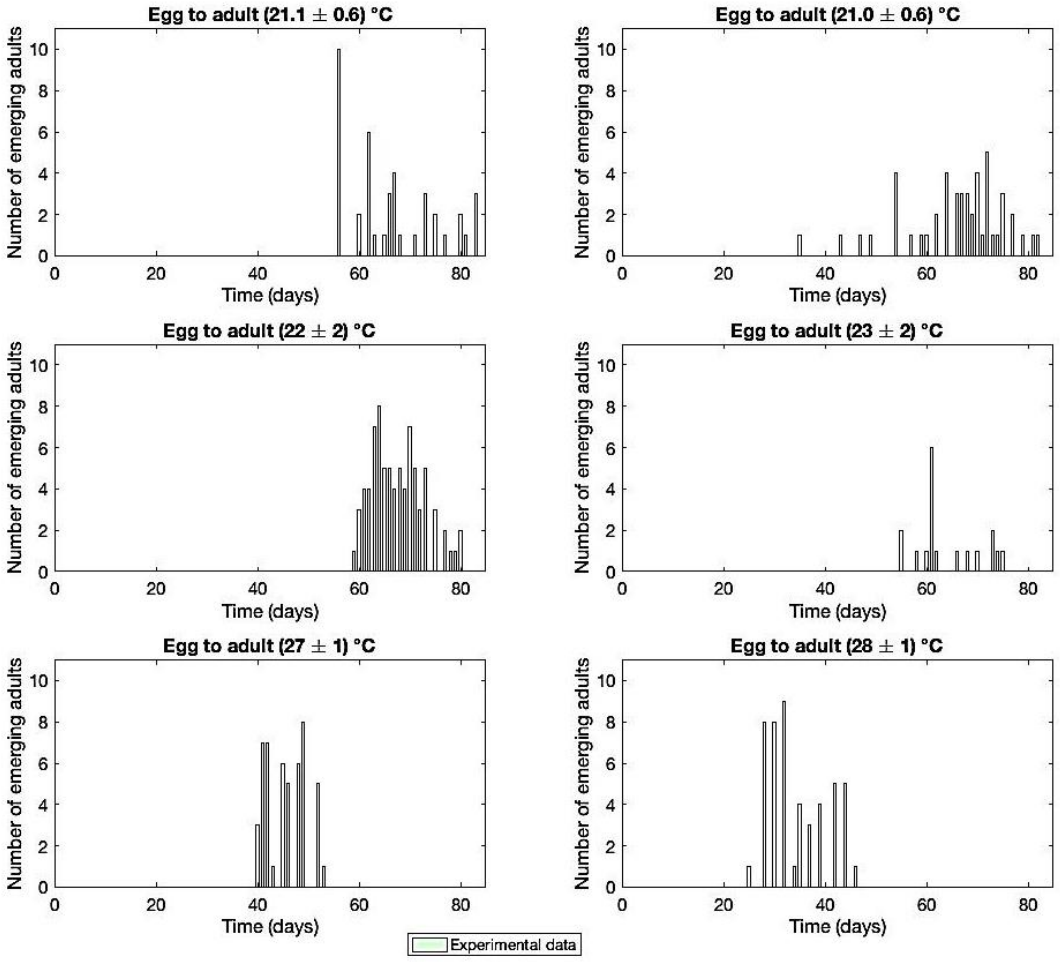


Figure 21: General plot of the distributions of eggs-adult development time under different constant temperatures

3.2 Thermal influence on sex ratios

Five new generations of *B. rufimanus* were obtained in different temperatures. The sex ratios of adults (F/M) are presented in **Figure 22**. No statistical differences were obtained from the Kruksal-Wallis test among five different temperatures ($\chi^2(4) = 5.47$, p=0.241).

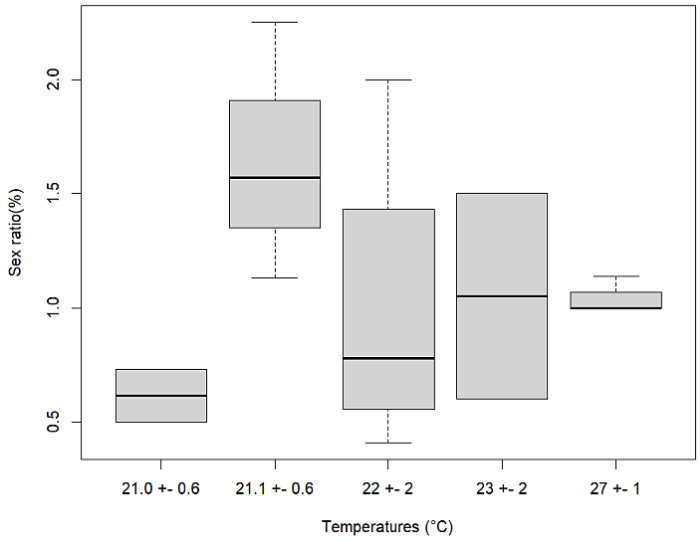


Figure 22: Sex-ratio (F/M) obtained under five different thermal conditions

3.3 Egg-to-larva and egg-to-adult development rate functions results

Among the four non-linear models tested, the Lactin function has been discarded from the analysis, given the non-convergence of the fitting algorithm after several attempts. The different values parameters that were obtained for the other models that could fit with the dataset (*i.e., Briere2, Logan, Sharp and Michele*) are presented in **Table 9**. Regarding the goodness of fit parameters and the biological interpretation of the models, it seems that the *Briere 2* model is the most reliable one for the thermal modelling of egg development and egg-adult development of *B. rufimanus*, with respective adjusted R^2 of 0.96 and 0.70.

Table 8: Thermal development characteristics of eggs and egg to adult development under different temperatures

			Development times					Degree-days				
Observation (n)	**Temp. (°C±SE)**	**Survival (%)**	**Mean (days±SE)**	**Mode (days)**	**Med. (days)**	**Kurt. (days)**	**Skew. (days)**	**Cumulative degreedays (°C·days±SE)**	**Mode (°C·days)**	**Median (°C·days)**	**Kurt. (°C·days)**	**Skew. (°C·days)**
Eggs (49)	12.4±0.4	44.90	35±3	34	35	2.26	0.26	85±8	81.6	84.0	2.26	0.26
Eggs (58)	14.9±0.9	87.93	27±1	27	27	3.04	0.03	133±5	132.3	132.3	3.04	0.03
Eggs (61)	17.7±0.7	95.08	13.9±0.6	14	14	10.50	−2.57	107±4	107.8	107.8	10.50	−2.57
Eggs (62)	20.5±0.6	100.00	10.8±0.8	10	11	2.00	0.43	114±9	105.0	115.5	2.00	0.43
Eggs (80)	22±1	100.00	9.6±0.6	10	10	2.55	0.16	115±7	120.0	120.0	2.55	0.16
Eggs (102)	23±2	98.04	8.4±0.5	8	8	1.05	0.23	110±6	104.0	104.0	1.05	0.23
Eggs (34)	23.4±0.6	100.00	7.8±0.6	8	8	2.64	0.07	105±8	107.2	107.2	2.64	0.07
Eggs (75)	28.0±0.6	100.00	5.9±0.3	6	6	10.58	−3.09	107±5	108.0	108.0	10.58	−3.09
Eggs (71)	31.1±0.4	81.60	4.1±0.3	4	4	6.70	2.38	87±7	84.4	84.4	6.70	2.39
Eggs (111)	35.0±0.4	84.69	3.3±0.5	3	3	3.73	1.32	83±12	75.0	75.0	3.73	1.33
Eggs (70)	40.0±0.4	0.00	–	-	-	–	–	-	-	-	-	-
Eggs-adults (38)	21.1±0.6	-	66±9	56	66	2.08	0.48	600±80	509.6	600.6	2.09	0.48
Eggs-adults (48)	21.0±0.6	-	66±10	72	68	4.09	−1.03	600±90	652.2	616.0	4.09	−1.03
Eggs-adults (79)	22±2	-	68±5	64	67	2.59	0.51	680±50	641.2	671.3	2.59	0.51
Eggs-adults (18)	23±2	-	64±6	61	61	1.89	0.40	740±70	702.7	702.3	1.90	0.40
Eggs-adults (49)	27±1	-	46±4	49	46	1.83	0.15	670±60	719.8	675.7	1.83	0.15
Eggs-adults (49)	28±1	-	35±6	32	32	1.89	0.43	550±90	513.9	513.9	1.90	0.44

Table 9: Best fit parameters of different models tested for the description of the thermal influences on egg incubation and total development of *B. rufimanus*

	Egg incubation		**Total development**	
Development rate function	**Best fit parameter (±SE)**	**Goodness of fit paramerers**	**Best fit parameter (±SE)**	**Goodness of fit paramerers**
Briere	$a=3.0 \cdot 10^{-4}$	SSE=0.24	$a=4.3 \cdot 10^{-5}$	SSE=0.003
	T_L=8.2 ± 0.5	RMSE=0.019	T_L=12	RMSE=0.003
	T_M=39.98 ± 0.01	R^2=0.96	T_M =33	R^2=0.70
	m=12 ± 2	NDF=704	m=5	NDF=280
Logan	$\psi=1.18 \pm 0.008 \cdot 10^{-2}$	SSE=0.23	$\psi=1 \pm 1 \cdot 10^{-3}$	SSE=0.003
	$\varrho=9.3 \pm 0.3 \cdot 10^{-2}$	RMSE=0.018	ϱ=0.12 ± 0.04	RMSE=0.0003
	T_M=42.2 ± 0.1	R^2=0.96	T_M=31 ± 3	R^2=0.67
	ΔT=3.0 ± 0.2	NDF=704	ΔT=1 ± 2	NDF=280
Sharpe and De	A=36	SSE=0.24	A=12	SSE=0.003
Michele*	B=5	RMSE=0.26	B=-210	RMSE=0.0034
	C=40	R^2=0.95	C=-6	R^2=0.69
	D=-30	NDF=702	D=-230	NDF=278
	E=57		E=-10	
	F=681		F=-300	

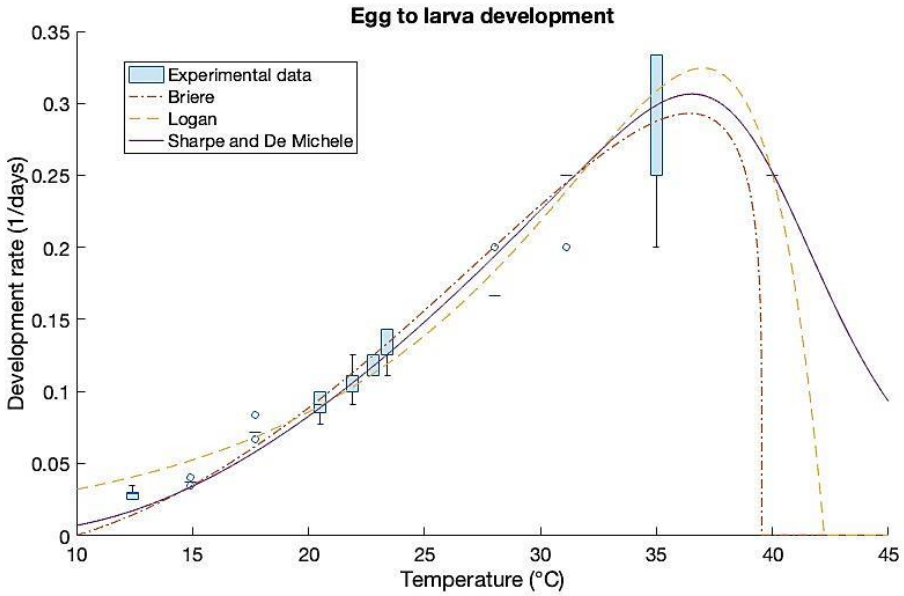
* The uncertainties were not reported because of non-reliable values.

Models of *Logan* and *Sharp and De Michele* did not reveal reliable development thresholds for both egg development and egg-adult development as represented by the fitted thermal development curves in **Figure 23.**

The lower developmental threshold (T_L) obtained for egg development was estimated at 8.2 ± 0.5°C with the model *Briere2*. The mean cumulative degree-day required for egg development computed from this T_L value ranged from 83 to 110 degree-days.

Concerning the egg-adult development, the developmental threshold was estimated at 12°C with the model *Briere2*. The mean cumulative degree-day required for egg development computed from this T_L value ranged from 550 to 740 degree-days.

(a)



(b)

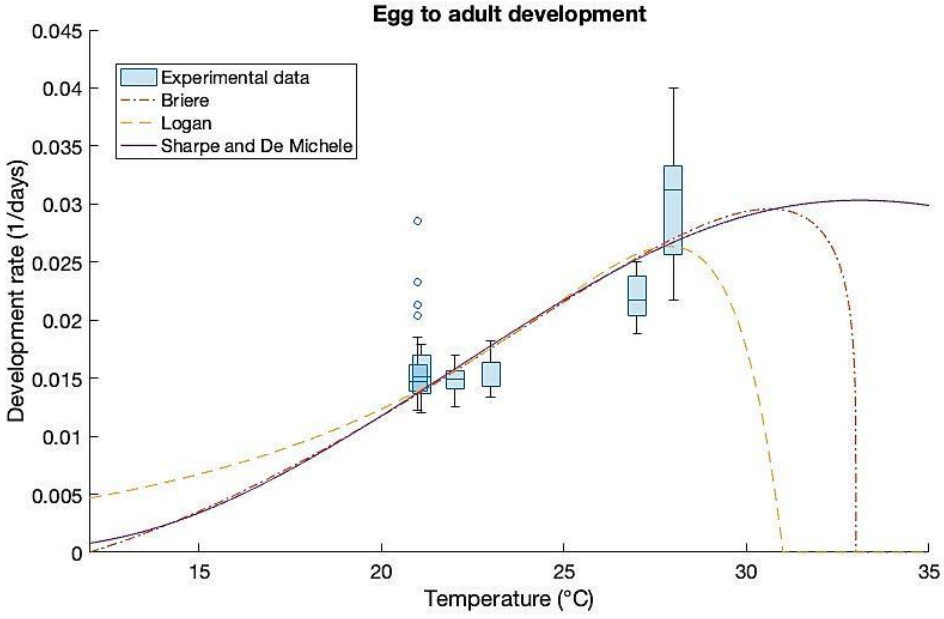


Figure 23: Thermal modelings of egg incubations (a) and egg to adult developments (b) of *B. rufimanus*

4 Discussion

4.1 Rearing procedure, limits and areas of improvements

This study provided the first rearing protocol for the bruchid species *B. rufimanus* emphasizing insight on the required conditions for key steps of the BBW and the host-plant developments, including the maintenance of overwintering adults, the termination of reproductive diapause depending on the host plant flowering, the mating and oviposition on subsequently formed pods, and the *V. faba* flowering and pod-setting under controlled conditions. These procedures were quite time consuming, and some improvements may be implemented by the development of two types of artificial diets given the distinct feeding behaviours of adults and larvae. Artificial diet for adults that enables the overcoming reproductive diapause (*cf.* nectar and pollen from the host plant) could be obtained by the settlement of itinerary beehive in field bean crops. Immature honey mainly collected from *V. faba* during the flowering of crops and pollen stains collected in pollen trap, would constitute an easy and large amount of necessary components for adults' overcoming diapause. Experiments led with an all-purpose mixture and pollen provided an effective nutritive resource to adults. However, the development of an artificial diet for feeding larvae should consider the maturation of seeds and the evolution of proteic/carbohydrates content all along the different larval stages. Korsvold (2020) provided quantification about the starch, protein and moisture seed content during the evolution of the faba bean seed development. The moisture seed content (MSC) was reported to vary from 82.87% (*cf.*, immature stage) to 32.7% (*cf.*, most matured observed stage). The highest starch concentration (SC) was observed at 40.5% on formed seeds presenting 55.42 % of MSC, while the lowest starch content was observed at 12.1% on forming seeds presenting MSC content of 82.87%. The protein concentration (PC) varied from 27.5% in formed seeds (*cf.*, MSC of 55.42%) up to 33.4% in immature seeds (*cf.*, MSC of 82.87%). These indications provide insight on the appropriate composition for two artificial diets that could be tested for the larval development: (i) an artificial diet for egg

hatching (*cf.*, L1 to L3 larval instars), and (ii) one artificial diet for ~ L3 larval instar up to the nymphosis.

Such artificial diets would resolve one of the major limits of this study, *i.e.* the determination of the end of the nymphosis. The cryptic post-embryonic development of bruchid often limits the thermal influence description from the oviposition to the adult emergence in order to avoid destructive samplings (Kutcherov 2020). In this study, a non-neglectable proportion of reared insects stayed inside the seeds (**Appendix B**). Moreover, the cryptical nymphosis prevented the determination of the end of the total development as adults may remain during an indeterminate time inside the seed before emerging. This is probably the reason of different development time distributions that were observed over different tested temperatures and the decreasing R^2 concerning the total development. This issue could also be resolved by inducing the adults emergence after treatments of seeds with the solution of NaOCl and chinosol during seven days at 30°C (Girsh *et al.* 1999), but this would induce a bias on the thermal developments.

4.2 Thermal development of *B. rufimanus*

The development time ranges that were observed for eggs and total development of *B. rufimanus* in controlled conditions corresponded to indications of the bio-ecological studies that reported incubation times of about 1-2 weeks, with most eggs hatching after 10 days (Boughdad 1994; Yus-Ramos *et al.* 2014; Roubinet 2016; Pölitz and Reike 2019). Total development times were reported to last about 90 to 110 days in field conditions (Boughdad 1994), here the total development was maximized at 68 ± 5 days under constant temperatures.

Thermal development modeling could be performed for other species of bruchids, such as *Bruchus pisorum* L., *Callosobruchus chinensis* L., *C. maculatus*, and *Acanthoscelides obtectus* (Say, 1831) or *A. macrophtalamus* (Park *et al.* 1991; Smith 1992; Wu *et al.* 2013; Mobarakian *et al.* 2014; Soares *et al.* 2015). Respective developmental threshold and the cumulative degree-days for eggs and total development were in line with these studies. For example, the

temperature threshold for *B. pisorum* egg development was estimated at 9.4°C which is similar to that of *B. rufimanus.* However, the cumulative degree-days for egg development were relatively lower with other studied bruchid species, *i.e.* 35.15 degree-days reported with *C. chinensis* and 47.2 degree-days reported for *B. pisorum*. Concerning the total development of *B. rufimanus,* the T_L value obtained and the degree-days (*i.e.*, 12°C and at least 550 degree-days) were in the range of that of other studied bruchid species, *i.e.*, 10.4°C and 526.3 degree-days with *C. chinensis*, 12.8°C and 632.8 degree-days with *A. macrophtalamus*, or 11.1°C and 685 degree-days for *A. obtectus*.

Recently, Gailis *et al.* (2022) assessed the thermal development of *B. rufimanus* in field conditions but their results lack of relevance due to methodological bias and inaccuracies of the environmental factors measurements. Indeed, they considered a base temperature of 0°C for the calculation of thermal development of *B. rufimanus,* which overestimates the necessary degree-days required for the insect development because bruchids present higher base temperatures as it could be highlighted on other bruchid species. Secondly, the temperature recordings were not performed within the faba bean crops, but with the "*closer weather station*". However, beyond the lack of precision regarding the distance separating field from recording weather station, microclimatic crop conditions of BBW post-embryonic development (*cf.*, pods at the base of the plants) may greatly differ from the temperature recorded by a standard weather station at 2m height (Durigon and de Jong van Lier 2013; Siebert *et al.* 2014).

4.3 Thermal development as potential management tools against *B. rufimanus*

The problem with the BBW management is related to the endophytic post-embryonic development as their feeding larvae remain inaccessible to phytosanitary treatments. According to some authors, implementing adapted cultural practices, such as early seed harvesting to prevent adult emergence and fumigating harvested seeds, can be an effective strategy to reduce insect populations from one year to the next (Mihiretu and Wale 2013; Bachmann *et*

al. 2020). Given the results of this laboratory study, at least 550 cumulative degree-days, calculated from the date of first ovipositions observation in crops (with a development threshold of 12°C) should determine the appropriate harvest time if seed maturation is sufficiently reached. These results must still be validated by field observations to confirm the potential use of results as management tool. Moreover, this strategy could be limited by the indeterminate growth of *V. faba*. In fact, this physiological aspect of faba bean causes the decline in seed maturation throughout pod formation, which competes with the retention of flowers within the plant (Patrick and Stoddard 2010). When the plant concentrates photoassimilate in the seed formation rather than in the flowers (*i.e.*, at the end of faba bean flowering), several pods are present on one plant while manifesting different maturation stages. As a result, the plant may present both mature and immature seeds, when the BBWs developing in these latter have reached the degree days required for their full development. Early harvesting of seeds with high relative humidity would be highly detrimental to seed storage due to the potential overheating of seeds or the appearance of contaminants such as mycotoxins (Acuña-Gutiérrez *et al.* 2022), which are not permitted by food or feed standards. In addition, when seeds are harvested above 16% of relative humidity, farmers incur increasing drying costs depending on the level of relative humidity of the seeds (Fegra and Synagra 2023).

In this sense, varietal effects on the earliness of seed maturity would be a potential advantage, ensuring that the majority of seeds mature before the onset of BBW emergence. As shown by the emergence patterns of BBWs in the sample of Beho 2021 (*cf.*, Chapter II), it is plausible that seeds maturity is reached before BBWs emergences, *i.e.,* there may exist some climate x cultivar associations that favor a faster plant development and seed maturation rather than BBWs, supporting the use of BBWs thermal development as a management tool to be implemented in IPM strategy. Future field experiments to validate the thermal model results should also characterize the seed relative humidity in parallel with the thermal development of *V. faba* to determine if an optimal harvest time could be identified.

Chapter IV

Varietal and environmental effects on the production of faba bean (*Vicia faba* L.) seeds for the food industry by confrontation of agricultural and nutritional traits with resistance against *Bruchus spp*. (Coleoptera: Chrysomelidae, Bruchinae)

Varietal and environmental effects on the production of faba bean (*Vicia faba* L.) seeds for the food industry by confrontation of agricultural and nutritional traits with resistance against *Bruchus* spp. (Coleoptera: Chrysomelidae, Bruchinae)

Taken from the following reference:

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Agriculture, Ecosystems & Environment, 327, 107831.

Abstract: Faba bean is a globally produced agricultural crop due to the high protein content in seeds. However, yields strongly vary depending on biotic and abiotic factors. Here, we evaluated the combined effect of faba bean varieties and climate on crop productivity, seed quality and *Bruchus spp*. infestation to identify most promising faba bean varieties for use in the food industry as local protein source. Varietal and year related factors were studied during two cropping years to explain variation of field yield, seed protein/ash/lipid content, protein production, and infestation rates. Fourteen varieties including nine winter varieties and five spring varieties were compared, from which one variety presented stable and promising yield, seed composition and low infestation rates. Annual effects significantly impacted field yield and protein production in contrast with the varietal effect that significantly impacted seeds protein content and infestation rates. Principal components analysis showed that infestation rate and yield were not correlated; thus, these two parameters could be optimized independently. The spring variety Fanfare exhibited the best and most stable results over the two

study periods. Winter varieties had higher yields, whereas spring varieties had higher seed protein content. The main parameters impacting bruchid infestations were variety, indicating the need to select certain varieties that reduce the impact of pests on seed quality. During 2020, a drought during growing season significantly impacted faba bean production, demonstrating the importance of developing drought-resistant varieties. Thus, fourteen faba bean varieties were characterized considering together key parameters for food uses, and were ranked to identify most interesting ones. We also highlighted most impacting parameters that should be taken into account for the future improvement of varietal resilience in European countries.

Keywords: Faba bean; Variety; Bruchus spp.; Chemical composition; Yield

1. Introduction

Producing indigenous plant proteins is essential for the sustainability of the European cropping systems, with interest being expected to increase in the coming decades given their multiple benefits provided to cropping systems, environment and ecosystems (European Parliament 2018). Leguminous plants constitute potential candidates to promote local plant protein productions (PPs). One such example is Vicia faba L., which is commonly named faba bean (as general denomination), but also broad bean, horse/field bean, and tic bean (depending on the traditional type of varietal groups). Faba bean crops provide many benefits to the environment by reducing the need for nitrogen fertilizers and, so reducing the greenhouse gases production associated with their production (Köpke and Nemecek 2010). Moreover, the cultivation of faba bean benefits crop rotations by improving the yields of successive crops, preventing and limiting the spread of pests and diseases, and favoring beneficial organisms, such as pollinators or natural enemies of arthropod pests, due to their extensive floral resources (Jensen *et al.* 2010; Karkanis *et al.* 2018).

The main asset of cropping faba bean is the production of seeds that contain high amounts of proteins, about 30% weight/weight (w w-1) of dry weight

(DW) (Rempel *et al.* 2019). Faba bean seeds have high nutritional value and a comparable amino acid (AA) score to other legumes (FAO/WHO/UNU 2007; Vioque *et al.* 2012; Pastor-Cavada *et al.* 2014; Wongsiri *et al.* 2015). Besides proteins, faba bean seeds are composed of starch (around 40% DW), minerals (~ 3–4% DW), and lipids (~ 2%) (Vioque *et al.* 2012; Landry *et al.* 2016). The relative percentages of each nutrient vary with variety (Micek *et al.* 2015).

Faba beans are used as food and feed. Food uses constitutes the most profitable outlet for growers, and was evaluated at 30–40 euros/t above the animal feed market (Biarnès *et al.* 2018; Bachmann *et al.* 2020). Food uses include seed consumption (whole or dehulled) in traditional meals (especially in Mediterranean countries). Transformation and formulation of new products in the food industry represents another food use opportunity for faba bean seeds, providing alternatives to traditional animal proteins. Their low lipids contents facilitate protein extraction, which leads to an improvement of the technological quality of proteins. Feed uses include pigs, poultry, and bovine feedings. The export of dehulled seeds for use in fish farms in Norway is currently rising (Biarnès *et al.* 2018). As feed, this crop provides a good alternative to imported soybean, and contributes to protein autonomy.

Despite these advantages, the promotion of faba bean is subject to sociological, technical, and logistical constraints (Simmen 2020). The greatest constraint in using faba bean seeds as food is infestation by *Bruchus* spp. (Coleoptera: Chrysomelidae, Bruchinae). This group of pests ravaging faba bean is commonly called broad bean weevils or bruchids, and include five species occurring in Belgium; namely, *Bruchus affinis* Frölich 1799; *B. atomarius* (L. 1761), *B. brachialis* Fåhraeus 1839, *B. rufimanus* Boheman 1833 and *B. pisorum* (L. 1758). *Bruchus rufimanus* is the most abundant species in faba bean crops (Zampetti and Ricci 2012; Baugnée *et al.* 2021). All five species are oligophagous univoltine species, the life cycle of which greatly depends on host plant phenology and climatic conditions (Segers *et al.* 2021). Adults overwinter in reproductive diapause in wooded sites, and colonize flowering crops during spring when temperatures exceed 15 °C (Hoffman 1962).

Individuals sexually mature while feeding on *V. faba* flowers, and reproduction occurs approximately two weeks after field colonization, with temperatures surrounding 20 °C (Tran and Huignard 1992). Gravid females oviposit on newly formed pods and hatching larvae directly bore through the pods pericarp (Pölitz and Reike 2019). The whole post-embryonic development, including four larval instars (L1–L4) and a nymphal stage, takes place inside one forming seed, and spans approximately 100 days. Most adults emerge when dry seeds are harvested (Boughdad and Lauge 1995). Emerging adults do not cause any further damage to stored seeds, as females cannot oviposit on dry seeds (Howe and Currie 1964). Adults overwinter in reproductive diapause until the next spring when faba bean crop starts flowering (Huignard *et al.* 2011).

Bruchids cause both qualitative and quantitative damage to seeds including a reduction in seed weight due to the consumption of the endosperm by developing larvae. This reduction ranges from 5.0% to 9.4% of the dry seed weight (Boughdad and Lauge 1995; Titouhi *et al.* 2015). The consumption of seeds by developing larvae also decrease the nutritional value and organoleptic properties caused by the accumulation of insects wastes such as (Huignard *et al.* 2011; Khelfane-Goucem and Medjdoub-Bensaad 2016). Esthetic quality is also strongly altered due to the perforation of the seed coat by emerging adults. In addition, perforated seeds are more susceptible to fungal infection in storage facilities or in crops after (Kaniuczak 2004; Ward 2018). These damage prevent the seed valorization in food market that only accept seed batches with < 2–3% infestation rates (*i.e.*, proportion of seeds presenting emergence hole of adults) (Bruce *et al.* 2011; Ward 2018). This target is difficult to meet, as broad bean weevils typically generate 3–100% infestation rates, depending on country and cultivar (Boughdad and Lauge 1995; Kaniuczak 2004; Roubinet 2016). As a consequence of bruchids infestations, the French export market of faba bean seeds to Egypt for traditional food uses (*i.e.*, most important food market) collapsed during the last decade (Lacampagne 2021). Solutions such as varietal selection must therefore be implemented to encourage this environment-friendly culture.

To date, few studies have assessed the susceptibility of different *V. faba* varieties to the development of bruchids, and underlined that no faba bean variety was yet able to support a seed production matching with food quality standards in Europe, as no resistance or tolerance could be observed (Roubinet 2016). However, the selection of varieties exhibiting some level of resistance represents the most promising way of controlling such pests. Some accessions were recently supposed to exhibit antibiosis or antixenosis resistance mechanisms to the development of bruchids (Szafirowska 2012; Seidenglanz and Hunady 2016; Carrillo-Perdomo *et al.* 2019). Further researches on the selection of candidate varieties for food industries (*i.e.*, producing stable and optimal protein quantity and quality), should focus on resistances against bruchids that decrease seeds quality, but also on resistance to abiotic stresses that greatly impact seeds quantity, and that are expected to increase with current climate change. However, to date, studies have not investigated the traits of varieties considering all of these factors simultaneously.

This study aimed to identify the potential faba bean varieties to produce viable seeds for use in the food industry. To accomplish this, 14 varieties were tested in field trials, and seeds were characterized according to bruchids injury level and seed composition to detect those with the maximal amount of protein and ash content, and minimal amount of fatty matter and bruchids infestation rate. Our results are expected to provide baseline information on the best varieties for seed breeders to grow and towards selecting more resistant faba bean cultivars for the future.

3 Material and methods

3.1 Plant material, field experiment and measurements

Fourteen faba bean varieties (including five spring varieties and nine winter varieties) were tested in Gembloux (Belgium) over two consecutive growing seasons (2018–2019 and 2019–2020). Geographical coordinates and altitude of the two field trials were 50◦49’91’’N, 4◦74’34’’E, 167.12 m (2018–2019), and

50◦50′34′′N, 4◦73′19′′E, 174.75 m (2019–2020). These locations had high bruchid population sizes based on previous faba bean infestation records. Thus, each variety was grown under hyper infestation conditions to discriminate sensitive and resistant varieties optimally. Spring varieties included commercial cultivars named "Bobas", "Fanfare", "Julia", "LG Cartouche" and "Tiffany." Winter varieties included commercial cultivars named "Augusta, "Axel", "Bering", "Bumble", "Diva", "Honey," "Irena", "Nebraska" and "Tundra" (**Appendix C**). Spring and winter varieties were tested in separate trials designed in four complete randomized blocks. The factor Type was used to discriminate winter and spring varieties. Faba beans were grown in plots of 14.0 m2 (2.0 × 7.0 m) at a density of 35 seeds per m2 (winter varieties) and 50 seeds per m2 (spring varieties). These different sowing densities were used to obtain the same stem density in winter and spring crops. Winter varieties of faba bean branch many times at the base of the plant during the winter period, whereas spring varieties have one to two upright stems per seed (unpublished data). External plots were placed around the test areas to attenuate border side effects. Plots were bordered by parcels of 27 m wide (called "generalized parcels"), which contained a single faba bean variety for winter and spring trials; namely "Nebraska" and "Fanfare," respectively. A representative scheme of this field set-up is presented in **Appendix D** in supplementary material. Under these trial conditions, we hypothesized that spring and winter varieties would grow in two distinct environments driven by respective weather conditions and pest pressure of the two years of study. No insecticides or fertilizers were applied in the experimental design. One fungicide treatment was applied at the bud-flowering stage of the crop to avoid any disease in the trial. Chemical weeding was performed after sowing. Seeds were mechanically harvested at maturity. The sowing and harvesting dates of spring and winter varieties are presented in **Table 12**.

Bruchid populations were monitored in parallel with meteorological conditions and varietal phenology. Climatic parameters of temperature and rainfall, which are recognized as the key factors driving bruchid infestation

(Carrillo-Perdomo *et al.* 2019), were recorded by a nearby weather station. The phenological traits measured for each variety included the flowering start date (*i.e.* 50.0% of flowering plants in plots) and the flowering duration, because both parameters strongly influence the oviposition periods of bruchids (Seidenglanz and Hunady 2016). Populations of bruchids were monitored using manual catches in generalized parcels, to avoid disrupting adult behavior (*i.e.,* feeding and oviposition), which could be influenced by preferences for certain varieties. This manual method of monitoring was preferred over semiochemical traps, because traps cannot efficiently detect the presence of adults during the flowering period, due to competition exerted by crop odor (Ward 2018; Segers *et al.* 2021). Manual catches were performed weekly in generalized parcels following a standard method, in which a single operator followed a fixed pathway (100 m length × 1 m width) at the same daily period (from 15:30 to 16:30), and caught active adults (*i. e.,* feeding or mating) in the flowers or apical leaves with a truncated cone reversed over a pill box. Insects were conserved in ethanol 70% (v v^{-1}) and brought to the laboratory for identification and counting. All these field measurements were computed in order to confront weekly bruchids field population with weekly averages of temperatures (°C, Tavg), cumulated rainfall (mm, Pcum), and varietal phenological stage.

3.2 Analyzes of harvested seeds

After harvesting the crop, agronomic traits including field yield (FY,kg ha^{-1}) and thousand seeds weight (TSW, g) were determined from a unique value based on the four plots pooled together for each variety. FY and TSW were determined at standardized 14% seed humidity. Seeds were then characterized based on damage and chemical composition analyses.

Damage analyses were performed on seeds stored at room temperature for one month to allow insects to complete their life cycle. Four randomly selected replicates of 50 seeds were sampled and seeds were ranked according to traces left by the post embryonic development of bruchids. The ranking approach implemented by Carrillo-Perdomo *et al.* (2019) was used: Class "HS" (*i.e.,*

"healthy seed") corresponded to non-infested seeds (**Figure 24-A**); class "SD" (*i.e.,* "surface damages") corresponded to infested seeds, presenting traces of necrosis on their tegument, which was left by bruchid larvae (**Figgure 24-B**); and class "EH" (*i.e.* "emergence hole") corresponded to infested seeds, presenting emergence holes of adults or "circular windows" on the seed coat, corresponding to the nymphal stage or adults remaining in the seed (**Figure 24 C–E**). Mean percentages of healthy seeds (HS), surface damage (SD), and emergences holes (EH) were computed from four replicates of 50 randomly selected seeds for each variety during the two year of study period.

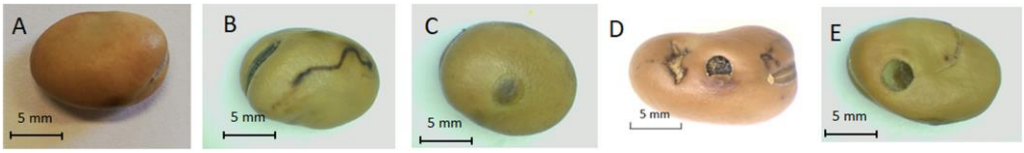

Figure 24: Seed damage classes based on traces left by the post-embryonic development of *B. rufimanus*: A – non-infested seed; B – infested seed with traces of bruchids larvae; C – seed with a "circular window" on the coat corresponding to the nymphal stage or adult stage that stayed inside the seed; D – seed with adult that stayed in the seed; E – seed with emergence hole left by emerging adults. © A. Segers (2020)

Chemical composition analyses were performed (each in triplicates) on faba bean flour produced on a laboratory mill (M20 IKA Labortechnik, Staufen, Germany). Dry matter (DM, %c $w.w^{-1}$) was determined according to the corresponding NREL method (Sluiter *et al.* 2008a). The nitrogen contents of the ground seeds were determined following the Dumas methodology using a Dumas Elementar Rapid N cube 161 15054 device (Donaustrasse, Germany). Crude protein contents (CP) were then calculated using the general factor 6.25 (Ben Amira *et al.* 2017), and were expressed as % (w/w) fresh matter (14% humidity). Protein productions (PP) were calculated as:

$$PP\ (kg\ proteins/ha) = (FY * CP)/100$$

Ash contents (AC, % $w.w^{-1}$ fresh matter) were quantified after mineralization in a muffle furnace (Nabertherm controller b180) at 575 ◦C for 4 h (Sluiter *et al.* 2008b). Crude lipid contents (CL, % w w^{-1} of dry matter) were determined after the hydrolysis of milled seeds (60 min in boiling 4 M HCl, solid-liquid

ratio of 1/5), in a Soxtherm equipment (Gerhard, Bonn, Germany), with the soxhlet method using petroleum ether (bp 40–60 ◦C). AC and CL were expressed as % of dry matter.

3.3 Statistical analyses

Univariate and multivariate analyses were performed using Rstudio software® v.1.3.959 and Minitab® v 19.2, to identify the best varieties for food industry. Preliminary descriptions of all varietal characteristics were performed with analyzes of variance (one-way ANOVA, α = 0.05) led on annual averages of CP, PP, AC, CL, HS, SD, EH. Varieties were then grouped with a Tukey *post-hoc* test for each growing year, independently. Then influences exerted by the factor years and variety (and their interaction) on seeds characteristics were assessed with a stepwise linear regression model (α = 0.1) using *Year*, *Variety*, and their interaction as input factors, and using FY, CP, PP, HS, SD, and EH as response factors. Winter and spring varieties were analyzed in two distinct models because: (i) the development of winter and spring faba bean crops occurred under different climatic conditions, due to differences in sowing and harvesting dates (**Table 12**), and (ii) the factor *Variety* was naturally nested in the factor *Type*, which would mask the effect of variety in the regression model (Schielzeth and Nakagawa 2013). The influence of the factor *Type* was assessed for each year separately with a one-way ANOVA test, and a Tukey *post hoc* test led on the annual average of each response factors (*i.e.*, average of FY, TSW, CP, PP, AC, HS, SD, and EH).

Multivariate analyses were performed to consider each quantitative variable combined (including agronomic, composition, and infestation characteristics of seeds) to rank varieties presenting the maximal PP/CP/HS and minimal CL/EH with biplot analyses. All scaled factors werelinearly combined, and Principal Component Analysis (PCA) were performed using the packages "FactoMineR" and "Factoextra" from Rstudio to summarize and visualize all the information of the 14 tested varieties (Kassambara and Mundt 2020; Husson *et al.* 2023). Principal components (PC) were retained according to their eigenvalue (> 1), total variance explained in the dataset (> 70%) (Kaiser

1961), and correlation with variables of interest. Biplot representation of individuals *(i.e., Variety*Year*) was performed, and groups were established according to the factors *Type* and *Year*. Each spring and winter variety was separately ranked according to the Euclidean distance to the point that optimally correspond to required characteristics for the food industry (*i.e.*, the point that maximize PP, CP, and/or HS according to result obtained within our dataset).

4 Results

The 14 commercial faba bean varieties tested over two growing seasons (2018–2019 and 2019–2020) exhibited different agronomic traits, seed composition, and bruchid infestation. Distinct differences were detected between varieties and when comparing certain results over the two years (**Table 10**). Comparison of each studied traits considering average per growing season (*i.e., Year * Type*) is presented in **Appendix E**.

Agronomic characteristics (*i.e.,* FY and TSW) declined from 2019 to 2020 across varieties. Only one variety (*cf.,* Fanfare) was more productive in 2020. The winter varieties performed best in 2019, included Axel, Bumble, Diva, Honey and Nebraska, which produced more than 5000 kg ha^{-1}. However, FY strongly decreased in 2020. The highest relative decline in FY between 2020 and 2019 was 53.2% and 44.8% for LG Cartouche and Honey, respectively. The worst performing varieties were Bobas in 2019 (3740 kg ha^{-1}) and LG cartouche in 2020 (2017 kg ha^{-1}). The greatest decline was observed for the best performing varieties during 2019; for instance, relative TSW decreased by 28.8%, 31.5% and 35.9% for Bumble, Honey and Bering, respectively. Fanfare and Irena had the best TSW stability during 2019 and 2020, with a slight relative increase of 0.2% and 3.1%, respectively.

Seeds composition characteristics (*i.e.,* CP, CL, and AC) varied between 2019 and 2020. CP ranged from 23.4% $w.w^{-1}$ (*cf.*, Diva 2020) to 28.4% $w.w^{-1}$ (*cf.,* Irena 2019) fresh weight. CP*s* were generally lower in 2020 (p-value = 0.000, difference = 1.02%, IC95% = [0.47; 1.56]). CL ranged from 0.88% to 1.76% $w.w^{-1}$

(mean: 1.36 ± 0.17%). LG Cartouche, Julia, and Fanfare had the lowest CL. Ash ranged from 2.19% to 3.55% w.w^{-1} (mean 2.96 ± 0.26%). On average, CL was statistically higher in 2020 (1.43%) compared to 2019 (1.30%) (p-value = 0.002). CL was higher in winter varieties (1.41%) compared to spring varieties (1.28%) (p-value = 0.007). However, this difference was very low when compared with the variability of other factors.

PP ranged from 538.2 kg ha^{-1} (*cf.*, LG Cartouche 2020) to 1418 kg ha^{-1} (*cf.*, Honey 2019). As a consequence of variation in FY and CP, PP also noticeably declined between 2019 and 2020. The most impacted varieties were LG Cartouche, Honey, and Bumble with relative decreases of 56.0%, 48.5% and 47.0%, respectively. These results were attributed to the major impact of FY and minor differences in CP across years.

Seed infestation characteristics (*i.e.*, HS, SD, and EH) strongly varied among and between varieties over the two years, with no clear correlations to seed composition. The mean proportion of healthy seeds (HS) ranged from 12% (*cf.*, Honey 2020) to 81% (*cf.*, Julia 2020). Surface damage (SD) ranged from 4.5% (*cf.*, Julia 2019) to 25.5% (*cf.*, Tundra 2020). For each variety, surface damage (SD) was inferior to emergence holes (EH), with major inter-annual variation being observed. SD increased in most varieties during 2020. Emergence holes (EH) ranged from 11.0% (*cf.*, Julia 2020) to 61.0% (*cf.*, Axel 2020). Overall, all varieties of faba bean had more healthy seeds (HS) in 2019 (61.3 ± 14.0%) compared to 2020 (53.8 ± 23.9%). Spring varieties had more healthy seeds (65.6 ± 8.7% in 2019 and 73.8 ± 7.3% in 2020) compared towinter varieties in both years (58.9 ± 15.8% in 2019 and 42.7 ± 22.7% in 2020).

4.1 Environmental influence on seed characteristics

When comparing the two years of study, quantitative differences were detected, so environment was assessed with pest pressure and climatic conditions (**Table 11**). Bruchid populations were monitored for up to 15 weeks (from early April to mid-July) in both growing seasons. In 2019, infestation started four weeks after flowering, and lasted six weeks (from week 23 to week 28). Overall, 308 adults were caught in generalized winter parcels

(GWP) and generalized spring parcels (GSP). Flowering periods in GWP and GSP occurred respectively from week 19 to 25 and week 22 to 26. In 2020, 288 adults were caught from week 19–30 in GWP and GSP, which bloomed from week 16 to 23 and week 22 to 26. bruchids were present in crops for longer in 2020 than 2019, with the flowering period lasting 8 and 11 weeks, respectively.

Cumulative daily temperature and precipitation of all the cropping periods are presented in **Table 12**. Cumulated temperature was similar in both years throughout the entire cropping period of spring varieties while winter varieties were grown under more cumulated temperatures in 2019 rather than 2020. Cumulated precipitation noticeably differed between 2019 and 2020, especially for spring varieties. Precipitations was below the average for the area in 2019, and was even lower in 2020, declining by 47.3% and 16.9% during the cropping period of spring and winter varieties, respectively.

Environmental observations (bruchids population + climatic condition) indicated that pest pressure (*i.e.,* bruchid population size) was generally constant between the two years, with longer pest presence in 2020 compared to 2019. Additional extreme climatic conditions of drought occurred during the sensitive phases of crop production in 2020 (*i.e.,* transient phase between flower and seed production). Spring varieties were besides affected by the occurrence of *Uromyces fabae* (Pers.) de Bary, 1879 during pods forming period in 2020.

Table 10: Field yield (kg.ha^{-1}, FY), thousand seeds weight (g, TSW), and mean ± standard deviation of seed protein content (% w.w^{-1}, CP), protein production (kg.ha^{-1}, PP), seed ash content (% w.w^{-1}, AC), seed fatty matter content (% w.w^{-1}, CL), mean healthy seeds (%, HS), infested seeds presenting surface damage (%, SD), and infested seeds with emergence holes (%, EH). Per column, numbers with different letters are statistically different (p-value < 0.05)

Year	Variety	Type	FY	TSW	CP	PP	AC	CL	HS	SD	EH
2019	**Augusta**	W	4652	527	26.5 ± 0.5 ^{c}	1234.5 ± 22.3 ^{de}	2.8 ± 0.0 ^{bc}	1.3 ± 0.1 ^{bc}	68.0 ± 14.79 ^{ab}	5.5 ± 3.8 ^{a}	26 ± 13.0 ^{abc}
	Axel	W	5533	500	25.6 ± 0.4 ^{c}	**1414.8 ± 20.7** ^{a}	3.0 ± 0.1 ^{ab}	1.4 ± 0 ^{ab}	42 ± 13.95 ^{b}	15.5 ± 11.4 ^{a}	42 ± 10.2 ^{ab}
	Bering	W	4571	593	26.8 ± 0.2 ^{bc}	1223.2 ± 8.1 ^{de}	**3.1 ± 0.0** ^{a}	1.3 ± 0.0 ^{bc}	52.5 ± 15.44 ^{ab}	7.5 ± 5.5 ^{a}	39 ± 13.5 ^{abc}
	Bumble	W	5264	626	25.8 ± 0.6 ^{c}	1356.4 ± 29.6 ^{ab}	2.7 ± 0.0 ^{c}	1.2 ± 0.1 ^{bc}	56.5 ± 18.79 ^{ab}	14 ± 4.3 ^{a}	28 ± 19.3 ^{abc}
	Diva	W	5250	478	25.7 ± 0.3 ^{c}	1348.7 ± 17.4 ^{ab}	3.0 ± 0.1 ^{ab}	1.3 ± 0.0 ^{b}	**70 ± 2.83** ^{a}	13.5 ± 6.6 ^{a}	16.5 ± 4.4 ^{c}
	Honey	W	5535	613	25.6 ± 0.2 ^{c}	**1418.3 ± 13.1** ^{a}	2.3 ± 0.1 ^{d}	1.0 ± 0.2 ^{c}	60 ± 9.93 ^{ab}	5.5 ± 3.0 ^{a}	33.5 ± 9 ^{abc}
	Irena	W	4731	454	**28.4 ± 0.7** ^{a}	1341.9 ± 35.4 ^{bc}	**3.1 ± 0.0** ^{a}	1.4 ± 0.0 ^{ab}	43 ± 5.77 ^{b}	9.5 ± 6.4 ^{a}	46.5 ± 8.2 ^{a}
	Nebraska	W	5103	447	23.5 ± 0.7 ^{d}	1200.1 ± 37.8 ^{e}	3.0 ± 0.1 ^{ab}	**1.6 ± 0.1** ^{a}	60.5 ± 7 ^{ab}	14.5 ± 8.0 ^{a}	25 ± 11.0 ^{abc}
	Tundra	W	4816	520	26.5 ± 0.6 ^{c}	1277.5 ± 29.4 ^{cd}	3.0 ± 0.1 ^{ab}	1.2 ± 0.1 ^{bc}	**78 ± 13.06** ^{a}	5 ± 1.2 ^{a}	16.5 ± 11.5 ^{c}
	Bobas	S	3740	452	25.8 ± 0.3 ^{c}	964.4 ± 9.8 ^{g}	2.9 ± 0.0 ^{abc}	1.3 ± 0.1 ^{b}	**71.5 ± 9.57** ^{a}	6 ± 7.7 ^{a}	22 ± 3.7 ^{abc}
	Fanfare	S	4120	402	25.4 ± 0.6 ^{c}	1045.2 ± 26.6 ^{f}	2.9 ± 0.0 ^{bc}	1.2 ± 0.2 ^{bc}	61.5 ± 5.26 ^{ab}	6 ± 7.1 ^{a}	30 ± 2.8 ^{abc}
	Julia	S	3892	382	26.1 ± 0.4 ^{c}	1014.4 ± 14.1 ^{fg}	2.9 ± 0.1 ^{bc}	1.2 ± 0.0 ^{bc}	**74 ± 2.31** ^{a}	4.5 ± 3.0 ^{a}	21 ± 3.8 ^{bc}
	LG cartouche	S	4314	469	28.3 ± 0.6 ^{ab}	1218.7 ± 25 ^{de}	3.0 ± 0.1 ^{ab}	1.0 ± 0.0 ^{c}	58 ± 3.27 ^{ab}	6.5 ± 6.6 ^{a}	34.5 ± 8.2 ^{abc}
	Tiffany	S	4101	401	25.3 ± 0.4 ^{c}	1037.1 ± 17.6 ^{f}	3.0 ± 0.1 ^{ab}	1.3 ± 0.0 ^{bc}	63 ± 10 ^{ab}	8.5 ± 6.4 ^{a}	28 ± 5.9 ^{abc}

(Continued)

Table 9 (continued) : Field yield (kg.ha^{-1}, FY), thousand seeds weight (g, TSW), and mean ± standard deviation of seed protein content (% w.w^{-1}, CP), protein production (kg.ha^{-1}, PP), seed ash content (% w.w^{-1}, AC), seed fatty matter content (% w.w^{-1}, CL), mean healthy seeds (%, HS), infested seeds presenting surface damage (%, SD), and infested seeds with emergence holes (%, EH). Per column, numbers with different letters are statistically different (p-value < 0.05)

Year	Variety	Type	FY	TSW	CP	PP	AC	CL	HS	SD	EH
2020	**Augusta**	W	3455	384	25.5 ± 0.8 ^{bcd}	881.3 ± 26.1 ^{d}	2.7 ± 0.1 ^{fg}	**1.2 ± 0.0** ^{a}	60.0 ± 8.49 ^{abc}	10.5 ± 6.61 ^{abc}	29.5 ± 4.43 ^{cd}
	Axel	W	3703	428	25.5 ± 0.3 ^{bcde}	942.4 ± 12.3 ^{bc}	2.9 ± 0.1 ^{efg}	**1.5 ± 0.1** ^{a}	19 ± 8.87 ^{de}	19.5 ±7.19 ^{abc}	61 ± 3.46 ^{a}
	Bering	W	3511	380	25.7 ± 0.6 ^{bcd}	897.5 ± 20.8 ^{cd}	2.9 ± 0.1 ^{efg}	**1.5 ± 0.0** ^{a}	41.5 ± 13.4 ^{bcd}	20 ± 4.32 ^{abc}	38 ± 12 ^{bc}
	Bumble	W	3049	446	23.6 ± 0.6 ^{g}	718.8 ± 17.1 ^{g}	2.8 ± 0.0 ^{fg}	**1.5 ± 0.1** ^{a}	36 ± 7.12 ^{cde}	24 ± 1.633 ^{a}	40 ± 7.66 ^{bc}
	Diva	W	3576	334	23.4 ± 0.6 ^{g}	835.5 ± 22.4 ^{e}	2.9 ± 0.0 ^{ef}	**1.5 ± 0.1** ^{a}	**70 ± 8.16** ^{a}	5.5 ±7.19 ^{c}	24.5 ± 5 ^{cd}
	Honey	W	3058	420	23.9 ± 0.6 ^{fg}	729.8 ± 18.5 ^{g}	2.8 ± 0.0 ^{fg}	**1.5 ± 0.1** ^{a}	12 ± 9.93 ^{e}	23.5 ± 9.15 ^{ab}	65 ± 4.76 ^{a}
	Irena	W	3552	468	**27.0 ± 0.2** ^{a}	960.1 ± 7.7 ^{b}	3.1 ± 0.1 ^{cd}	**1.1 ± 0.0** ^{a}	37 ± 18.8 ^{cde}	11 ± 7.75 ^{abc}	52 ± 12.96 ^{ab}
	Nebraska	W	3776	377	24.2 ± 0.3 ^{defg}	916.2 ± 10.2 ^{bcd}	2.9 ± 0.0 ^{def}	**1.5 ± 0.1** ^{a}	**71.5 ± 5** ^{a}	5.5 ± 3.79 ^{c}	22.5 ± 4.12 ^{cd}
	Tundra	W	3724	402	23.9 ± 0.2 ^{fg}	889.5 ± 6.6 ^{d}	3.1 ± 0.0 ^{cde}	**1.5 ± 0.1** ^{a}	37 ± 20.8 ^{cde}	25.5 ± 11.82 ^{a}	37.5 ± 14.08 ^{bc}
	Bobas	S	3517	410	25.2 ± 0.3 ^{cdef}	886.3 ± 11.6 ^{d}	3.3 ± 0.0 ^{bc}	1.5 ± 0.0 ^{bc}	72.5 ± 3.79 ^{a}	6 ± 2.83 ^{c}	21.5 ± 4.12 ^{cd}
	Fanfare	S	4420	403	25.8 ± 0.2 ^{abc}	**1141.4 ± 9.02** ^{a}	2.7 ± 0.1 ^{g}	**1.3 ± 0.1** ^{a}	**75.5 ± 1** ^{a}	7 ± 3.83 ^{c}	17.5 ± 4.43 ^{d}
	Julia	S	2679	338	26.6 ± 0.5 ^{ab}	713.3 ± 12.8 ^{g}	**3.5 ± 0.0** ^{a}	**1.3 ± 0.0** ^{a}	**81 ± 5.29** ^{a}	8 ± 6.93 ^{bc}	11 ± 9.59 ^{d}
	LG cartouche	S	2017	415	26.7 ± 0.3 ^{ab}	538.2 ± 11.18 ^{h}	3.4 ± 0.0 ^{ab}	1.3 ± 0.1 ^{ab}	64.5 ± 6.81 ^{ab}	6.5 ± 3 ^{c}	29 ± 4.76 ^{cd}
	Tiffany	S	3267	338	24.1 ± 0.3 ^{efg}	786.4 ± 8.8 ^{f}	**3.5 ± 0.1** ^{a}	**1.5 ± 0.0** ^{a}	**75.5 ± 7.55** ^{a}	5.5 ± 3 ^{d}	17.5 ± 6.61 ^{d}

Table 11: Climatic condition and faba bean phenology associated with bruchid population dynamics. Climatic conditions were quantified from the average temperature (Tavg, °C) and cumulated precipitation (Pcum, mm) per week. Bruchid populations were quantified from the the number of individuals caught in generalized winter parcels (GWP) and generalized spring parcels (GSP) in parallel to faba bean phenology (F = flowering, P = Pods setting).

	2019					2020				
Week n°	**Tavg (°C)**	**Pcum (mm)**	**Phenology GWP**	**Phenology GSP**	***Bruchus spp.* (GWP+GSP)**	**Tavg (°C)**	**Pcum (mm)**	**Phenology GWP**	**Phenology GSP**	***Bruchus spp.* (GWP+GSP)**
16	13.2 ± 3.16	0.1				10.39 ± 3.06	4.8	F		
17	12.43 ± 3.53	7.7				12.6 ± 1.94	0	F		
18	8.24 ± 2.35	19.6				11.14 ± 1.69	11.2	F		
19	9.37 ± 1.91	17.2	F			13.2 ± 2.76	2.3	F+P		1 (1 + 0)
20	11.34 ± 1.49	19.6	F			8.93 ± 1.84	0	F+P		3 (3 + 0)
21	13.57 ± 1.81	26.8	F+P			16.06 ± 2.29	1.8	F+P		2 (2 + 0)
22	16.17 ± 3.96	8.7	F+P	F		16.1 ± 0.59	0	F+P	F	78 (78 + 0)
23	14.96 ± 2.25	26.1	F+P	F	15 (15 + 0)	14.63 ± 3.75	21.3	F+P	F	5 (5 + 0)
24	15.29 ± 1.40	33.7	F+P	F+P	113 (87 + 26)	15.14 ± 2.73	9.7	P	F+P	6 (6 + 0)
25	18.26 ± 2.45	10.3	F+P	F+P	97 (57 + 40)	17.14 ± 1.05	30	P	F+P	29 (24 + 5)
26	21.8 ± 2.75	0	P	F+P	53 (21 + 32)	20.24 ± 2.62	7	P	F+P	28 (20 + 8)
27	16.87 ± 1.53	0.4	P	P	28 (2 + 26)	16.69 ± 0.92	15.5		P	58 (26 + 32)
28	15.99 ± 2.13	3.7	P	P	2 (0 + 2)	15.73 ± 1.76	10		P	61 (5 + 56)
29	17.59 ± 2.36	0.4		P		16.89 ± 1.70	10		P	12 (0 + 12)
30	23.61 ± 4.80	22.2		P		17.29 ± 1.69	12.9		P	5 (0 + 5)
tot	**228.69**	**196.5**			**308 (182+126)**	**222.17**	**136.5**			**288 (170 + 118)**

Table 12: Climatic conditions from sowing to harvesting of spring and winter faba bean varieties during the two years of the study (cumulated daily average temperature, Tcum; cumulated daily precipitation, Pcum)

	Winter varieties				**Spring varieties**			
	Sowing date	**Harvesting date**	**Tcum (°C)**	**Pcum (mm)**	**Sowing date**	**Harvesting date**	**Tcum (°C)**	**Pcum (mm)**
2018-2019	**08/11/2018**	**12/08/2019**	**2,703.9**	**551,4**	**28/03/2019**	**22/08/2019**	**2,179.7**	**289,9**
2019-2020	**21/11/2019**	**30/07/2020**	**2,566.0**	**458,3**	**09/04/2020**	**20/08/2020**	**2,183.5**	**152,6**

Multivariate linear modeling (stepwise regression analyses) was separately performed for spring and winter varieties to identify the respective influence of input factors *Year*, *Variety*, and their respective interactions on response factors FY, CP, PP, HS, SD and EH (**Table 13)**. The interaction factor was not determined as significant in any studied model.

For winter varieties, *Year* (84.61%) contributed more compared to *Variety* (7.86%) in the FY model (R^2adj = 0.90). The CP model was less explanatory

(R^2adj = 0.78), with *Variety* contributing strongly (47.24% compared to 36.35% for *Year*). This model had the highest error contribution (16.42%). *Year* variance (88.50%) contributed the most to the PP model, and had the lowest error contribution (5.77%). This last model was strongly impacted by FY data, which explained their high similarity.

Table 13: Regression analysis modeling the contributions of the factors Year, Variety (and their interactions) on FY, CP, PP, HS, SD, and EH. NS = not selected by the model

		FY		CP		PP		HS		SD		EH	
		p-value	Contribution (%)	p-value	Contribution (%)	p-value	Contribution (%)	p-value	Contribution (%)	p-value	Contribution (%)	p-value	Contribution (%)
Winter	**Year**	<0.001	84.61	<0.001	36.35	<0.001	88.50	<0.001	14.92	*NS*	*NS*	0.002	8.54
	Variety	0.005	7.86	<0.001	47.24	0.006	5.77	<0.001	44.86	*NS*	*NS*	<0.001	55.58
	Error (%)		7.54		16.42		5.73		40.22		*NS*		35.89
	Adjusted R^2		**0.9004**		**0.7831**		**0.9243**		0.5073		*NS*		0.5604
Spring	**Year**	0.005	35.00	<0.001	15.17	0.003	37.68	0.012	20.65	*NS*	*NS*	0.013	20.68
	Variety	*NS*	*NS*	0.009	64.57	*NS*	*NS*	0.022	31.20	*NS*	*NS*	0.022	31.21
	Error (%)		65.00		20.26		62.32		48.14		*NS*		48.20
	Adjusted R^2		0.3158		0.7299		0.3440		0.4091		*NS*		0.4085

Variety was the highest contributing factor to infestation factors (44.86% and 55.58% for HS and EH, respectively). Annual differences exhibited significant, but lower, contributions to models based on the evaluated parameters and related interactions. No model could be produced for SD, as the inadequacy of the model was significant (p-value = 0.013).

Models based on the spring varieties were of lower quality compared to the winter varieties. The highest R2adj was 0.73 (CP model), in which *Variety* contributed the most (64.57%), as obtained for the winter varieties. FY and PP models (R^2adj of 0.32 and 0.34, respectively) differed to those of the winter, as *Year* was the sole contributing factor (< 37.7%), leading to a high error contribution (> 62.3%). The infestation-related models for spring varieties were of poorer quality. The highest contributions were related to *Variety* (31.2%), but this contribution was lower compared to that for winter varieties. The contribution of *Year* was higher for spring varieties, accounting for 20.7% of variance. As with the winter varieties, no SD model could be produced. The

lower quality of the spring varieties models may be explained, at least in part, by the lower number of studied varieties in this group (5 compared to 9 winter varieties).

Overall, these models indicated that CP and seed infestation parameters were more impacted by *Variety*, whereas FY and PP were more related to environmental conditions. Differences were also observed between spring and winter types in terms of factors implicated in the models, their individual contributions to the models, and so model quality.

4.2 Grouping and ranking of varieties for the food industry

Three first principal components (PC) computed from the linear combination of scaled factors explained more than 80% of total variance combined (**Figure 25 - a**) with respective PC1, PC2 and PC3 explained variances of 40.5%, 27.9%, and 15.1%. The contribution of each factor to all computed PCs is presented in **Appendix E**. The two first PCs (*i.e.,* Dim1 and Dim2) presented a biplot representation of each pair (*i.e., Variety* * *Year*), with PC1 being strongly correlated with PP, FY, and TSW, and PC2 being strongly correlated with HS and EH (**Figure 25 - b**). These two axes clearly separated the varieties with respect to protein production and bruchid infestation rate (*i.e.,* lower right region of **Figure 25 - c** containing optimal varieties). Other biplots for PC1–PC3 and PC2–PC3 did not present any additional explanatory information.

Groupings of each cultural cycle (*cf., Type* × *Year*) are presented in **Figure 26**. The influence of *Year* and *Type* on seed characteristics was visualized, extending stepwise regression modeling. The differentiation winter and spring varieties according to the year of study are more pronounced along PC1 illustrating the general decrease of the productivity-related factors (*i.e.,* FY and PP). In comparison, differentiation of years of study according the type of faba beans was more pronounced along PC2, showing that winter varieties were more impacted by bruchids compared to spring varieties. This result supported that winter varieties could achieve higher FY compared to spring varieties.

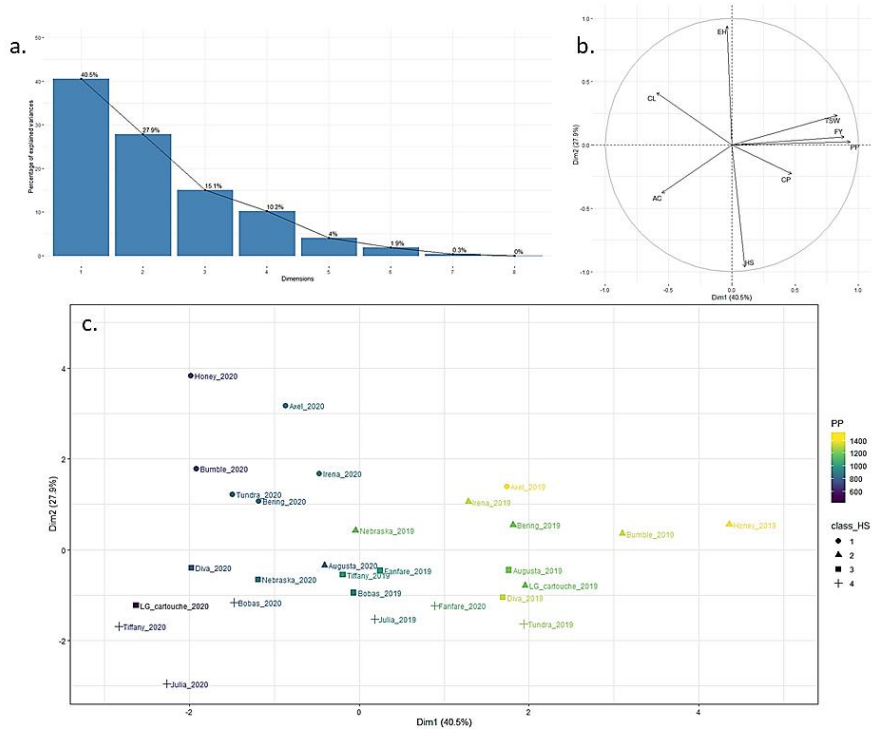

Figure 25: Principal component analysis (PCA) results: a. Scree plot indicating the percentage of explained variance with each PCs; b. Correlation circle indicating the contribution of each factor with PC1 and PC2; c. Biplot representation of individuals according to Dim 1. (PC1) and Dim. 2 (PC2). Groupings were made according to quartiles of HS (1 to fourth quartile) and protein production (kg ha^{-1}).

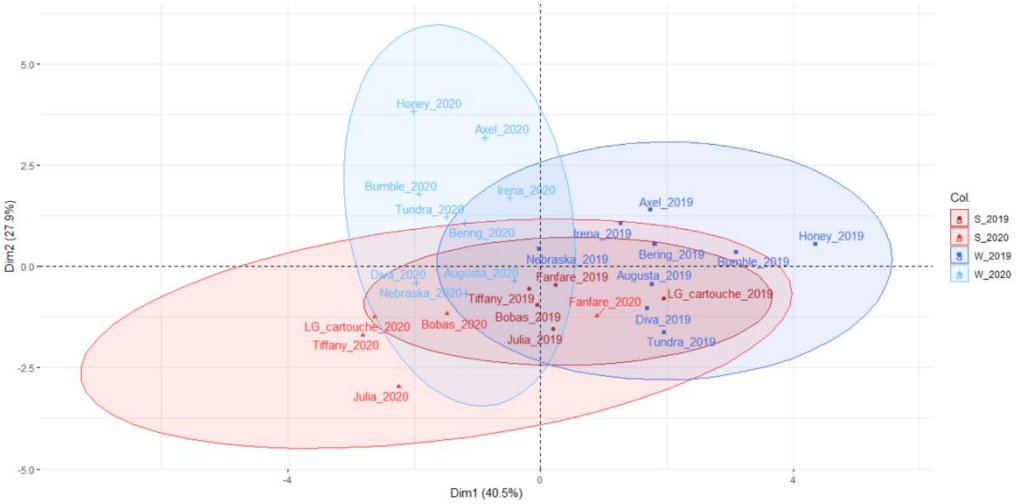

Figure 26: Individual groups according to the year of study and type of faba bean (W = winter varieties; S = spring varieties).

Based on these groups, separated means comparisons (on the factor *Type*) were performed (**Table 14**). Winter varieties had higher FY in 2019 (difference = 1017 kg ha^{-1}, CI95% = 814; 1220) compared to spring varieties, but differences were minimal in 2020. The major difference in FY between spring and winter varieties led to a major difference in PP in 2020 (256.8 kg ha^{-1}, CI95% = 201.6; 312.0). In the same year, spring varieties had higher CP (difference = 0.958%, CI95% = 1.730; 0.186), highlighting the strong influence of annual variation in FY on PP.

Table 14: One-way ANOVA p-value comparing the significance of the Type parameters in 2019 and 2020 on CP, FY, and PP. ° = p-value < 0.1, * = p-value < 0.5, *** = p-value < 0.001

	CP (% w/w)	**FY (kg/ha)**	**PP (kg/ha)**	**HS (%)**	**SD (%)**	**EH (%)**
2018-2019	0.785	<0.001***	<0.001***	0.088	0.044*	0.355
2019-2020	0.016*	0.081°	0.271	<0.001***	<0.001***	<0.001***

The optimal varieties for the food industry (*i.e.,* producing high and stable amounts of protein, and low bruchid infestation) were located in the lower right region of the biplot (**Figure 25 - c)**. The point that maximized these factors (named "optimal point") corresponded to biplot coordinates (5; - 3). Each spring and winter variety was ranked according to mean Euclidean distance computed from this point in the two years (**Figure 27**). Varietal Euclidean distances ranged from 4.93 ± 0.65 (*cf.,* Fanfare) to 6.99 ± 2.16 (*cf.,* Axel). While no statistical differences were found, varieties were ranked according to the characteristics of interest for food industries. The most stable varieties over the two years had the lowest standard deviations, as observed for Fanfare.

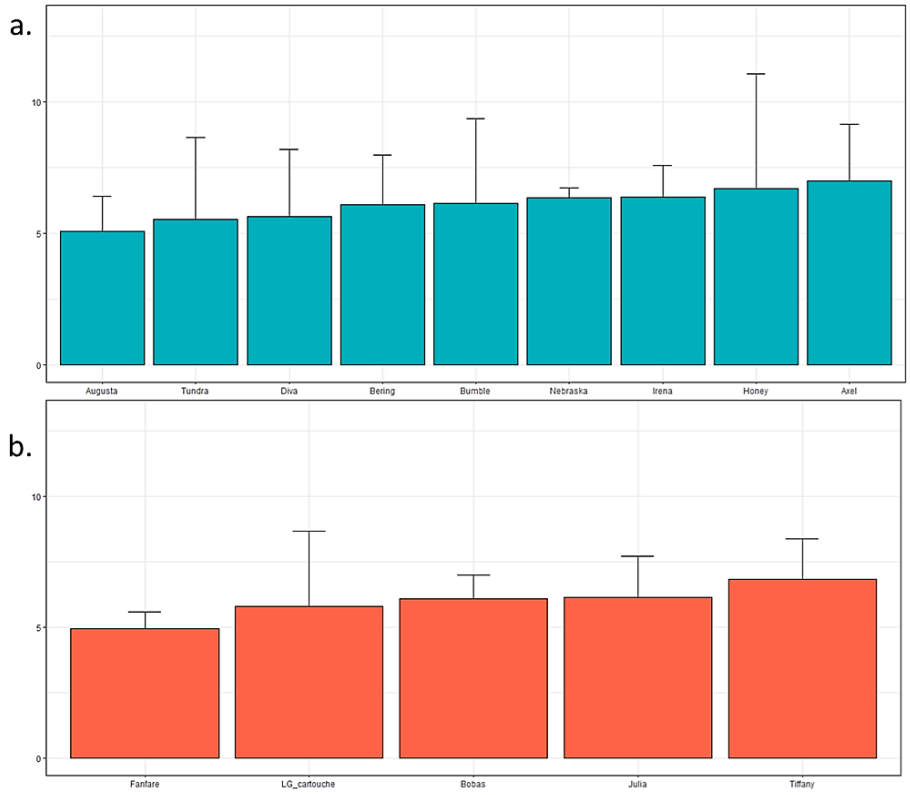

Figure 27 : Varietal ranking according to mean Euclidean distances computed between each individual and optimal point from the graph of individuals (*i.e.*, coordinates [5; -3]). (a) Winter varieties. (b) Spring varieties

Individually, some varieties demonstrated high performance. For example, in 2019, the varieties Honey had the highest FY/PP, while this cultivar was the worst performing one during 2020. Mean Euclidian distances may hide such extreme results, which are more expressed by important standard deviations. Equally, Tundra 2019 seems to be the closer sample to the hypothetical ideal point (5; -3). However, its overall quality was diminished when taken into account the performance of this variety in 2020. These highlights once more the level of annual differences demonstrated in previous analysis and underlines the need to consider overall performances (including bruchids infestation and PP) on several years of test.

5 Discussion

It is important to promote indigenous leguminous crops with high PPs to support European cropping systems; however, biotic and abiotic factors cause variable crop quality and quantity in food industry production. Here, we screened the quality (*i.e.,* bruchids resistance) and quantity (*i.e.,* protein production) of 14 commercial faba bean cultivars (including five spring varieties and nine winter varieties) over two years with distinct climatic conditions. Multivariate analyses showed that meteorological conditions (*Year*) strongly influenced FY and PP, whereas *Variety* strongly influenced CP and infestation rates. Biplot analysis discriminated (though not significantly) the most promising cultivars with respect to seeds quality and quantity. Our results provide preliminary information on field infestations by *Bruchus spp*. in the Walloon region (Belgium), and a methodological baseline is proposed for the characterization of varieties based on productivity, bruchid resistance and seeds composition.

5.1 *Bruchus* spp. infestation in relation to faba bean variety

Bruchid infestation of faba bean crops has been described for several countries, along with assessments of resistance mechanisms in different varieties (Boughdad 1994; Szafirowska 2012; Medjdoub-Bensaad *et al.* 2015; Seidenglanz and Hunady 2016; Carrillo-Perdomo *et al.* 2019; Pölitz and Reike 2019). Bruchid populations occur in regions where faba beans are harvested as dry seeds, allowing the completion of the life cycle and rise in pest populations during the following growing season (Ward 2018). Adults overwinter in wooded sites, and start colonizing fields when temperatures reach 15 °C, before or during the crop flowering period. This pattern was observed in Belgium, in an area presenting high infestation rates of *Bruchus* spp. (**Table 11**). Field infestation started when crops were flowering and mean temperatures were about 15 °C. The bruchid population peaked at week 24 (*cf.*, year 2019) and week 22 (*cf.*, year 2020), supporting observations made in neighboring countries, like Germany (2018) where infestation peaked in week 25 (Pölitz and Reike 2019).

Pest pressure (*i.e.,* total amount of individuals recorded during monitoring sessions) was stronger in 2019 (308 individuals) compared to 2020 (288 individuals), with bruchids remaining in crops longer during 2020 compared to 2019 (12 weeks *vs.* 7 weeks respectively), leading to greater subsequent damage to harvested seeds in 2020. This phenomenon could be explained by the longer flowering period observed in 2020, which provided food supplies and extended the egg laying period, as suggested by Seidenglanz and Hunady (2016). Pest pressure was stronger on winter varieties during both years, resulting in greater subsequent damage to seeds. This phenomenon was also reported by Seidenglanz and Hunady (2016), who suggested that earlier flowering is a stronger driver when early and late flowering varieties are grown together. Early flowering varieties attract females earlier and oviposition lasts longer, as food supply is extended by late flowering varieties, leading to greater subsequent damage. This hypothesis can now be confirmed by the monitoring of pest populations proving that bruchids populations remained longer and in greater numbers on winter varieties. However, such differences of infestation could not be observed among varieties from winter or spring type, *i.e.,* no clear relation could be established concerning the precocity-duration of flowering (**Appendix E**) and the subsequent damages observed.

Although spring varieties were less impacted by bruchids (lower infestation) in our study, these varieties did not necessarily produce seeds of better quality and quantity for the food industry. In our field trials, winter and spring varieties were grown side by side, with the delayed phenology of spring varieties impacting earlier flowering winter varieties. However, when spring and winter varieties are grown in distinct geographical locations, winter varieties flower earlier during the cooler and rainier periods. Thus reducing the risk of bruchid infestation, which is favored by high temperatures and sunny climate (Szafirowska 2012; Simmen 2020). Winter varieties tending to have an early and short flowering period, are more recommendable compared to spring varieties.

Damage clearly varied across the 14 varieties. During both years, the most resistant commercial cultivars (with > 70% of non-infested seeds) were Julia, Bobas, and Diva. In contrast, Axel, Honey, and Irena had < 40% of non-infested seeds. Regression models showed that the most important factor influencing variation was *Variety* (44.86% for winter varieties and 31.20% for spring varieties), followed by *Year* (14.92% for winter varieties and 20.65% for spring varieties). This result supports recent studies showing that the infestation rates of cultivars are driven by different potential resistance mechanisms, such as antibiosis and antixenosis, which develop at different stages of the pest life cycle. The attractiveness of plants to adult bruchids may vary depending on odor profiles for feeding and oviposition (Ferry *et al.* 2004; Carrillo-Perdomo *et al.* 2019). The composition of epicuticular wax on faba plant pods might also influence the acceptance of oviposition substrate by gravid females. Zhao *et al.* (2019) showed that the profiles of epicuticular wax on *V. faba* pods vary with cultivars. Furthermore, the fatty acid profile of epicuticular wax impacted the oviposition preference of other species of bruchids (*i.e., Callosobruchus maculatus* (Fabricius 1775)). Higher relative quantities of oleic acid tend to reduce oviposition (Parr *et al.* 1998). Plants might also have resistance mechanisms to penetration by hatching larvae, such as physical barriers (*e.g.*, trichrome of pods) and the production of biochemicals, like lectins, polyphenols, proteinase inhibitors, and alpha amylases inhibitors (Mitchell *et al.* 2016; Mishra *et al.* 2018). Doss *et al.* (2000) provided evidence of plant induced defenses against *B. pisorum* by the neoplasmic growth of pod tissues caused by lipid compounds (named "*bruchins*"), which prevented larvae from penetrating pods. The present study explored post-embryonic resistance mechanisms by analyzing seed damage, rather elucidating the mechanisms that influence the oviposition of adults. Our results did not provide insights on resistance mechanisms of faba to the development of larvae. This is because larval mortality (SD) was considered less important than the emergence of adults (EH). SD also strongly varied, preventing us from drawing any statistical conclusions. These results support the observations of Seidenglanz and Hunady (2016), who found that

polyphenol content (*i.e.,* tannins, vicine, and convicine content) did not affect the mortality of *B. rufimanus*. However, Lattanzio *et al.* (2005) showed that *C. maculatus* mortality was linked to the polyphenol content, while Carrillo-Perdomo *et al.* (2019) identified an accession of *V. faba* (Quasar) that had superior SD to EH. Thus, future studies should focus on identifying the presence of biochemicals other than tannins, vicine, and convicine to elucidate resistance mechanisms in *V. faba* cultivars. Differences to HS among the 14 tested cultivars in the current study should be based on oviposition preferences rather than detrimental biochemicals for larval development.

5.2 Field yield and protein production

The FY (mass of grain harvested per hectare) of faba beans varies considerably in the world, ranging from 2.3 to 6.8 t ha^{-1}. Maximal theoretical yield was estimated as high as 6.56 t ha^{-1} or 7.7 t ha^{-1}, respectively under Mediterranean conditions with irrigation or temperate conditions (Mínguez and Rubiales 2021). In Belgium, mean yields of 4 t ha^{-1} were obtained for *V. faba* between 2014 and 2019. These yields increased after 2017, with maximal yields of 4.9 t ha^{-1} in 2019. Faba yields in Belgian are part of the most performing ones compared to other countries in Europe and globally, which have yields of 3 t ha^{-1} and 2 t ha^{-1}, respectively (FAOSTAT, 2021). The current study obtained highly variable FY (3.8–5.5 t ha^{-1}), but remained consistent with mean yields in Belgium, despite both years having lower precipitation than usual (798 mm and 731 mm in each year, respectively (IRM, 2021). Axel, Bumble, Diva, Honey, and Nebraska had the highest FY. However, slight changes to climatic conditions in the two years resulted in lower FY. Fanfare had the highest PP stability under water limited environments, and should be promoted for use due to its tolerance to low precipitation.

Stepwise regression analyses (**Table 13**) showed that *Year* (summarizing meteorological changes) strongly affected FY and PP, particularly for winter varieties. This phenomenon might be attributed to the flowering and grain filling physiology of faba beans being strongly influenced by environmental conditions. The flowering and grain filling stages (*i.e.,* key growing stages

influencing yield) relies on several environmental and intra-plant factors (Patrick and Stoddard 2010). On average, just 24.0% of produced ovules will develop mature seeds (Rowlands 1960). This lack of flowers retention is caused by inefficient fertilization and pollination, which occur when flowers are 6-days old only (Stoddard 1986; Stoddard and Bond 1987). Flower retention is highly sensitive to transient stresses, such as drought or high temperatures, which make flowers abort (Khan *et al.* 2010). Intra-plant competition between reproductive structures and vegetative/reproductive structures for assimilate availability also hinders the retention (Jaquiery and Keller 1980). Consequently, climatic conditions and transient stresses during the fertilization of flowers and subsequent seed formation strongly affect yield. In the current study, pollinator activity was consistent in both years (unpublished field observations), whereas climatic conditions (particularly rainfall) distinctly differed at the onset of flowering, with dryer conditions occurring in 2020 compared to 2019. During 2019, winter and spring faba beans received 36.8 mm and 34.8 mm rainfall, respectively, during the first two weeks of flowering before pod formation. In 2020, rainfall did not exceed 4.8 mm during the flowering period of winter faba bean crops, which probably contributed to the loss of the first flowers that were produced (*i.e.*, pod formation was delayed by three weeks after flowering). In comparison, the spring varieties received 21.3 mm rainfall during the second week of flowering. This suggests that drought was more impacting on FY than temperatures as temperatures were quite similar. Early flowering varieties would therefore be preferable as it would delay the sensitive phase of flower fertilization and seed formations with risk of drought in Belgium and should contribute to the field yield (Duc 1997; Link *et al.* 2010; Korsvold 2020). Besides, cool temperatures of the early growing season in Belgium (and other North-Western European countries) will slightly affect FYs of faba beans as it is considered as part of the "cool-season species'' (Mínguez and Rubiales, 2021) on one hand, and it should delay the pod formation period with the period of bruchids presence on the other hand.

The favorable precipitation conditions (1078 mm in 2002, a typical year) found in Belgium during the flowering and grain filling stages promotes the cultivation of faba with high PPs (FAOSTAT, 2021). The frequency and intensity of extreme events (such as heat waves) are predicted to increase during spring-summer in future years in Belgium. Winter precipitation is also projected to rise over the 21st century, whereas summer precipitation is projected to decline (Hoyaux *et al.* 2010). Thus, it would be necessary to adapt existing faba bean varieties or develop new ones that correspond to changing climatic conditions in Belgium and Europe, in general, to maintain optimal production. The genes involved in the early initiation of flowering (genes *E*), response to vernalization (gene *HR*), and late flowering (gene *LF*) have already been identified for the pea, but not yet faba beans. Thus, these genes need to be identified to allow breeders to develop cultivars that are better adapted to stressful environments (*i.e.,* with early and short duration flowering periods) (Patrick and Stoddard 2010).

The wide variety of faba bean cultivars available on the market in many countries makes difficult the comparison of results across studies, as no single cultivar has been defined as a general control over the years. Only a few varieties that were evaluated here have also been assessed in previous papers, such as "Fanfare." Compared to the current study, this variety had higher yields (5 t ha^{-1}) with lower variability in Denmark and Finland (Skovbjerg *et al.* 2020). The PP recorded in our study during 2019 was higher compared to previous studies, whereas the PP recorded in 2020 was comparable, and even higher (Stoltz *et al.* 2013; Micek *et al.* 2015; Reckling *et al.* 2018; Barłóg *et al.* 2019) Even though the varieties that performed well in 2020 did not reach the level of production obtained in 2019, they still matched or exceeded the PPs listed in the literature over past decades. Higher PP was only obtained in a study conducted in Serbia, demonstrating that this parameter can be enhanced in specific locations (Mihailović *et al.* 2010; Mínguez and Rubiales 2021).

5.3 Seed composition

CP ranged from 23.4% to 28.4% ($w.w^{-1}$) of fresh seed weight (*cf.*, 14.0% HR), supporting the findings of Sharan *et al.*(2021). Regression analysis confirmed that year had a low impact on CP (1% $w.w^{-1}$ difference in seeds between 2019 and 2020; **Table 13)**, contributing to 36.35% and 15.17% variability in winter and spring varieties, respectively. In contrast, *Variety* strongly impacted seed composition (61.79% of variability in CP), reaffirming the need for research to optimize varieties. As protein function is minimally impacted by changes of varieties (Singhal *et al.* 2016), new varieties should be developed that enhance CP, in parallel to improving yield and resistance to infestation. The CP of winter and spring varieties differed. The CP of winter varieties was 24.3% higher compared to that of spring varieties (**Table 14)**. Thus, winter varieties should be developed in Belgium and similar temperate countries, due to their lower sensitivity to drought and higher or equivalent field yields compared to spring-sown varieties (Neugschwandtner *et al.* 2015, 2019).

To improve existing varieties, it is necessary to develop new genetic resources by more sequencing (Khazaei *et al.* 2020). This is highlighted by the interesting results of the 14 varieties investigated in the present study; yet, only some have been referenced in the literature, and no genetic information is available. Various studies have sought to enhance the CP of faba bean genetically. Proteins found in the cotyledons are mainly non-enzymatically active, and serve as nutrients for the future embryo. Storage proteins belong to multiple families, including globulins (major family) and albumins. Globulins are separated in two groups with different functions, depending on their sedimentation coefficients: legumins-types (11S) and viciline-types (7S) (Warsame *et al.* 2018). The legumin/vicilin ratio affects certain properties of faba bean when developing end-products for consumers (Singhal *et al.*, 2016). Higher legumin content is preferred to improve protein function; thus, their accumulation was investigated. Legumins are stored at the end of protein storage in seeds, after vicilin. Therefore, the legumin/vicilin ratio is initially impacted by pedoclimatic conditions. However, different genes are involved in producing these two types of proteins. Improved genotypes can be used to

enhance legumin storage in the protein bodies of the cotyledon. The nutritional value of faba bean can be enhanced by modifying genes and increasing the repetition of coding for different protein types and amino acid profiles. Increasing sulfur-containing amino acids and tryptophan content could increase the nutritional value of the faba beans (Singh *et al.* 2012; Warsame *et al.* 2018). Studies have begun to map the faba bean genome, and to link genes to phenotypes; however, further research is needed to obtain a complete understanding (Cooper *et al.* 2017)). Databases must be constructed to gather genetic information on different faba bean varieties, which could then be used to guide genetic research to enhance FY, CP, and *Bruchus* resistance.

In our study, most varieties had equivalent ash content, except for Bumble, Augusta, Fanfare, and Honey, which had lower ash content. The overall obtained ash content was in the lower range of that presented in published literature (Gasim *et al.* 2015), indicating that mineral intake during ingestion by humans would be low. Legumes usually have high mineral content, even though their bioavailability is not always favorable. The presence of anti-nutritional factors (such as phytic acid) in faba bean lowers the availability of minerals for assimilation in the digestive tract (Zhang *et al.* 2020). CLs were very low and did not vary enough to be used as a discriminating factor between varieties or types. The very low lipid content meant that it would not present any issues when faba bean seeds are transformed to processed food or feed.

5.4 Biplot analysis

The strong influence of *Year* on FY in the regression analysis was the consequence of extreme climatic conditions encountered during the second year of study. No correlation was observed between infestation factors and FY (data not shown). This phenomenon might be attributed to (i) the climatic requirements of pests for development and reproduction being fulfilled in both years of study; (ii) pest pressure being similar in 2019 and 2020, and (iii) quantitatively low of seed weight caused by bruchids (from 5.0% to 10.0% of

dry seed weight). This weak quantitative loss was due to the development of endophytic pests occurring in forming seeds, and so did not impact the physiology and yield of host plants (Boughdad and Lauge 1995; Shearman *et al.* 2005; Titouhi *et al.* 2015; Roubinet 2016; Chapelin-Viscardi *et al.* 2019). Such losses became insignificant when considering variation in FY caused by extreme climatic conditions. Unlike previous studies, we obtained no correlation between FY and CP (Skovbjerg *et al.* 2020). Separate analysis for spring and winter varieties did not reveal any partial correlation, with an FY-CP correlation of 0.17 and 0.37, respectively.

PCA (**Figure 25**) and individual biplots (*i.e., Variety*Year*) on PC1 and PC2 provided information on the contributing characteristics (**Figure 25 and Figure 26**). For instance, infestation characteristics (HS and EH) were independent of productivity characteristics, and were strongly correlated with PC1 and PC2 (**Figure 25 - b**). Moreover, these first two PCs present eigenvalues > 1 (**Appendix F**), meaning they account for more variance than accounted by each individual variable from standardized data. The two first components were retained because the third computed PC (which was also correlated with productivity characteristics) could not provide a more efficient and precise separation of individuals. Despite the quality of this representation, a limitation of this study relies in the gathering of all observation by their annual means for each variety which may induce a loss of information. However, this still indicates global trends considering all seeds characteristics, and this allow a better visualization of the influence exerted by the years of study (*cf.*, the climate that occurred during growing seasons) and the type of varieties. Future studies should consider the same number of replicates in order to represent all individual observations in the biplot space, which would allow a better characterization of the varietal stabilities in different environment.

These biplots were particularly useful while grouping varieties according to year of study and the type of faba bean, visually demonstrating the greater productivity and infestation of winter faba beans and influence of climate on

all tested varieties. This approach allowed us to visualize stable varieties in relation to environmental influences, and to classify varieties based on the productivity and quality of seeds. The ranking of varieties based on Euclidean distances led to more nuanced results than by considering seed infestation and protein production separately. Best performing varieties seemed to be Fanfare, Augusta and Tundra, instead of Julia, Bobas and Diva according to bruchid resistances, or Axel, Irena and Fanfare according to protein productivities. The variability of Euclidean distances is essential in the characterization of varietal stabilities which are not expressed by the averages. This was typically the case of variety Honey that presented best performing PP in 2019 and worst performing PP in 2020. In this study, we highlight yield stability in water limited environment (*cf.,* climatic condition of year 2020) by computing distances from an optimal point based on the variation of our dataset which does not suggest quantitative prerequisites. Each experiment performed under these conditions will have its own point of interest depending on the data set itself.

Fanfare was slightly better ranked than Nebraska (difference of 0.14 in mean Euclidean distances) but Fanfare was the most stable variety with a lower standard deviation. As water limitation is considered to be the most important environmental constraint to crop productivity, and is expected to increase in both frequency and intensity (Khan *et al.* 2010), Fanfare was the best adapted cultivar for the quality, quantity, and stability of seed production in Belgium (out of the 14 tested cultivars). However, infestation rates still exceeded export quality standards for human consumption. Despite this, valorization opportunities may arise for fractioning processes for food industries where visual quality standards are expected to be less constraining (Traore and Simmen 2021). Following clarification on the safety concerns of seed infestation before seed fractioning, the method of ranking varieties represents a promising direction for future characterizations.

6 Conclusion

This study investigated the ability of 14 faba bean varieties to meet the standards required for use in the food industry. Deciding factors included bruchid infestation rate, seed composition, field yield, and protein production. To study year-dependent variations, the 14 varieties were studied over 2 years and were mainly impacted by climatic conditions variation. Variation in infestation rate was strongly associated with faba bean cultivar. The best performing cultivars were Julia, Bobas, and Diva. Damage analyses excluded post embryonic defense mechanisms, and suggest probable oviposition preferences of bruchids on the 14 tested cultivars. Because damage differed between winter and spring varieties, future cultivars should have early and short flowering periods. Our results on field yield and protein production confirmed *V. faba* as a promising crop for local protein production. However, environmental conditions exerted a much stronger influence compared to the effect of variety. Winter varieties were generally more productive, and should be preferentially selected over spring varieties. However, only five spring varieties were compared to nine winter ones. More varieties need to be compared to confirm our findings. Different levels of stability in yield were observed under water limited environments; thus, stable varieties, such as Fanfare, should be preferentially selected over high yield performing but less stable varieties, such as Honey. This study proposed a new tool for ranking varieties by considering multiple characteristics of interest for food industries. PCA showed little to no correlation between infestation rate and productivity parameters; thus, these characteristics could be improved separately. This knowledge is of great importance for breeders, as they could implement complementary improvements on both bruchid resistance and seed productivity, with no anticipated co-effects. This tool could be improved by adding complementary factors related to other pests and disease resistance, seed processability, protein quality (*e.g.*, amino acid profile, anti-nutritional factors content, digestibility), and protein functionality (*e.g.*, ability to form foam, emulsion, dough). This study also highlighted the importance of developing varieties that fit current and future European climatic conditions.

Based on the presented results, future studies should focus on yield stability over years and the resistance of varieties to bruchids, with Fanfare being promising. However, the capacity for genetic improvement should be investigated for winter varieties, in terms of early flowering and seed filling stages, which enhance field yield (*cf.*, quantity), as well as bruchid resistances and protein composition (*cf.*, quality). Such advances could potentially supply cropping systems in Belgium and other countries in Europe with sustainable plant protein production.

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Appendix A. Supporting information Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agee.2021.107831

Chapter V

Impacts of semiochemical traps designed for *Bruchus rufimanus* Boheman 1833 (Coleoptera: Chrysomelidae) on nontarget beneficial entomofauna in field bean crops

Impacts of semiochemical traps designed for *Bruchus rufimanus* Boheman 1833 (Coleoptera: Chrysomelidae) on nontarget beneficial entomofauna in field bean crops

Taken from the following reference:

Segers, A., Noël, G., Delanglez, L., Caparros Megido, R., & Francis, F. **(2023).** *Impacts of Semiochemical Traps Designed for Bruchus rufimanus Boheman 1833 (Coleoptera:Chrysomelidae) on Nontarget Beneficial Entomofauna in Field Bean Crops.* Insects, 14(2), Article 2.

Abstract: Broad bean weevils (BBWs–Coleoptera: Chrysomelidae) are serious pests of field bean seeds that hamper the promotion of this crop in the diversification of European cropping systems. Recent research has identified different semiochemical lures and trap devices for the development of semiochemical-based control strategies of BBWs. In this study, two field trials were carried out in order to provide necessary information supporting the implementation of sustainable field use of semiochemical traps against BBWs. More particularly, three principal objectives were followed including (i) the identification of the most efficient traps for BBWs capture and the influence of trapping modality on BBWs sex-ratio, (ii) the assessment of eventual collateral effects on crop benefits including aphidophagous and pollinator insects such as Apidae, Syrphidae and Coccinellidae, (iii) the assessment of the crop developmental stage influence on the capture by semiochemical traps. Three different semiochemical lures were tested in combination with two trapping devices across two field trials in early and late flowering field bean crops. The crop phenology and climate parameters were integrated into the analyses to interpret the spatiotemporal evolution of the captured insect populations. A total of 1380 BBWs and 1424 beneficials were captured. White pan traps combined with floral kairomones were the most efficient traps for the capture of BBWs. We demonstrated that the crop phenology (*cf.*, the flowering stage) exerted strong competition on the attractiveness of semiochemical traps.

Community analysis revealed that only one species of BBWs was captured in field bean crops (*i.e.*, Bruchus rufimanus), and no trend was highlighted concerning the sex ratios according to the trapping devices. The beneficial insect community included 67 different species belonging to bees, hoverflies and ladybeetles. Semiochemical traps manifested a strong impact on beneficial insect communities that included some species under extinction threats and need to be further adapted to minimize such collateral effects. Based on these results, recommendations are provided for the implementation of the most sustainable BBWs control method that minimizes the impact on the recruitment of beneficial insects, which is an important ecosystem service for faba bean crops.

Keywords: faba bean; Vicia faba; bruchids; community structure; Apoidea; Syrphidae; Coccinellidae

1 Introduction

One of the biggest challenges of current agriculture is to ensure food security while reducing its impact on the environment at the same time (Foley *et al.* 2011; Bedoussac *et al.* 2015). More sustainable cropping systems can be implemented at the national level by increasing crop diversity and promoting beneficial synergies between neighboring/successive crops in rotations (Power 2010; Renard and Tilman 2019). In this sense, the introduction of legume crops such as *Vicia faba* L. (Fabaceae), commonly named field bean, faba bean or horse bean, is expected to greatly contribute to the sustainability of European feed/food production (Mínguez and Rubiales 2021). This culture provides many benefits to agroecosystems (Jensen *et al.* 2010). First, the plants have root symbioses with *Rhizobium* bacteria (*i.e.*, nodules) that fix atmospheric nitrogen (N) and transform it into an uptaking form for plants. This natural green manure provides N fertilizers to the subsequent crop (Preissel *et al.* 2015) and decreases greenhouse gases emission related to the production, transport and spreading, of synthetic fertilizers (Köpke and Nemecek 2010; Jensen *et al.* 2012). The main advantage of cropping field beans is the production of seeds with high starch or protein contents that can be used as feed or food. This

contributes greatly to plant-protein autonomy in Europe and may replace unsustainable soybean imports from South America (Voisin *et al.* 2014; European Parliament 2018; Rempel *et al.* 2019). Finally, cropping field bean crops increases the abundance and the diversity of beneficial arthropods (*i.e.*, biocontrol agents and pollinators) by providing a large amount of pollen and nectar (Schulz-Kesting *et al.* 2021). Pollinators such as bumblebees, honeybees and wild bees feed on these floral resources. Predators of aphids such as lady beetles (Coleoptera: Coccinelidae), hoverflies (Diptera: Syrphidae), or some parasitoid species may also feed on the nectar provided by extrafloral nectaries (Mondor and Addicott 2003; Nuessly *et al.* 2004; Jamont *et al.* 2013; Everwand *et al.* 2017).

The promotion of field bean introduction should, however, be economically reliable to meet sustainable development criteria. Yet, field bean crops show irregularities in seed quality and quantity due to biotic stresses such as root or leave diseases and pests (Johansen *et al.* 1994; Duc 1997; Abras *et al.* 2015). One of the most important groups of pests for the valorization of seeds in the human food market is named broad bean weevils (BBWs). This group of pests corresponds to small Coleoptera belonging to the Chrysomelidae family (sub-family Bruchinae) and greatly impact the field bean seed quality. Five species (closely related morphologically and difficult to distinguish) were recorded in Belgium including *Bruchus rufimanus* Boheman 1833 (the most common one in field bean crops), *B. affinis* Frölich 1799, *B. atomarius* (L. 1761), *B. brachialis* Fåhraeus 1839 and *B. pisorum* (L. 1758) (Zampetti and Ricci 2012; Baugnée *et al.* 2021). The pest stage of BBWs is the larvae developing inside forming seeds (Hoffmann 1945). Quantitative damage consists in a loss of seed dry mass that may range from 5 to 9.4% (Boughdad 1997; Roubinet 2016; Titouhi *et al.* 2017) and qualitative damage includes a decrease in the seed germination capacity (Khelfane-Goucem and Medjdoub-Bensaad 2016), a fall in nutritional and tasty properties caused by the accumulation of waste and feces by feeding larvae (Huignard *et al.* 2011), and a decrease in the aesthetic quality caused by the perforation of emerging adults (Segers *et al.* 2021). These injured seeds are then more susceptible to the development of phytopathogenic fungi in storage

commodities such as *Penicillium* spp. or *Aspergillus* spp. (Boughdad and Lauge 1995; Kaniuczak 2004). Seed batches presenting infestation rates (*i.e.*, the proportion of seeds presenting traces of post-embryonic development of BBW) higher than 2–3% are rejected from the food market (Garrabos *et al.* 2007; Bruce *et al.* 2011), which is the more profitable outlet for growers exceeding by 30 euros/t the prices from feed markets (Biarnès *et al.* 2018; Bachmann *et al.* 2020).

The univoltine life cycle of BBWs starts in spring. Overwintering adults colonize crops at the blooming stage when temperatures exceed 15 °C (*i.e.*, the activity threshold of flying adults). These adults are in reproductive diapause and need to feed on the nectar and pollen of host plants for sexual maturation (Tran and Huignard 1992). Mating and oviposition are favored by temperatures exceeding 20°C. Gravid females lay eggs on young pods and hatching larvae complete the whole post-embryonic development inside a single forming seed by endosperm consumption (Pölitz and Reike 2019). The emergence of the diapausing adults occurs either at the end of summer when mature seeds are harvested, *i.e.*, in storage commodities or in the field depending on the timing of harvest (Boughdad and Lauge 1997), or the next spring when infested seeds are sown (Medjdoub-Bensaad *et al.* 2007). Around fifty percent of adults are reported to emerge during the harvesting period (Gailis *et al.* 2022). It should be noted that no secondary infestation impacts stored seeds as females are (i) in reproductive diapause and (ii) unable to lay eggs on dry seeds (Howe and Currie 1964).

The control of BBWs is difficult because the damaging stage is out of reach of any phytosanitary intervention and because the adult's behavior in crops is closely related to phenological and climatic factors (Ward 2018; Hamidi *et al.* 2021; Segers *et al.* 2021). European conventional methods of control consist of phytosanitary interventions with pyrethroids targeting adults before oviposition (Garrabos *et al.* 2007; Ward 2018). However, this method of control has shown increasing inefficiency over the last decades (Ward 2018; Simmen 2020) and led to increasingly massive infestations since 2013. In consequence,

the export market of seeds to Egypt for food uses collapsed (Lacampagne 2021) which has considerably decreased the field bean popularity (Ward 2018; Gailis *et al.* 2022).

New alternatives were recently suggested on the basis of chemical processes underlying the attraction of BBWs to the host plants. The volatile organic compounds (VOCs) emitted by faba bean flowers that attract adults searching for food resources (*i.e.*, flower kairomone) were identified as a blend of (R)-limonene, (E)-ocimene, (R)-linalool, 4-allylanisole (*i.e.*, estragol), cinnamyl alcohol, cinnamaldehyde, $\alpha$and β -caryophyllene (Bruce *et al.* 2011). Three of these compounds were demonstrated to be effective for BBWs attraction in field experiments, *i.e.*, (R)-linalool (17.7 mg/day), cinnamyl alcohol (0.4 mg/day) and cinnamaldehyde (0.77 mg/day). Later studies have identified another kairomonal signal emitted by pods having an attractive effect on gravid females searching for an oviposition site as a blend of cis-3-hexenyl acetate (30–40%), ocimene (15–20%), linalool (10–20%), α and β caryophyllene (10–20%), and limonene (15–20%) (Frerot *et al.* 2015; Leppik *et al.* 2016). No pheromone was found to be effective in the field (Segers *et al.* 2021). Semiochemical lures based on flower and pod kairomones were produced to develop semiochemical control strategies against BBWs, including monitoring and mass trapping strategies (Montagné *et al.* 2018; Ward 2018). Two designs of traps can be used with these lures, a white pan trap with a transparent cylinder and a green funnel trap with barrier crossbars. Information about the influence of lures and trap design on the capture of BBWs is lacking while they play a strong influence on the trapping efficacy according to the insect's behavior (Renkema *et al.* 2014; Fountain *et al.* 2017). No information is provided on the influence of crop developmental stages (which may reduce the attractiveness of semiochemical traps), or the potential impact of these semiochemical traps on beneficial insect communities.

In this study, two field trials were carried out in winter field beans and spring field beans with four principal objectives: (i) comparing the effectiveness of different semiochemical traps (two trap designs and three semiochemical

lures) to identify the best-suited trap for the control of BBWs population, (ii) assessing the influence of semiochemical trap design on BBWs sex-ratios, (iii) determining whether the attractiveness of semiochemical traps reproducing the crop odor were not in competition with the crop itself and (iv) characterizing the potential collateral effect of semiochemical traps on beneficial insects. This study is the first integrative and comparative semiochemical trapping trial in field bean crops.

2 Material and methods

2.1 Plant material

Two field trials were carried out during the cropping season 2020–2021 for the comparison of trapping modalities. Two cultures of field bean were cropped in Gembloux (Belgium) at geographical coordinates 50°50'34''N, 4°73'19''E and an altitude of 174.75 m. This area was known to have high BBWs populations according to previous studies (Segers *et al.* 2022), *i.e.*, field beans were cropped under hyper-infestation conditions for the optimal discrimination of trap efficiency. These two crops included a winter field bean variety (WFB) named "Nebraska" and a spring field bean variety (SFB) named "Fanfare". WFB and SFB were sown, respectively, on 17 November 2020 and 24 March 2021 at respective densities of 35 seeds/m2 and 50 seeds/m2. This difference in seed density ensures the same density of stems because WFB branch many times at the base of the plants during the winter period while SFB has one to two upright stems per plant (Segers *et al.* 2022). In these conditions, we assumed that both crops are growing in two distinct environments driven by (i) the time laps in phenological development, (ii) the different weather conditions during the respective developmental stages of both crops and (iii) the different beneficial and pest communities at each crop developmental stage. No insecticides or fertilizers were applied in the trials. One fungicide treatment was applied at the bud-flowering stage to avoid the occurrence of diseases during the experiment. Chemical weeding was performed after sowing.

2.2 Traps, lures and experimental design

Two designs of traps **(Appendix H**) were associated with three types of kairomonal lures, *i.e.*, six trapping modalities were tested during field experiments. Green funnel pan traps with barrier cross bars (PV-Pherobank B.V., Wijk bij Duurstede, The Netherlands) are commonly used in the monitoring or mass-trapping of moths and beetles. These traps are composed of a transparent bucket closed by a green funnel below two perpendicular green cross bars. Semiochemical lures are placed in a cage at the center of the crossbars. The second type of trap was a white pan trap with a transparent cylinder (PB-AgriOdor, Rennes, France). This trap presents a white container surmounted by a transparent cylinder for the interception of flying insects. Semiochemical lures are deposited on a central receiver inside the cylinder. Both of these traps were filled with water containing an odorless surfactant (TRITON X-100, 0.1% v/v) reducing the surface tension to drown insects falling into the trap (Baroffio *et al.* 2018). As non-lured semiochemical traps were already demonstrated to be non-attractive to BBWs (Bruce *et al.* 2011), four repetitions of control traps, consisting of a sticky transparent surface (measuring 14.8 cm 21 cm), were displayed in both crops (**Appendix H**). Semiochemical lures tested in traps included three different types of kairomones: two kairomones commercialized by AgriOdor reproducing the pod odors (AGDG) and the flower odors (AGDF). These lures were dispensed inside polyethylene vials. The third kairomone was commercialized by International Pheromone System Ltd. (Neston, UK) and reproduces the odor of flowers (IPSF). This lure consists of wax plugs that are impregnated with 1.32 g of floral kairomones combined with 0.36 g of extenders and 0.07 g of antioxidants.

The experimental site (**Figure 28**) covered an area of about 4 hectares and included varietal trials of field beans, lupines and peas. Traps were placed at the center of border parcels 27 m wide. The density of BBWs was assumed to be homogeneous in these parcels. Four replicates of all trap types and attractants were placed in each crop in cross combinations which means that traps were positioned in doublets associating pan and funnel traps, both lured

with the same attractant. This pairing of traps aimed to avoid trapping interferences that would have been induced by different semiochemical lures within pairs of traps. Inter-trap distance in each doublet was 5 m and the doublets were spaced at a distance of 20 m from the others. Repetitions of doublets were randomly placed in both crops, a total of 24 traps were placed per crop. Traps were placed during the flower bud stage and removed at the end of crop fructification. Semiochemical attractants were renewed every two weeks. To assess the presence of insects in crops, manual catches were conducted once a week following a standard procedure. A single operator prospected a fixed pathway of 100 m length x 1 m width at the same daily period (from 15:30 to 16:30) and captured/counted adults of BBWs in the flowers or apical leaves using a truncated cone reversed over a pill box.

2.3 Insects collection, preparation and identification

The total amount of captured insects was collected weekly from the traps and stored in ethanol 70% (v/v). After an initial sorting, beneficial insects belonging to Apoidea (Hymenoptera), Syrphidae (Diptera), Coccinellidae (Coleoptera) as well as pests belonging to the subfamily Bruchinae (Coleoptera: Chrysomelidae) were selected and prepared for identification. Beneficial insects were selected for their key role as biocontrol agents and/or pollinators in field bean crops. The method used for the preparation of insects followed the guidelines of Mouret *et al.* (2007) and Fagot *et al.* (2022). Morphological keys of lady beetles (Roy *et al.* 2013), hoverflies (Baugnée and Branquart 2000) and wild bees were used (Patiny and Terzo 2010; Rasmont and Terzo 2010; Falk 2015; Pauly 2019). Bruchid species were separated by sex and identified by J. Fagot using the key of Zampetti and Ricci (Zampetti and Ricci 2012). Identifications were cross-checked by morphological comparison with reference collections of the Conservatory of Functional and Evolutionary Entomology (Gembloux Agro-Bio Tech, University of Liège, Belgium) (Francis and Haubruge 2012).

2.4 Monitoring of field bean phenology

As the host plant phenology and climatic parameters play an important role in BBWs population dynamics (Carrillo-Perdomo *et al.* 2019), climatic parameters were followed in parallel with the phenological development of WFB and SFB during the whole period of the experiment. From 19 April (*i.e.*, the 21st week) to 2 August 2021 (*i.e.*, the 32nd week), WFB and SFB were checked weekly to assess their developmental stages by recording the total number of nodes, the number of nodes bearing inflorescence and the number of nodes bearing pods on 20 randomly selected stems. Mean values of the nodes were used to characterize the growth and the blooming and/or fructifying stages of crops. Climatic parameters, including the maximal daily temperature (°C) and rainfall (mm), were recorded by a nearby weather station. The influence exerted by the different developmental stages on the manual or semiochemical captures was qualitatively assessed by comparing the mean number of nodes with the total abundances of captured insects in each crop.

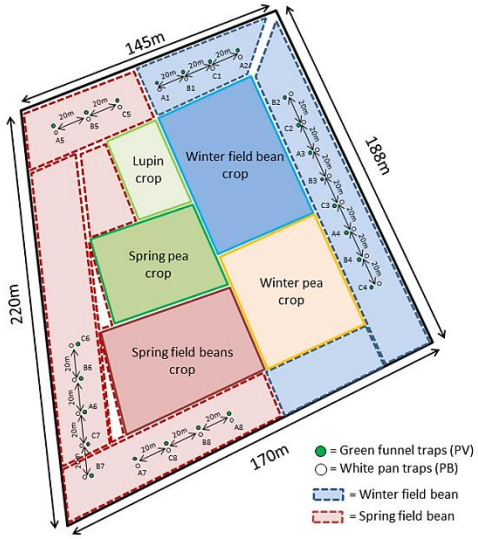

Figure 28 : Experimental site and trap disposition in winter (WFB) and spring field bean (SFB) parcels. Each lure is set in doublets of each type of trap (PV and PB). A1 to A8: replicates of pod kairomones from AgriOdor (AgdG) in winter and spring field beans; B1 to B8: replicates of flower kairomones from AgriOdor (AgdF) in winter and spring field beans; C1 to C8: replicates of flowers kairomones from International Pheromone System (IPSF) in winter and spring field beans. © A. Segers 2021

2.5 Statistical analysis

Statistical analyses were performed with the software RStudio (version 2022.07.1+554) and R (version 4.1.2). First, descriptive statistics and graphs were displayed with the ggplot2 R package (Wickham 2016) in order to describe (i) the population dynamics (*cf.*, manual and semiochemical catches) according to the two respective crop phenologies and (ii) the timing of eventual collateral effects on beneficial insects. The comparison of trapping modalities was then performed by considering winter and spring varieties together in order to provide a holistic consideration of insect communities according to the precocity of both crops. Generalized Linear Mixed-Models (GLMMs) fitted with a Poisson error distribution was used in the comparison of trapping modalities and sex ratios using the lme4 R package (Bates *et al.* 2014). The logarithmic transformations of abundances of BBWs and beneficial insects (Apoidea, Coccinelidae and Syrphidae), were used as variables to explain. Each trap was assumed statistically independent and the location of the trapping site (*i.e.*, trap lure; **Figure 28**) was specified as a random effect to minimize the pseudo-replication of the repeated sampling at each trapping site (Harrison *et al.* 2018). The collection date (*i.e.*, incorporating the crop phenology), the trapping modalities, and the interaction between the trapping devices and semiochemical lures were specified as fixed effects. Multiple comparisons of means based on Tukey's all-pairs comparisons for the trapping modalities of BBWs and beneficial insects were performed using a multcomp R package (Hothorn *et al.* 2008) with Bonferroni's correction (adjusted p-value < 0.05). Sex ratios (count of male/count of female) were compared by ANOVA for each trapping modality.

Regarding the community analysis, abundance ranks per trapping modality were displayed using the package BiodiversityR (Kindt and Coe 2005). Alpha diversity metrics within the captured insect dataset were characterized using Hill's framework (Hill 1973; Roswell *et al.* 2021) at each sampling site. More particularly, species richness, Hill–Shannon and Hill–Simpson indexes were estimated by standardizing their coverage (*i.e.*, selecting the lowest coverage value) using iNEXT R package (Hsieh *et al.* 2016). Classical Shannon and

Simpson indexes show linearity and replication biases which are inherent in their algebraic formulae (Jost 2006). Both these indexes are based on the relative abundance of each taxon and are influenced by the dominance-rarity pattern of the sampling. Both these indexes were mathematically transformed becoming Hill–Shannon and Hill–Simpson indexes (Hill 1973) to better calculate the mean rarity of the species (Roswell *et al.* 2021). The coverage was used to equalize samples instead of equal effort or rarefaction standardization because this method provides a better balance of the expected species richness against the true diversity of our defined sampling system (Roswell *et al.* 2021). Linear Mixed-Models (LMMs) were performed to compare the generated alpha diversity metrics with the lme4 R package (Bates *et al.* 2014). As for the abundance comparisons, the trapping site was set up as a random effect. Multiple comparisons of means based on Tukey's all-pairs comparisons for the trapping modalities of the alpha diversity metrics were performed using the multcomp R package (Hothorn *et al.* 2008) with Bonferroni's correction (adjusted p-value < 0.05).

Last, beta species diversity was analyzed within the dataset of captured insects according to all semiochemical trapping modalities (PBAGDF, PBAGDG, PBIPSF, PVAGDG, PVAGDF and PVIPSF) using the Bray–Curtis dissimilarity matrix and Principal Coordinate Analysis (PCoA) in order to show these dissimilarities at each sampling site. Distance based redundancy analyses (dbRDA) were then performed on the Bray–Curtis dissimilarity matrix with the capscale R function setting up trapping modalities and sampling sites as explanatory variables. Afterward, ANOVA with 999 permutations was performed to test the influence of trapping modalities and sampling sites on the dissimilarities of the observations. These analyses were performed using the vegan R package (Oksanen 2011). Multi-level pattern analysis (MLPA) was performed to identify the indicator species of trapping modality using the indicspecies R package (Cáceres and Legendre 2009). MLPA generates the IndVal index between the species and each trapping modality and then calculates the highest association value by incorporating a correction for unequal group (*i.e.*, trapping modality) sizes (Dufrêne and

Legendre 1997). The significance of the association which is calculated with a permutation test (n = 999) was set up at 0.1.

3 Results

3.1 Host plant phenology and population dynamics of BBWs and beneficial insects

Traps were installed from the onset of flowering up to the ripening pods of both crops. The developmental stages of WFB and SFB are, respectively, represented in **Figure 29 a and 30 b**. Climatic parameters recorded in parallel with crop phenologies are presented in **Appendix I**. The winter field bean crop started flowering from the 19th week (*i.e.*, flower bud stage) but the crop development was strongly slowed down by the cold temperatures observed during the month of May (from the 19th to the 22nd weeks). The flowering of WFB increased after the 22nd week and lasted up to the 25th week with a flowering peak during the 23rd week when young pods began to form at the base of plants. The pod formation and grain filling of WFB lasted until the 31st week. Spring field bean crops started flowering during the 23rd week and lasted until the 28th week with a flowering peak observed during the 25–26th weeks. The pod formation started in the 25th week and pods were mature in the 32nd week.

Following this phenological development, twenty-four semiochemical traps were installed with four control sticky traps and were weekly collected from the 15 of April 2021 (*i.e.*, 17th week) until the 29 July 2021 (*i.e.*, 31st week) in WFB and from the 6 of June 2021 (*i.e.*, 24th week) until the 2 August 2021 (*i.e.*, 32nd week) in SFB. Manual catches/countings were also performed weekly during these periods to check the presence of BBWs in crops. The BBWs and beneficial population dynamics in WFB and SFB are, respectively, presented in **Figure 29 b and 30 b**.

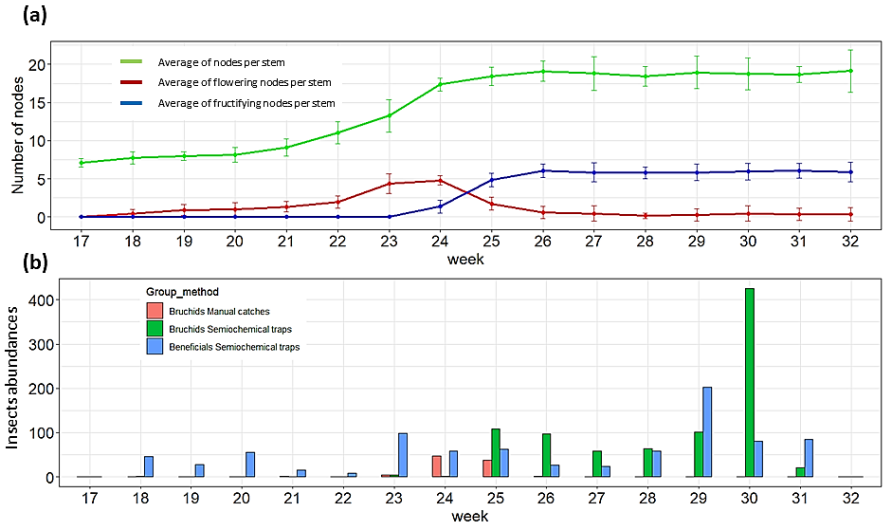

Figure 29: Population dynamics of broad bean weevils (BBWs) and beneficial insects (Apoidea, Syrphidae and Coccinellidae) in WFB according to the crop development. (a) Development of WFB: mean number of nodes per stem (± standard deviation-green), mean number of flowering nodes per stem (± standard deviation-red) and mean number of fructifying nodes per stem (± standard deviation-blue). (b) The temporal abundance of BBWs and beneficial insects according to trapping method: manual trapping (red), captured-semiochemical BBWs (green) and captured semiochemical beneficials (blue)

A total of 1,380 BBWs were captured in semiochemical traps , 882 and 498 in WFB and SFB crops, respectively. No BBWs were captured by sticky control traps while manual catches/countings registered a total of 236 BBWs, 93 in the WFB crop and 143 in the SFB crop, respectively. A single bruchid capture was recorded in a semiochemical trap during the 19th week in WFB, but most of the adults were recorded from the 22nd week at the end of May by manual catches/countings, which coincided with the peak of the crop flowering (**Figure 29 b**). Semiochemical traps started to capture BBWs from the 25th week which corresponds to the decreasing of flower amounts in crops when young pods began to form. This trend was also observed in SFB: manual caches/counting indicated the presence of BBWs during the peak of flowering (*cf.*, 25th week), while semiochemical traps captured BBWs at the end of flowering (*cf.*, from the 26th week) when the young pods were formed. Concerning the influence of climate, maximal daily temperatures exceeded the

flying threshold of BBWs (*i.e.*, 15 °C) from the 20th week but no semiochemical captures of BBWs were observed until the 25th week.

A total of 1,424 beneficials were captured in semiochemical traps (**Appendix J**), 571 in WFB and 853 in SFB, respectively. No beneficials were captured by semiochemical sticky traps that presented other groups of insects such as Diptera species, Hemiptera species, and Ichneumonoidea wasps. No trend seemed to be highlighted concerning the field bean crops phenologies, but the temperatures and precipitations were more likely to influence the beneficial abundance in semiochemical traps (*i.e.*, weeks presenting high temperatures and low precipitations). During the 17th to the 25th weeks, semiochemical traps captured more beneficials than BBWs in the WFB crop. In the SFB semiochemical traps, beneficials were more abundant than BBWs during the 24th to the 26th weeks. After the blooming of both crops, BBWs were more abundant in semiochemical traps than beneficials. Peaks of BBWs capture are both observed at the end of WFB and SFB blooming.

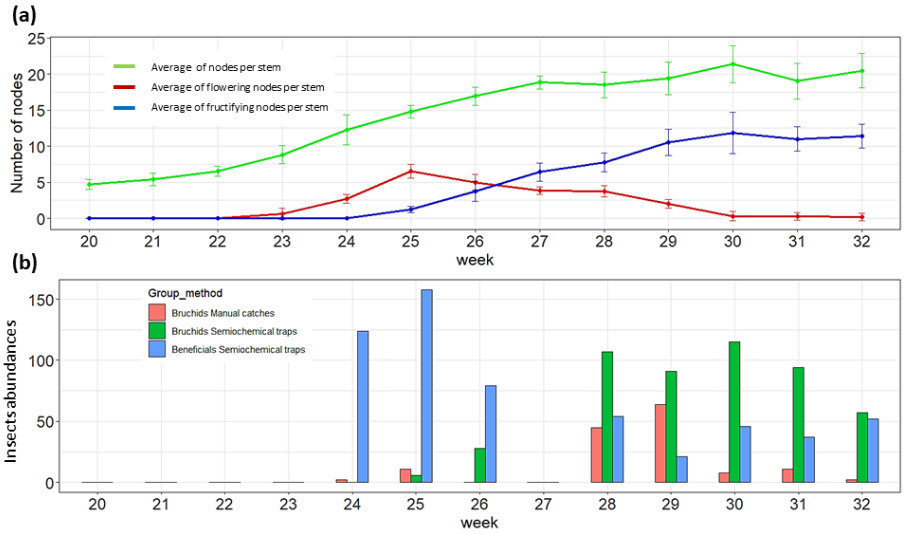

Figure 30: Population dynamics of broad bean weevils (BBWs) and beneficial insects (Apoidea, Syrphidae and Coccinellidae) in SFB according to the crop development. SFB according to crop development. (a) Development of WFB: mean number of nodes per stem (± standard deviationgreen), mean number of flowering nodes per stem (± standard deviation-red) and mean number fructifying nodes per stem (± standard deviation-blue). (b) The temporal abundance of BBWs and beneficial insects according to trapping method: manual trapping (red), captured-semiochemical BBWs (green) and captured-semiochemical beneficials (blue).

3.2 Influence of trapping modalities on the capture of BBWs and beneficials

The results of the GLMMs and Tukey's pair comparisons with Bonferroni's correction are provided in **Figure 31**. BBWs catches, **Figure 31 a**, were significantly higher with white traps lured with floral kairomones of IPS (PBIPSF) rather than all other green trapping modalities (PVAGDF-PBIPSF z-value = -4.15; PVAGDG-PBIPSF z-value = -4.48; PVIPS-PBIPSF z-value = -3.12; all adjusted p-values < 0.05). No significant differences in terms of BBWs captures were highlighted within the different associations of white or green traps with different semiochemical lures. The association of pods kairomones with the white trap (PBAGDG) captured significantly more BBWs than its equivalent green trap (PVAGDG) (z-value = -2.99; adjusted p-value < 0.05). Concerning the capture of beneficials, **Figure 31 b**, the PBIPSF modality was significantly more efficient than PBAGDG and all the green trapping modalities (PBIPSF–PBAGDG z-value = 3.14; PVAGDF-PBIPSF z-value = -5.12; PVAGDG-PBIPSF z-value = -5.44; PVIPSF-PBIPSF z-value = -4.38; all adjusted p-values < 0.05). Both AgriOdor lures (AGDG and AGDF) were significantly more attractive in white traps than in the green traps (PVAGDF–PBAGDF z-value = −3.46; PVAGDG–PBAGDF z-value = −3.80; adjusted *p*-values < 0.05). Sex ratios of BBWs were calculated for trap capture in each trap combination (**Figure 32**). No statistical differences were observed. (df = 5; F-stat = 0.46; *p*-value = 0.80).

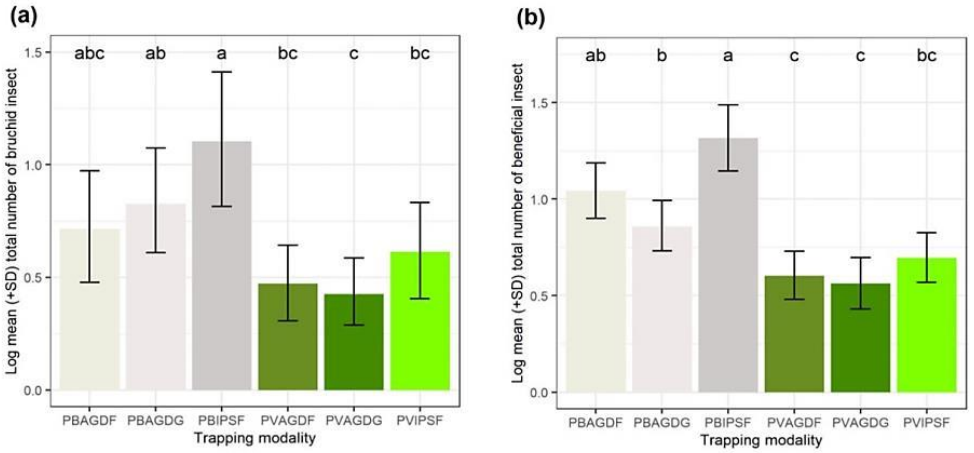

Figure 31: Log of the mean number (± SD) of captured insects per trapping modality. (a) Broad bean weevils (BBWs); (b) beneficial insects. In (a) and (b) graphics, the shades of grey correspond to the white traps and the shades of green correspond to the green traps. Log of the means with different letters are significantly different (Bonferroni's adjusted *p*-value < 0.05).

3.3 Analyses of communities

All the BBWs that were captured during the experiment (*cf.*, semiochemical and manual traps) corresponded to the species *B. rufimanus*. Consequently, no further diversity analyses were performed on BBWs. Concerning beneficials, the community of 1,424 insects captured by semiochemical traps included 20.4% of lady beetles (Coleoptera: Coccinelidae), 22.24% of hoverflies (Diptera: Syrphidae), and 57.4% of social and solitary bees (Hymenoptera: Apoidea). Bees encompassed 49 species, Coccinellidae included six species and Syrphidae included 12 species (**Appendix J**). *Apis mellifera* Linnaeus, 1758 (n = 321), *Bombus terrestris* (Linnaeus, 1758) (n = 275), *Coccinella septempunctata* Linnaeus, 1758 (n = 191) and *Episyrphus balteatus* (De Geer, 1776) (n = 129), were the most captured species. The abundance rankings of these dominant species according to trapping modalities are represented in **Figure 33.**

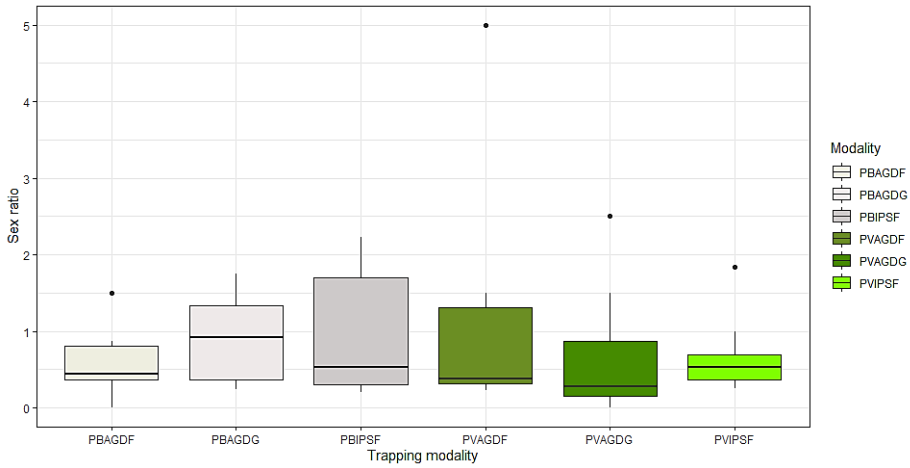

Figure 32: Boxplots of sex-ratios led to BBWs captures according to trapping modalities. The black dots are outliers, the bold horizontal line in each boxplot corresponds to the median of sex-ratio between the upper and lower quartile (*i.e.*, the interquartile range or the middle 50% scores) and the upper and lower whiskers (*i.e.*, the score outside the middle 50%).

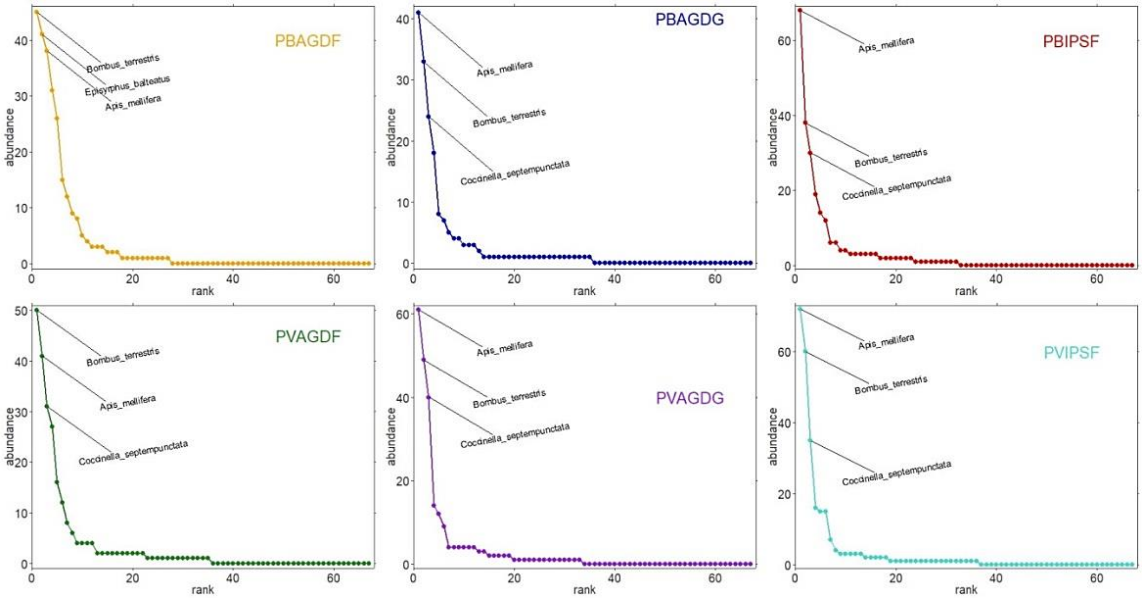

Figure 33: Abundance rank analysis. The top three species are labeled in each graphic for each trapping modality: orange curve for PBAGDF; blue curve for PBAGDG; red curve for PBIPSF; green curve for PVAGDF; purple curve for PVAGDG; turquoise curve for PVIPSF.

The comparison of alpha diversity according to each trapping modality is represented in **Figure 34**. At coverage-standardized samples, the richness of beneficial insects (**Figure 34 a**) is significantly higher in PBAGDG than in the PVAGDF modality (z-value = −3.01; adjusted *p*-value < 0.05). For Hill–Shannon and Hill–Simpson alpha metrics (**Figure 34 b,c**), the values are significantly higher in PBAGDF than in PVAGDG and PVAGDF (Shannon PBAGDF–PVAGDF z-value = −3.21; Shannon PBAGDF–PVAGDG z-value = −3.50; Simpson PBAGDF–PVAGDF z-value = −3.47; Simpson PBAGDF–PVAGDG z-value = −3.59; adjusted *p*-values < 0.05). The percentage of missing species oscillated from 34.66% to 62.22% among the trapping modalities (**Table 15**). Compared to the actual species richness estimated by the Chao1 index, PBAGDF would do the most collateral damage for beneficial insects, with 74.89 ± 17.44 different species captured, while the same lure combined with green traps would have the least impact on the beneficial communities, with 28.02 ± 12.92 different species captured.

Table 15: Species richness and chao1 index per trapping modality.

Trapping Modality	Species Richness	Chao1 Index ± SE	Potential Estimation of Missed Taxa Proportion [%]
All the experiment	67	114.88 ± 28.50	41.68
PBAGDF	45	74.89 ± 17.44	39.91
PBAGDG	34	52.03 ± 12.07	34.66
PBIPSF	37	68.62 ±23.06	46.08
PVAGDF	16	28.02 ± 12.92	42.90
PVAGDG	18	47.65 ± 28.08	62.22
PVIPSF	24	38.88 ± 12.28	38.28

Finally, the communities associated with all repetitions of trapping modalities are represented on the biplot space of the PCoA analysis in **Figure 35**. Overall variance explained by two principal components accounted for 29.1%. The communities were mainly structured along two principal gradients. The first gradient is the type of trap that seems strongly correlated with the first principal component that encompasses 17.4% of the explained variance. The second gradient is the type of crop (WFB or SFB) which is correlated with the second principal compound accounting for 11.7% of the explained variance.

Dissimilarities of species assemblage between each observation are significantly driven by the used trapping modality (df = 5; F = 3.62; *p*-value < 0.01) and the type of crops (df = 1; F = 4.01; *p*-value < 0.02).

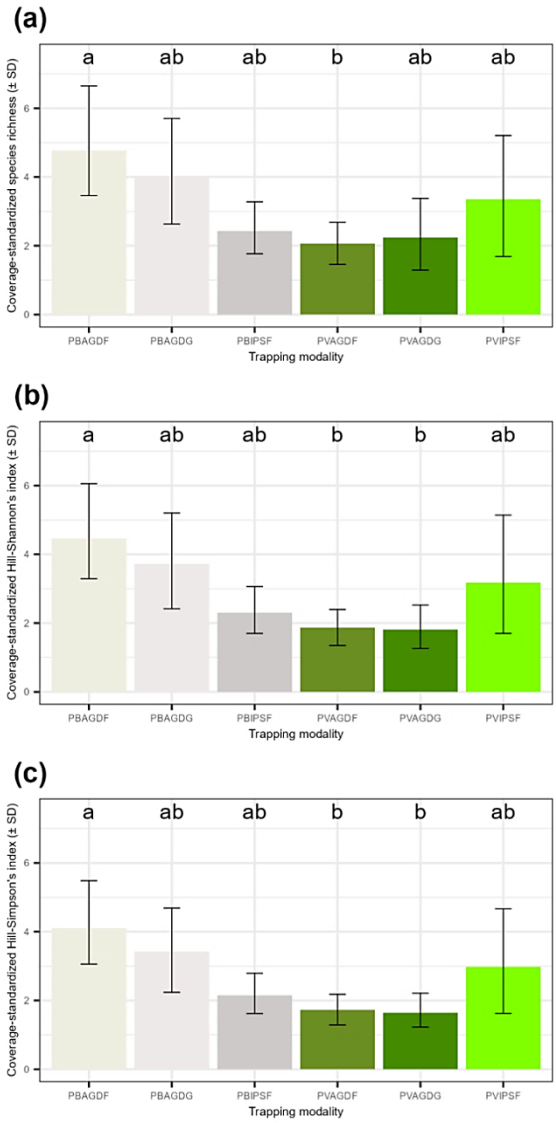

Figure 34 : Alpha diversity metric comparisons per trapping modality for beneficial insects: (a) coverage-standardized species richness, (b) Hill–Shannon index and (c) Hill–Simpson index. In (a) (b) and (c) graphics, the shades of grey correspond to the white traps and the shades of green correspond to the green traps. Alpha diversity means with different letters are significantly different (Bonferroni's adjusted *p*-value < 0.05).

Within these communities, MLPA analysis identified indicator 11 species that are significantly (alpha at 0.1) associated with a trapping modality, or group of trapping modalities (**Table 16).** The six categories incorporate six bee species (three *Bombus* spp.), three hoverfly species and two lady beetle species.

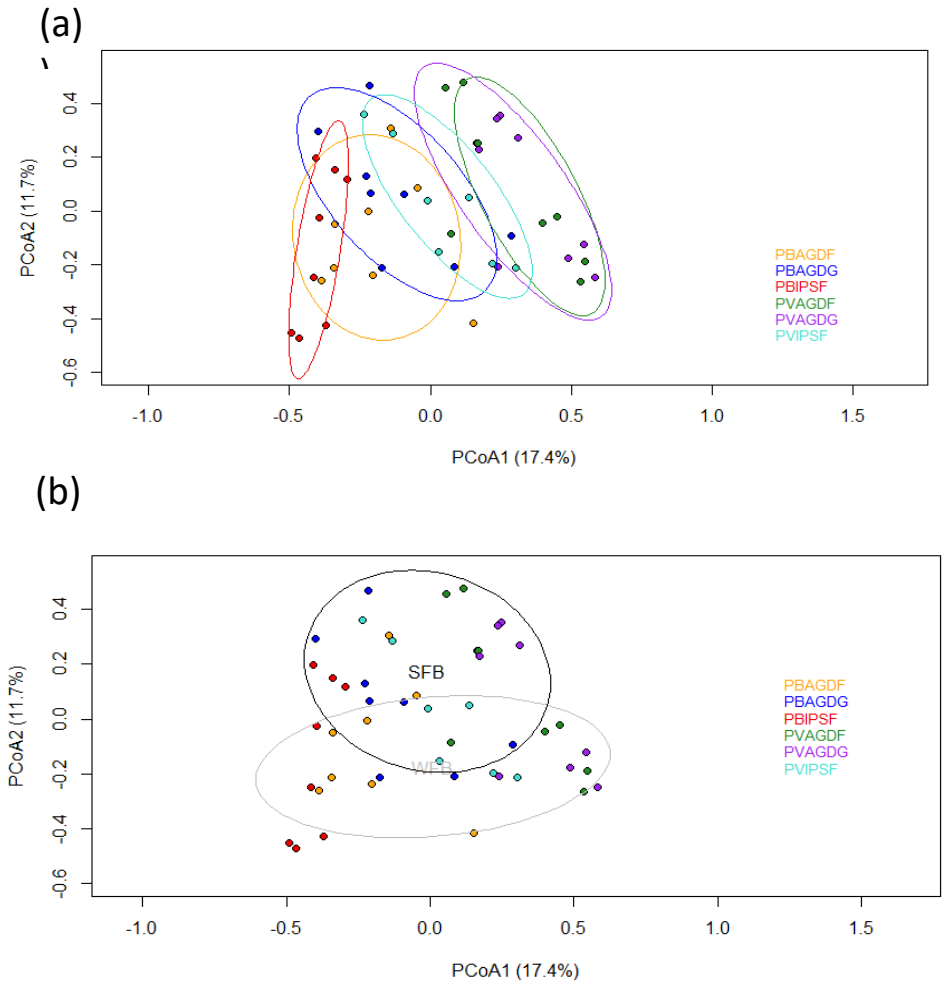

Figure 35: Principal Coordinates Analysis (PCoA) of trapping modalities at each sampling sites. Each dot color corresponds to a trapping modality (*i.e.*, lure x trap): red = PBIPSF; orange = PBAGDF; blue = PBAGDG; turquoise = PVIPSF; purple = PVAGDG; green = PVAGDF. (a) Ellipses show the 75% confidence interval of the locations grouped by trapping modality with the same correspondence color of the dots. (b) Ellipses show the 75% confidence interval of the locations grouped by Winter Field Bean culture (WFB) in grey and Spring Field Bean culture (SFB) in black.

4 Discussion

A total of 48 traps were tested in two crops under hyper-infestation conditions. Data collection included insect sampling in semiochemical traps, manual catches, phenological and climatic records during a period of fifteen weeks in one site. A total of 2,804 insects of interest including 68 different species were identified. All these data were integrated to interpret major factors (biotic and abiotic information) driving population dynamics that may interfere with the traps comparison. This sampling effort successfully discriminated against the most efficient type of traps and provided a local assessment of collateral effects on selected groups of beneficials. The crop phenology was also demonstrated to manifest an influence on the attractiveness of BBWs. These local observations are discussed in the following sections.

Table 16 : Indicator species of captured communities in function of trapping modalities. '*' indicates p-value < 0.05.

Trapping Modality	**Taxa**	**Indicator Statistic**	***p*-Value**
PBAGDF	*Lasioglossum minutissimum*	0.48	0.07
	Andrena subopaca	0.44	0.05 *
PBIPSF	*Apis mellifera*	0.76	>0.01 *
	Melanostoma mellinum	0.54	>0.01 *
	Lasioglossum laticeps	0.44	0.08
PVAGDF + PVAGDG	*Harmonia axyridis*	0.44	0.02 *
	Coccinella septempunctata	0.43	0.04 *
PBAGDF + PBIPSF	*Episyrphus balteatus*	0.59	>0.01 *
	Spaerophoria scripta	0.53	>0.01 *
PBIPSF + PVIPSF	*Bombus hortorum*	0.60	>0.01 *
PBAGDF + PBIPSF + PVIPSF	*Bombus pascuorum*	0.48	>0.01 *

4.1 Most efficient trap for the capture of bruchid, phenological influences on catches and impact on beneficials

The trap modality that was statistically the most efficient for the capture of BBWs was the combination of IPS kairomonal lures (*i.e.*, flowers kairomones) with a white pan trap. White traps were more effective than green traps with all semiochemical lures while previous studies suggested that green cone traps baited with flower kairomones were efficient for the capture of BBWs (Ward 1999; Bruce *et al.* 2011). In regards to the sex ratio, IPSF lures would have been expected to present more males than females, because males were demonstrated to be more attracted by flower kairomones (Bruce *et al.* 2011). The AGDG lures would also have been expected to capture more females than males, as gravid females are attracted by pod kairomones for oviposition (Leppik *et al.* 2016). These hypotheses were not validated as more females were captured by flower kairomones on the one hand and no statistical difference could confirm the more abundant catches of females (*i.e.*, less important sex ratio) with pod kairomones on the other hand.

The field bean phenology played an important role in the number of BBWs that were recorded in traps. In both cultures, the number of trapped BBWs increased as the number of flowers decreased while (i) manual catches/counting attested the presence of BBWs in crops, and (ii) maximal daily temperatures were superior to the flying activity threshold of BBWs. This observation shows a strong competition of the crop odor with the attractiveness of the semiochemical traps that decrease their efficiency, as suggested by previous studies (Ward 2018). The most important capture of BBWs in WSB and SFB crops (more than 500 BBWs) occurred during the 30th week which corresponds to the end of SFB flowering which is supposed to trigger the departure of adults from both field bean crops in search of a food source (Medjdoub-Bensaad *et al.* 2007; Huignard *et al.* 2011; Hamidi *et al.* 2021; Segers *et al.* 2021) and may drastically increase the attractiveness of semiochemical lures. Manual catches/countings were maximal during the peak of flowering and continued until the end of blooming before dropping sharply. This can be explained by two factors. First, the peak of crop flowering

is reported as the period of highest BBW density (Gailis *et al.* 2022). Secondly, the concentration for feeding and mating occurs in the flowers which are located on the upper parts of the plants, the individuals found there are easily visible by the operator performing the manual catches/countings. After flowering, the males leave the crop and the females spread into the crop to lay eggs at the base of plants and became difficult to be caught/counted.

Beneficials considered in this study focused on bees, hoverflies and lady beetles. Populations were not as dependent on crop phenologies as BBWs because they were captured by semiochemical traps during the whole experimental period. An increase in captures is nevertheless observed during the flowering periods of field bean crops. This is consistent with other studies stating that crop flowering is a good predictor of the beneficials abundance (Nayak *et al.* 2015). Climatic factors also influenced captures, *i.e.*, warm and low precipitation which is common for poikilothermic flying organisms (Hodkinson *et al.* 1998). Respectively, 312 and 415 bycatches were observed in WFB and SFB before the BBWs captures exceeded the captures of beneficials. This constitutes an important impact on auxiliaries before the BBWs infestation. The impacts of trapping modalities are contrasted in terms of abundance and diversity. PBIPSF was the most effective trap regarding the abundance of beneficials, but PBAGDF was the most effective one on species richness. Thus, the combination of white traps and floral attractants had the most important effect on beneficial communities.

4.2 Analyses of communities of BBWs and beneficials: balanced impacts according to functional groups and their ecology

Analyses of the BBWs community revealed that only one population of *B. rufimanus* was present in field bean crops. None of the four other BBWs recorded in Belgium that develop in faba bean seeds were observed (Zampetti and Ricci 2012; Baugnée *et al.* 2021). More particularly, no *B. pisorum*, an important pest of pea that may use seeds of *V. faba* as larval host (Zampetti and Ricci 2012; Reddy *et al.* 2018), was captured in semiochemical traps despite the presence of pea parcels close to the trials. This may suggest that

the semiochemical lures established on the basis of electroantennographic studies on *B. rufimanus* are specific to this pest. Other BBWs species could be more sensitive to other specific VOCs or to a different ratio in ubiquitous volatiles emission such as (Z)-3-hexenyl acetate, limonene, caryophyllene, linalool, myrcene (Bruce *et al.* 2005; Bruce and Pickett 2011).

The community of beneficials (bees, hoverflies and lady beetles) observed in semiochemical traps included 67 species belonging to 31 different genera and eight families (**Appendix J**). The most abundant species were *A. mellifera*, *B. terrestris*, *C. septempunctata* and *E. balteatus*, which are commonly observed in field bean crops (Nation 2010; Garratt *et al.* 2014). They include three functional groups according to the ecology of different species: (i) pollinators (bees and hoverflies), (ii) aphid predators (lady beetles and hoverflies) and (iii) pollinators and aphid predators (hoverflies). Ecological differences within and between these groups induce different vulnerabilities of the populations to semiochemical traps that must be considered in the assessment of their impact on ecosystem services. The pollinator community is important for crop yield as cross-pollination increases the number of pods per plant, the number of seeds per pod, and seed weight, which can increase total yield by up to 185% compared to self-pollination (Nayak *et al.* 2015). However, not all species contribute equally to cross-pollination. Among dominant species of pollinators, it was reported that bumblebees contributed more efficiently to the fertilization of bean flowers (*i.e.*, the number of pods was significantly higher) in front of honey bees and hoverflies (Garratt *et al.* 2014). Additionally, temporal variation in population dynamics is a key determinant of semiochemical trap impacts on pollinator communities. Indeed, the annual colonies of some bumblebees develop from founding queens whose flight periods cover the months of March, April and May. Therefore, the abundance of *Bombus* spp. captured during these periods underestimate the impact on their population because they represent potential colonies of 100 to 400 individuals (Leadbeater and Chittka 2008). It should be noted that not all the *Bombus* species present the same pollination efficiency, some being nectar robbers such as *B. terrestris* workers (Leadbeater and Chittka 2008) while

others being cuckoos of other bumblebee species such as *B. vestalis* (Lhomme and Hines 2019).

Unfortunately, the semiochemical traps captured bee species that are exposed to extinction issues in Belgium which could be one of the most detrimental impacts of this IPM alternative. *Andrena ovatula* (Kirby, 1802), *A. wilkella* (Kirby, 1802), *B. hortorum* (Linnaeus, 1761) (indicator species in IPSF traps), *B. vestalis* (Geoffroy, 1785) are near threatened species. *Lasioglossum minutulum* (Schenck, 1853) and *Stelis signata* (Latreille, 1809) are classified as vulnerable in Belgium and *Nomada fuscicornis* Nylander, 1848 (the cuckoo bee of *Panurgus calcaratus* (Scopoli, 1763) which was not present in our sampling) is endangered [889]. Semiochemical traps are also damaging for oligolectic bee species such as *Chelostoma campanularum* (Kirby, 1802), *Megachile ericetorum* Lepeletier, 1841 and *Melitta leporina* (Panzer, 1799). Only *M. ericetorum* strictly forages in Fabaceae family, mainly *Lotus* spp. and *Lathyrus* spp. flowers (Westrich 2018; Vujić *et al.* 2022). None of the captured hoverfly species are exposed to a European extinction threat (Vujić *et al.* 2022). Most of the species show aphidophagous behavior at the larval stage except for *Eristalis tenax* (Linnaeus, 1758) and *Xylota lenta* Meigen, 1822 whose larvae show microphagous and saproxylic behavior, respectively (Speight 2010).

Concerning aphid predators, *E. balteatus* and *C. semptempunctata* were the most abundant taxa in all trapping modalities. These two species are reported to be the most abundant aphidophagous in the agrosystems of Europe and Belgium (Francis *et al.* 2001). *E. balteatus* larvae and adults have different diets. Adults feed on pollen and nectar while larvae feed on aphids. *Coccinella semptempunctata* is a polyphagous predator, both larvae and adults are known to feed on Aphidoidea, Psylloidea, Coccoidea and mites (Singh *et al.* 2004). Larvae of *E. balteatus* may consume up to 396 aphids in field conditions, and up to 1,322 under laboratory conditions (Tenhumberg 1995), while the respective consumption of *C. semptempunctata* larvae (third instar) and female adults are, respectively, 277 or 204 aphids per day (Xue *et al.* 2009). No study clearly compared the predation rate of lady beetle and hoverfly larvae, and

their consumption rate depends on many factors such as temperature, host plant, aphid species and experimental conditions. Ecological factors such as prey densities and intra-guild predation may also impact the co-existence of these aphidophagous species (Hindayana *et al.* 2001). Both of these two species should remain the least impacted by semiochemical traps. Our results showed that hoverflies were more impacted by white traps and that lady beetles by green traps, as reported by other studies (Rodriguez-Saona *et al.* 2012; Atakan and Pehlivan 2015; Kemp and Cottrell 2015).

4.3 Maximising *B. rufimanus* trapping and minimising beneficial insect trapping: a dilemma

Semiochemical traps offer a sustainable pest management tool that can contribute to the promotion of grain legumes for European crop diversification, but this tool should not impact the ecological services of field bean crops. In this study, the ratio of captured beneficials per captured BBWs was nearly higher than 1, which emphasizes that these semiochemical traps present a similar impact on beneficials and BBWs. This excludes, therefore, a potential mass trapping strategy against BBWs that spares collateral effects on organisms of agronomic interest. To mitigate the capture of beneficial insects, trap design could be improved and their spatiotemporal deployment could be adapted to the influence of flowering on the capture of BBWs.

Adaptation measures that could improve the selectivity of semiochemical traps and their implementation in the field are multiple. Firstly, other types of traps can be used instead of water-containing traps. These traps are subjected to drying in sunny field conditions which imply repeated field interventions to maintain the trapping devices which are costly in time and energy for growers. Sticky white delta traps are currently being studied in alternative to white pan traps (Ené Leppik, personal communication). Then, the trap selectivity can be increased by (i) the placement of a grid preventing larger insects to reach the trap killing agent (Fountain *et al.* 2017), (ii) the improvement of semiochemical attractiveness (improved or new semiochemical) (Segers *et al.* 2021), and (iii) the displaying of a more selective

killing agent in trapping devices, such as entomopathogenic fungi or double-stranded RNA (Segers *et al.* 2021; Chen *et al.* 2021). The reduction in trapping periods by displaying traps at the appropriate moment, *i.e.*, from the flowering peak, would also limit the captures of beneficials. Finally, an integrated pest management strategy that can be considered is the trap crop approach. This method takes advantage of the effectiveness of semiochemical traps and the influence of crop flowering. It consists in attracting BBWs in an early flowering cultivar and capture them with semiochemical traps to protect late flowering cultivar crops (George *et al.* 2019).

5 Conclusions

The multiple advantages of introducing leguminous plants in crop rotations are well documented in the literature. However, European farmers rarely opt for their introduction in cropping systems which is mainly due to biotic sensitivities causing irregular and uncertain harvests in terms of quality and quantity. Research is needed in the development of better-performing cultivars, innovative and efficient technical itineraries for the sustainable control of pests and the producing food of a quality that could be valorized in national markets. In this study, a potential new biocontrol strategy was investigated by testing and comparing semiochemical traps for the capture of BBWs as well as their collateral effects on beneficial insects in two field bean crops (*i.e.*, winter field bean and spring field bean). Key factors of the agrosystem influencing BBWs population dynamic (*i.e.*, the phenology and the climate) were followed in parallel to interpret the evolution of captured pests. The best-suited traps for the capture of BBWs were identified as well as their impact on beneficial communities. The strong influence exerted by the flowering of crops on the capture of BBWs was described and a complete description of captured communities is provided. Recommendations and guidance are suggested on this background for the development of optimal biocontrol strategies that minimize the impact on beneficial insects. These results support the development of new control methods in legume crops which are essential crops for the diversification of European cropping rotations and for the resilience of the food production system.

Supplementary Materials: The following supporting information can be downloaded at: https:// www.mdpi.com/article/10.3390/insects14020153/s1. Table S1: The general database of Beneficials. Table S2: The general database of BBW.

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Chapter VI (part. 1)

Biotechnological control - Hypocrealean Entomopathogenic Fungi (HEF)

1 Introduction

This chapter deals with two specific methods of control against bruchids including entomopathogenic fungi (EF) and RNA interference (RNAi). The objective is to investigate whether these adopted methods can be destined to the formulation of potential biopesticide. The first part of this chapter deals with entomopathogenic fungi and the second one addresses RNA inferterence in bruchids.

Entomopathogenic fungi are naturally occurring micro-organisms that can offer effective microbial control agents (MCA) against insects and remplace the use of synthetic insecticides (Lacey *et al.* 2001). They are included in arthropod pathogens with viruses, bacteria, and nemathodes and can be used in multiple strategies of biological control like classical biocontrol, conservative biocontrol and augmentative biocontrol (Lacey *et al.* 2015). The biocontrol strategy that will be covered in this chapter is focused on the innundative release of Hypocealean Entomopathogenic Fungi (HEF) against *B. rufimanus*, because this strategy is the most effective for pest control (Lacey *et al.* 2015).

Entomopathogenic fungis (EFs) number between 700 and 800 species (Regnault-Roger *et al.* 2005). According to Kirk *et al.* (2008), EFs are found in the divisions of Chytridiomycota, Zygomycota, Ascomycota from the Kingdom of Fungi, and in the subdivision Oomycota from the Chromista Kingdom. Most of the EFs used for biocontrol research are included in the Entomophtorals orders (Zygomycota) and Hypocreals order (Ascomycota) (Shah and Pell 2003). HEF produce conidia, asexual spores that will infect insect trough the host cuticle. As conidia enter in contact with the cuticle of the insect, which is ensured via van der Waals connections with epicuticular hydrocarbons and apolar conidial walls, it will germinates within a few hours and initiate the pathogenesis cycle (Samson *et al.* 1988). The infection of their host via external cucticle contact is a particular characteristic to EF, other entomopathogen agents such as bacteria or viruse infect their host via mid-gut penetration.

The pathogenesis cycle is divided in three principal steps (Samson *et al.* 1988; Jaronski 2014). Fistrly, the phase of contact with insect cuticle triggers the spore's germination. Then, the fungi will penetrate the insect's cuticle by production of several enzymes and by mechanical pressure. Finally, the fungus will develop in the hemocoele by growing partitioned hyphae and colonize their host, which generally results in the deaths within four to fourteen days. After the host death, the fungus rapidly transforms into mycelium and emerges from intersegmental areas of their hosts. The fungus emergence is favored by prolongated period under high humidity conditions.

The main reason justifying the EFs interest in faba bean crop destined to food market is their selectivity and supposed minimal risks to beneficial entomofauna including pollinators, predatory beetles and parasitic wasps that is greatly attracted by faba bean crops flowers (Brownbridge and Glare 2007; Lacey *et al.* 2015). Moreover, they provide efficient and low cost alternative minimizing risks for human compared to conventional chemical insecticides (Zimmermann 2007).

Studies about the EFs potential to control insect pests of faba are scarce (Jaronski 2018), and in consequence, almost no registered MCAs are available concerning the different pests in faba bean crops. The only study assessing the EFs potential against *B. rufimanus* was provided by Sabbour *et al.* (2007), which tested three EF strains (*Beauveria bassiana, Metarhizium anisopliae and Verticillium lecanii*) in laboratory and in field trials against *B. rufimanus*. Their successful results suggested that EFs (especially *B. bassiana* and *M. anisopliae*) could constitute effective MCAs to be formulated into biopesticides. However, the methodological information did not indicated the quantities of conidia administrated per surface units in field spraying assays as well as the application method responsible of their success (*i.e.*, canopy spraying or seed coating treatment). Moreover, no subsequent studies highlighted the efficiency of these MCAs with classical spraying method in large scale faba bean crops. These elements underline the need of additional information about the EFs potential and their method of application for the control of *B. rufimanus* infestations.

In this chapter, five EFs strains were screened for their lethality on two bruchids species (*B. rufimanus* and *C. maculatus*). More precisely, lethal (LT 50) and sub-lethal effects (oviposition inhibition) were characterized in laboratoty bioassay. Results are then discussed for the potential inclusion of EFs in IPM programs against *B. rufimanus*.

2 Material and methods

2.1 Fungal cultures

Five EFs strains were used in lethality assessment bioassay carried out on *C. mabulatus* and *B. rufimanus*:

- *Beauveria bassiana* ((Balsamo-Crivelli) Vuillemin) strain GHA isolated from the commercial product BotaniGard 22WP® (Certis Europe, Bruxelles, Belgium) – **Figure 36 a**;
- *Aspergillus flavus* Link, 1809 strain MUCL 55276 isolated from larvae of *Agriotes lineatus* (L.) (Coleoptera: Elateridae) – **Figure 36 b-c**;
- *Metarhizium acridum* ((Driver & Milner) JF Bischoff, Rehner & Humber) strain IMI330189 isolated from Green Muscle® - **Figure 36 d**;
- *Metarizium brunneum* strains V275 and USDA 4556 that were obtained from Swensea University – **Figure 36 e-f**.

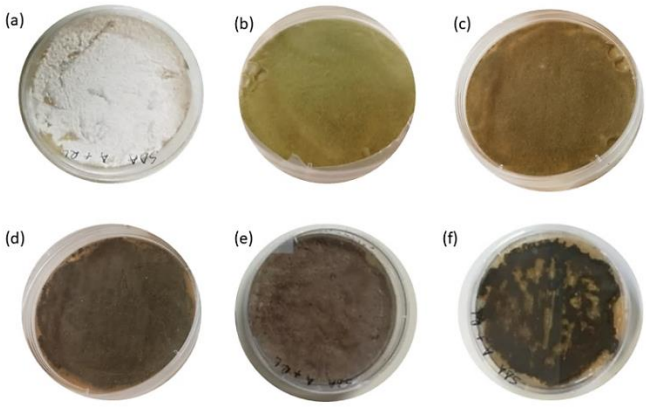

Figure 36: Sporulating EFs used in bioassays agains *C. maculatus* and *B. rufimanus.* (a) *B. bassiana* (strain GHA) sporulating after 15 incubation days, (b) *A. flavus* (strain MUCL 55276) after nine incubation days, (c) *A. flavus* (strain MUCL 55276) after 15 incubation days, (d) *M. acridum* (strain IMI330189) after 15 incubation days, (e) *M. brunneum* strain USDA 4556, and (f) strain V275 after 15 incubation days

Conidial suspensions were obtained on two types of media during the experiments. Firstly fungal strains of *B. bassiana* (strain GHA), *M. acridum* (strain IMI330189) and *A. flavus* (strain MUCL 55276) were grown on 10 mm petri dishes containing Potato Dextrose Agar (39g/l PDA; Merck KGaA, Darmstadt, Germany) with 0.005% chloramphenicol (Sigma-Aldrich, Saint-Louis, Missouri). They were incubated for 15 days at 25°C up to sporulation and recovered in 4 ml of sterile water solution containing tween 80 (0.05% v/v) (Merck KGaA, Darmstadt, Germany). The cultures were scraped and filtered to separate conidia from mycelium fragments and diluted to constitute a discriminatory single concentration of 1 x 10^8 conidia/ml set with Neubauer hemocytometer on 100 fold dilutions. Secondly, fungal strains of *B. bassiana* (strain GHA), *M. brunneum* (strain V275) and *M. brunneum* (strain USDA 4556) were grown on a solid culture medium containing Sabouraud Dextrose Agar (SDA), prepared by dissolving a mixture of 12g Sabouraud Dextrose Broth (SDB) and 6g bacteriological agar in 400 ml distilled water. Fungal strains were incubated for 15 days at 25°C. After incubation, conidia were removed from the cultures by following the same scrapping-filtering procedure.

Each strain were tested for conidia's viability by spreading 100 µl of conidial suspension at 10^5 CFUs/ml on petri-dishes containing PDA/SDA medias. After incubation at 23°C during 24h, three random replicates of 1 cm^2 were mounted on slides to count the number of spores with germ tubes under the microscope. Conidia were considered to have germinated when the length of the germ tube was at least twice the diameter of the conidium (**Figur 37**).

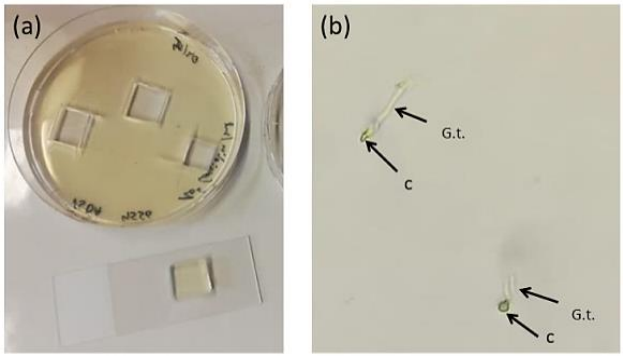

Figure 37: Viability test design (a) and (b) germ tube observation for conidial viability assessment (G.t. = germ tube; C = conidia)

2.2 Bruchids rearings and experimental design

Adults of *B. rufimanus* used in bioassay were collected from harvested field bean seeds cultivated in the region of Gembloux (Belgium). These adults were maintained in reproductive diapause in climatic chamber reproducing overwintering conditions (see Chapter III) where adults can survive up to six months (Tran *et al.* 1993).

Adults of *C. maculatus* used in experiments were reared on *V. unguiculata* seeds at 27 ± 2 °C and 60 ± 10% of relative humidity in total darkness. These insects came from Lincoln University (UK). Emerging adults were immediately collected from the rearing for exposition to conidial suspensions.

Pre-experimental tests were performed to compare two modes of sprayings, *i.e.* the Potter tower sprayer (Potter 1952) and the manual spraying (**Figure 38**). Three doses of one milliliter containing 10^8 CFUs of *A. flavus* conidial suspensions were sprayed on three replicated batches of 30 adults of *C. maculatus* that were anesthesiated with CO_2 to prevent their movement during spraying. Sprayed insects were displayed on a filter paper in 10 mm petri dishes. The potter tower spraying parameters were programmed at a pressure of 40 kPA (Rodríguez-González *et al.* 2017). Manual sprayings were performed under a truncated cone to avoid the spread of conidial suspensions out of the petri dish and standardize the height of the sprayer above insects. Both of these procedures allows the estimation of the number of conidia applied on insects by determining the volume of suspension applied to the petri dish (*i.e.*, the weighting of petri dish before and after spraying) and the relative area covered by the insects (Inglis *et al.* 2012). Insects were then transferred in 10 × 10 × 5 cm boxes in an incubator at constant temperature of 23°C and total darkness to reproduce the seed storaging conditions where *C. maculatus* infestations occure. Mortalities were checked at 48h of intervals. Results (presented in next section) validated the manual spraying for further bioassays led on *B. rufimanus* and *C. maculatus*.

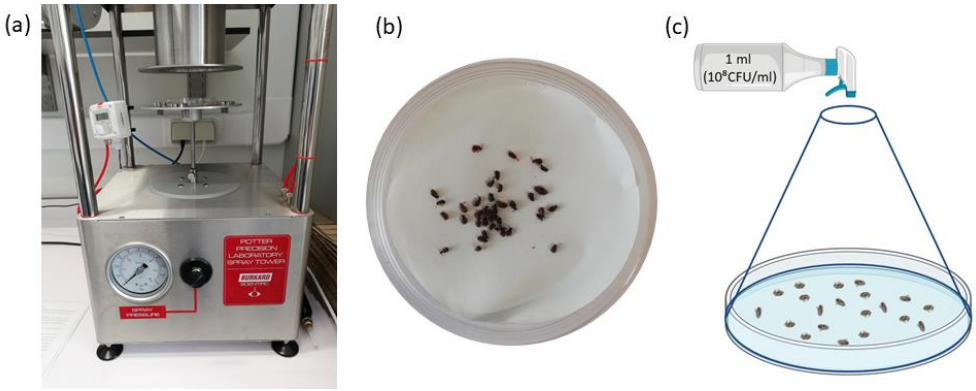

Figure 38: Potter tower sprayer (a), anesthesiated adults of *C. maculatus* on a petri dish+filter paper for spraying (b) and manual spraying disposftiv (c)

Two bioassays were respectively performed on batches of 20 or 30 adults of *B. rufimanus* and *C. maculatus*. First bioassay consisted in the spraying of three fungal strains, *i.e.*, *B. bassiana* (strain GHA), *A. flavus* (strain MUCL55276) and *M. acridum* (strain IMI330189) on non-anesthesiated baches of 30 adults. One milliliter of conidial suspensions containing 10^8 CFUs/ml was applied on adults following the manual spraying procedure described here above. A negative control consisting in the spraying of 1 ml of 0.05% Tween 80 solution (v/v) was tested in parallel. In the second experiment, three fungal strains, *i.e.*, *B. bassiana* (strain GHA), *M. brunneum* strain (USDA4556) and *M. brunneum* (strain V275) were tested. One milliliter of conidial suspensions containing 10^8 CFUs were applied on anasthesiated adults following the same manual spraying procedure. In parallel, one chemical control treatment was performed (spraying of 1 ml of λ-cyhalothrin solution at 400µl/l) as well as a negative control (spraying of 1 ml of 0.05% Tween 80 solution).

After treatment, adults of *B. rufimanus* were immediately transferred to plastic boxes and kept in an incubator at 75% of relative humidity, a temperature of 20 °C a photoperiod of 12h, and fed with 10% sucrose solution. Adults of *C. maculatus* were kept in total darkness in plastic boxes in an incubator at 23°C. Each modality was repeated three or five times. Survivals were assessed at 48–h intervals during ten or fifteen days.

Dead individuals were counted and disinfected by soaking in 70% v/v ethanol, followed by three soaks in distilled water. To confirm fungal infection, cadavers were placed on moist filter papers in incubators at 25°C until the intersegmental emergence of entomopathogenic fungi (**Figure 39**).

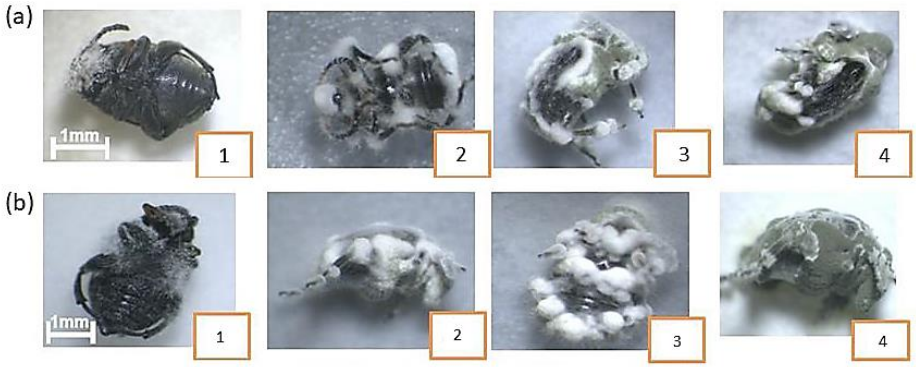

Figure 39: Emergence of *M. acridum* V275 (a) and *M. acridum* USDA 4556 (b) from infected cadaver of *B. rufimanus* during the four days following the insect death © R. Lugendo

The sublethal effect of each treatment on oviposition of *C. maculatus* was assessed via the sexing of adults (each batches presented a sexe ratio of 1:1) and the placement of 10 g of cowpea seeds in plastic boxes. The ovipositions were regularly checked and the average number of eggs was punderated with survival rate. The reproductive diapause of *B. rufimanus* adults prevented the evaluation of sublethal effects. Experimental specificities of the five bioassays are summarized in **Table 17**.

2.3 Statistical analyses

The median lethal times (LT_{50}) were estimated using a non-parametric method (Kaplan-Meier), which assigns a probability of survival based on the observed survival time (Kaplan and Meier 1958). The resulting survival curves were then compared using a non-parametric test approximately distributed as a chi-square test (log-rank test) to assess significant differences between the treatments and the control. All these statistical analyses were performed using RStudio software (Version 1.3.959) with the "*survival*" and "*survminer*" packages.

Table 17: Synthesis on the different EFs lethality assessments carried out on *C. maculatus* and *B. rufimanus.* PT = application with Potter Tower; MS = application by manual spraying; IO = Inhibition of ovipostion

Exp. Id	Bruchid species	Treatment	Medium	Biological replicates	N bruchids	Exp. time	CO_2 Anesthesia	Spraying method	Amount of CFUs/insects	Observation
1	*C. maculatus*	No treatment	-	3	90	15	No	-	-	LT50 + IO
		A. flavus (strain MUCL55276) 1 ml - 10^8 CFUs/ml	PDA + Chloramphenicol	3	90	15	Yes	PT	4.76×10^3	LT50
		A. flavus (strain MUCL55276) 1 ml - 10^8 CFUs/ml	PDA + Chloramphenicol	3	90	15	Yes	MS	1.15×10^5	LT50 + IO
2	*C. maculatus*	*B. bassiana* (strain GHA) 1 ml - 2×10^8 CFUs/ml	PDA + Chloramphenicol	3	150	15	No	MS	$> 2.30 \times 10^5$	LT50
		A. flavus (strain MUCL55276) 1 ml - 10^8 CFUs/ml	PDA + Chloramphenicol	5	150	15	No	MS	$> 1.15 \times 10^5$	LT50
		M. acridum (strain IMI330189) 1 ml - 10^8 CFUs/ml	PDA + Chloramphenicol	3	90	15	No	MS	$> 1.15 \times 10^5$	LT50
		Tween 80 (0.05%) - 1 ml	-	3	90	15	No	MS	-	LT50
3	*B. rufimanus*	*B. bassiana* (strain GHA) 1 ml - 2×10^8 CFUs/ml	PDA + Chloramphenicol	3	150	15	No	MS	$> 5.12 \times 10^5$	LT50
		A. flavus (strain MUCL55276) 1 ml - 10^8 CFUs/ml	PDA + Chloramphenicol	5	150	15	No	MS	$> 2.56 \times 10^5$	LT50
		M. acridum (strain IMI330189) 1 ml - 10^8 CFUs/ml	PDA + Chloramphenicol	3	90	15	No	MS	$> 2.56 \times 10^5$	LT50
		Tween 80 (0.05%) - 1 ml	-	3	90	15	No	MS	-	LT50

(Continued)

Table 13 (continued): Synthesis on the different EFs lethality assessments carried out on *C. maculatus* and *B. rufimanus*. PT = application with Potter Tower; MS = application by manual spraying; IO = Inhibition of ovipostion

Exp. Id	Bruchid species	Treatment	Medium	Biological replicates	N bruchids	Exp. time	CO_2 Anesthesia	Spraying method	Amount of CFUs/insects	Observation
4	*C. maculatus*	*B. bassiana* (strain GHA) 1 ml - 10^8 CFUs/ml	SDA	3	60	10	Yes	MS	1.15×10^5	LT50 + IO
		M. brunneum (strain USDA4556) 1 ml - 10^8 CFUs/ml	SDA	3	60	10	Yes	MS	1.15×10^5	LT50 + IO
		M. brunneum (strain V275) 1 ml - 10^8 CFUs/ml	SDA	3	60	10	Yes	MS	1.15×10^5	LT50 + IO
		λ-cyhalothrin (400 µl/l) - 1 ml	-	3	60	10	Yes	MS	-	LT50 + IO
		Tween 80 (0.05%) - 1 ml	-	3	60	10	Yes	MS	-	LT50 + IO
5	*B. rufimanus*	*B. bassiana* (strain GHA) 1 ml - 10^8 CFUs/ml	SDA	3	60	10	Yes	MS	2.56×10^5	LT50
		M. brunneum (strain USDA4556) 1 ml - 10^8 CFUs/ml	SDA	3	60	10	Yes	MS	2.56×10^5	LT50
		M. brunneum (strain V275) 1 ml - 10^8 CFUs/ml	SDA	3	60	10	Yes	MS	2.56×10^5	LT50
		λ-cyhalothrin (400 µl/l) - 1 ml	-	3	60	10	Yes	MS	-	LT50
		Tween 80 (0.05%) - 1 ml	-	3	60	10	Yes	MS	-	LT50

3 Results and discussion

3.1 Lethality assessment on *C. maculatus* and *B. rufimanus*

The mortality of 1830 bruchids was monitored up to fifteen days during these five bioassays including (i) 270 sexed adults of *C. maculatus* for the comparison of two application methods and the inhibition of oviposition, (ii) 780 adults of *C. maculatus* for the assessment of LT50 against five EFs strains, *i.e.*, *B. bassiana* (GHA), *M. acridum* (IMI1330189), *A. flavus* (MUCL 55276), *M. brunneum* (V275) and *M. brunneum* (USDA4556) and their potential inhibitory effects on oviposition, and (iii) 780 adults of *B. rufimanus* for the assessment of LT50 against five EFs strains, *i.e.*, *B. bassiana* (GHA), *M. acridum* (IMI1330189), *A. flavus* (MUCL 55276), *M. brunneum* (V275) and *M. brunneum* (USDA4556). Survival curves of each treatment are presented in **Figure 40**. Parameters related to the Kaplan-Meier survival curves estimation and the log-ranks tests are presented in **Table 18**.

In general, conidial suspensions have quicker lethal effects on *C. maculatus* compared to *B. rufimanus*. For both bruchid species, the best results in terms of lethality (*i.e.*, shortest LT50 values) are achieved with *B. bassiana* (GHA) with LT50 of five days ($\chi^2_{(0.95,\ 1)}$ =103, p<0.001) on *C. maculatus* and four days ($\chi^2_{(0.95,\ 1)}$ =101, p<0.001) on *B. rufimanus*. Regarding other strains, *M. brunneum* V275 and USDA 4556 also demonstrated interesting LT50 values on *C. maculatus*, with LT50 estimated at six days ($\chi^2_{(0.95,\ 1)}$ =64, p<0.001 ; $\chi^2_{(0.95,\ 1)}$ =92.3, p<0.001), while on *B. rufimanus* their respective LT50 were estimated at eight days ($\chi^2_{(0.95,\ 1)}$ =53.5, p<0.001) and six days ($\chi^2_{(0.95,\ 1)}$ =71.4, p<0.001). *A. flavus* and *M. acrifum* seemed not as effective as other strains but this could be explained by the different growing media. Indeed, *B. bassiana* (GHA) exhibited TL50 values on *B. rufimanus* that were twice as long when cultivated on PDA+chloramphenicol compared to SDA. SDA therefore seems to improve the virulence of EFs rather than PDA, which is a commonly used media for EFs screening experiments. Surprisingly, the chemical control, lambda-cyhalothrin at 400 µl/l, had a longer LT50 than *B. bassiana* (strain GHA) on *C. maculatus*. This could be either due to a resistance mechanism acquired by the

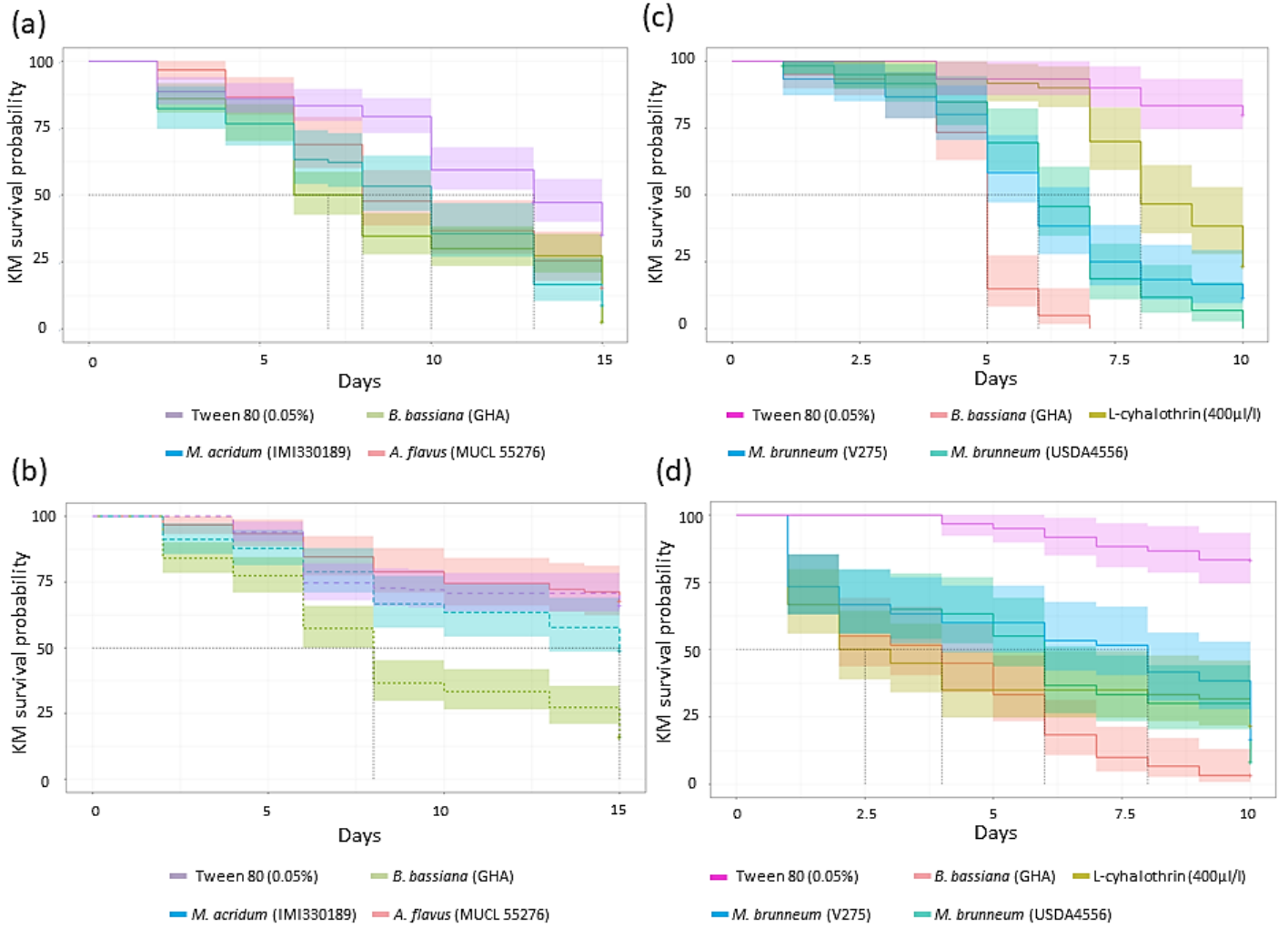

Figure 40: Survival curves after treatments performed on *C. maculatus* in bioassays 2 (a) and 4 (c); Survival curves after treatments performed on *B. rufimanus* in bioassays 3 (c) and 5 (d). LT_{50} estimations are reported by the dotted lines

Table 18: Synthesis of EFs lethality bioassay results (LCL = lower confidence interval; UCL = upper confidence interval; Chisq = the chi-square statistic of the log-rank test of equality; Df = degrees of freedom related to of the log-rank test of equality; P = p-value related to the log-rank test of equality)

			Kaplan-Meier survival estimates					Log-rank test *VS* control			Log-rank test all treatments		
Exp. Id	**Bruchid species**	**Treatments**	**n**	**Events**	**Median**	**0.95LCL**	**0.95UCL**	**Chisq**	**Df**	**Pval**	**Chisq**	**Df**	**Pval**
1	*C. maculatus*	*A. flavus* (MUCL 55276) - MS	90	59	10	10	12	24.9	1	<0.001			
		A. flavus (MUCL 55276) - PT	90	53	14	10	>15	15.5	1	<0.001	26.2	2	<0.001
		Tween 80 (0.05%) - control	90	26	>15	>15	>15	/	/	/			
2	*C. maculatus*	*A. flavus* (MUCL 55276)	90	76	**8**	8	10	17	1	<0.001			
		B. bassiana (GHA)	150	146	**7**	6	8	51.2	1	<0.001	57.9	3	<0.001
		M. acridum (IMI330189)	90	82	**10**	8	10	29.2	1	<0.001			
		Tween 80 (0.05%) - control	150	97	13	13	15	/	/	/			
3	*B. rufimanus*	*A. flavus* (MUCL 55276)	90	29	>15	>15	>15	0.1	1	0.8			
		B. bassiana (GHA)	150	125	**8**	6	8	72.4	1	<0.001	105	3	<0.001
		M. acridum (IMI330189)	90	46	15	13	>15	6.3	1	0.01			
		Tween 80 (0.05%) - control	150	51	>15	>15	>15	/	/	/			
4	*C. maculatus*	*B. bassiana* (GHA)	60	60	**5**	5	5	103	1	<0.001			
		L-cyhalothrin (400µl/l)	60	46	**8**	8	10	36.1	1	<0.001			
		M. brunneum (USDA 4556)	60	59	**6**	6	7	92.3	1	<0.001	199	4	<0.001
		M. brunneum (V275)	60	53	**6**	5	7	64	1	<0.001			
		Tween 80 (0.05%) - control	60	12	>10	>10	>10	/	/	/			
5	*B. rufimanus*	*B. bassiana* (GHA)	60	58	**4**	2	5	101	1	<0.001			
		L-cyhalothrin (400µl/l)	60	47	**2.5**	2	4	54	1	<0.001			
		M. brunneum (USDA 4556)	60	55	**6**	5	7	71.4	1	<0.001	105	4	<0.001
		M. brunneum (V275)	60	50	**8**	4	10	53.5	1	<0.001			
		Tween 80 (0.05%) - control	60	10	>10	>10	>10	/	/	/			

insect, which has a relatively high number of generations per year, or the high temperature (23°C) that reduced the effectiveness of the active substances.

3.2 Sublethal effects on *C. maculatus*

Sublethal effects on the of *C. maculatus* are presented in **Figure 41.** Globally, all EFs strains induced a reduction of the punderated ovipositions (*i.e.*, nb of ovipositions/survival rate), except for the treatment *B. bassiana* (GHA) after six days of the fourth bioassay. This may be explained by the high mortalities observed in the treated insects that were below 10% of surfical rate, which increased the puderated oviposition more than the real insect fecundity **Figure 41 c**. In the first experiment (**Figure 41 a**), the total punderated ovipositions in the *A. flavus* threatment was 221.80 eggs *vs* 485.47 in the control, *i.e.*, a reduction of fecundity of 54.31%. During the fourth experiment **(Figure 41 b)**, the total punderated oviposition in each treatments were 393.33 eggs for *B. bassiana* (GHA), 241.15 eggs for λ-cyhalotrin (400µl/l), 303 eggs for *M. brunneum* (USDA 4556) and 336.02 for *M. brunneum* (V275) *vs* 654.91 eggs in the control, corresponding to respective fecundity reductions of 39.94%, 63.17%, 53.73% and 48.69%.

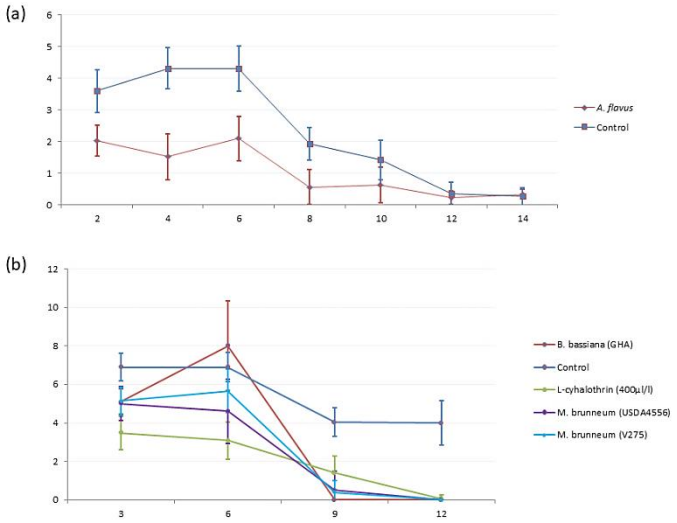

Figure 41: Inhibitory effects of different treatments on *C. maculatus* during the exeriments 1 (a) and the experiment 4 (b), *i.e.*, averages and standard deviations of punderated egg-countings on ten randomly selected seeds

3.3 EFs as potential biopesticide or biocontrol agents against *B. rufimanus*

Optimal time for EFs to kill their host should be inferior to five days (Shah and Pell 2003). Given the lethality results, most promising strains for *B. rufimanus* control may be ranked as follow: *B. bassiana* (GHA), *M. brunneum* (USDA4556), *M. brunneum* (V275). Furhter works should be carried out on other strains amonfg these EFs species that are numerous and may present more specific virulence against bruchids (Jaronski 2020, personal communications). For example, some European strains of EFs were proved to show better lethality on *C. maculatus*, *i.e., B. bassiana* (strain 0305 (1186)) originating from France and conserved at the USDA-ARS Collection of Entomopathogenic Fungal Cultures, Ithaca, New York (Cherry *et al.* 2005). However, some strains of *B. bassiana* were also pointed for their collateral effects on the honey bee nestmate recognition behavior that should promote risks of forager drift and desease spread across colonies (Cappa *et al.* 2019). The assessments of most specific pests should therefore ensure the absence of collateral effects on beneficial entomofauna of faba bean crops.

Based on these preliminary results, it could be assumed that innundative delivery methods of entomopathogenic fungi such as large scale crop spraying to target adults before oviposition could be effective. However, their application on field should take into consideration (i) the biology of the pest that oviposit at the base of the plants, (ii) the potential unfavorable field factors decreasing conidia viability, *i.e.* UV light, desiccation, and high temperature ranges at the top of the canopy, (iii) the dense canopy of *V. faba* preventing the sprayed to reach the target parts of the plants, and (iv) the economic viability of the control method. For example, the standard label rate of *B. bassiana* (GHA) corresponds to 2,5 x 10^{13} conidia/ha (Wraight and Ramos 2002; Lacey and Kaya 2007). In this sense, large scale field application methods such as dropleg sprayings would correspond to these requirements by targeting the plants organs that are directly attractive to ovipositing *B. rufimanus* females while reducing the expositions of the upper flowers attractive to beneficials (Hausmann *et al.* 2019).

Another potential strategy for the use of EFs as biocontrol agents of *B. rufimanus* would be the innoculative approach based on *attract and infect* strategy. *Attract and infect* strategy against phytophagous insects consist in the co-formulation of insects pathogens with some attractive volatile organic compounds (VOCs) such as sexual/aggregation pheromones or host plant kairomones (El-Sayed *et al.* 2009). Previous chapter highlighted that semiochemical attractants such as floral kairomones could be the most attractive for flying adults of *B. rufimanus*. The coformulation of the most specific EFs against bruchid in adapted devives favoring the insect contact with dry conidia would therefore benefit from potential autodisseminations of conidia adults of *B. rufimanus* and conspecific contamination during mating such as observed with other coleopteran pests devices where they would enter in contact with dry conidia was already successfully performed for other coleopteran pests (Vega *et al.* 1995; Klein and Lacey 1999). Moreover, the specificity of the EFs strain combined with kairomonal attractants would transform the lack of semiochemical specificity, *i.e.*, the collateral attractivity towards pollinators before BBWs infestations (see conclusion from previous chapter), into a considerable asset whether the autodissemination of EFs conida in faba bean flowers (*i.e.*, the bruchids feeding resource) would be increased by co-attracted flying pollinators before the BBWs field colonization. Such phenomenon could already be observed for other pests, *i.e.* entomovectoring (Smagghe *et al.* 2020).

Finally, another potential use of EFs as biocontrol agents to explore would be their potential endophiytic activity. Endophytic fungi are described as mutualistic microbes living in healthy plants and protecting them against insect herbivory and diseases (Stone *et al.* 2004; Lacey *et al.* 2015). Control method based on endophytic fungi against BBW would consist in colonization of *V. faba* tissue by the fungus providing protection against herbivorous stages of this pest (*i.e.* seminovorous larvae), as a result of feeding deterrence, antibiosis phenomena, or triggering host plant defenses (Lacey *et al.* 2015). This method would be interesting as it targets larval development which is the

most sensitive and out of reach development stage of BBWs (Seidenglanz and Hunady 2016).

4 Conclusions

Five entomopathogenic strains were tested in laboratory conditions against two bruchid species, *i.e.*, *C. maculatus* and *B. rufimanus*. Most lethal strain agains *B. rufimanus* was *B. bassiana* (GHA) and all EFs strains showed inhibitory effects on *C. maculatus* ovipositions. These preliminary results may suggest two potential strategies of EFs crop applications to avoid *B. rufimanus* ovoposition including dropleg spraying (*cf.*, innundative strategy) or attract and infect dispositive (*cf.*, innoculative strategy). Further researches such as endophytic fungi could also provide suitable applications to the pest biology

Chapter VI (part. 2)

Biotechnological control – Gene silencing with RNA interference

1 Introduction to chapter VI (part 2.)

In this second part, a new emergent selective tool for pest management strategies is addressed *i.e.*, the RNA interference (RNAi). This biotechnological method of control has enormous potential applications extending to viruses, fungi, bacteria, insects or plants. This technic relies on the cellular biological process of transcriptional silencing which is trigerred by specific double stranded RNA (dsRNA) (Zhang *et al.* 2022).

Recently, RNAi technique lead to the development of new generation of biopesticides against coleopteran pest like *Leptinotarsa decemlineata* (Say 1824) or *Diabrotica virgifera* (J. L. LeConte 1868) but none studies assessed yet the portential of RNAi with bruchids. Necessary prerequesite in the elaboration of gene silencing is the knowledge of the target pest genome and/or transcriptome for the synthesis of dsRNA. Unfortunately, the genome *B. rufimanus* could not yet be sequenced. To our knowledge, the only genomic information about bruchid species concerns *C. maculatus*, for which the transcriptome was recently made available by Sayadi *et al.* (2016). This chapter therefore developed micro-injection method for the administration of dsRNA to adults of *C. maculatus.* The post-injections gene expressions patterns were assessed via RT-qPCR and revealed for the first time a specific gene silencing in a bruchid species, which offers new possibilities en the areas of bruchids control.

2 Gene Silencing of *laccase 1* induced by double-stranded RNA in *Callosobruchus maculatus* (Fabricius 1775) (Coleoptera: Chrysomelidae) suggests RNAi as a potential new biotechnological tool for bruchid's control

Taken from the following reference:

Segers, A., Carpentier, J., Francis, F., & Caparros Megido, R. **(2023)**. *Gene Silencing of laccase 1 Induced by Double-Stranded RNA in Callosobruchus maculatus (Fabricius 1775) (Coleoptera: Chrysomelidae) Suggests RNAi as a Potential New Biotechnological Tool for Bruchid's Control.* Agriculture, 13(2), Article 2.

Abstract: Bruchids are the most important pests of leguminous seeds in the world. In this study, the focus was done on *Callosobruchus maculatus*, a serious pest of *Vigna unguiculata* seeds. As no efficient control methods preventing collateral effects on beneficials currently exist, this study investigated whether RNA interference (RNAi) could provide a new biotechnological and selective tool for bruchids control. Three principal objectives were followed including (i) the identification of all RNAi machinery core components and a key protein to silence in *C. maculatus* genome (*cf.*, *dicer-2*, *argonaute-2*, *R2D2*, and *laccase 1*), (ii) the identification of suitable reference gene for RT-qPCR analyses, and (iii) the micro-injection of dsRNA coding for *laccase 1* to adults of *C. maculatus* to assess gene expression levels by RT-qPCR and potentially related mortalities. Phylogenetical analyses performed from transcriptomic information successfully identified all necessary proteins in the RNAi mechanism and also the open reading frame of *laccase 1* in *C. maculatus*. A new reference gene was identified (i.e., *alpha-tubuline 1*) and coupled with *glutiathone S transferase* for RT-qPCR analyses. Double-stranded RNAs coding for *laccase 1* and *green fluorescent protein* (control) were produced and 400 ng of each dsRNA were micro-injected into *C. maculatus* adults. RT-qPCR analyses revealed a stable significant decrease in *laccase 1* expression in about 80% of adults treated with

laccase 1 dsRNA after three days post-injection. No significant mortalities were observed which is probably related to the non-exposure of adults to anti-nutritional factors that are usually regulated by laccase. Further research should focus either on the feeding larval stage which is directly exposed to anti-nutritional factors, or on other target genes to induce dead phenotypes. This study is the first gene silencing report on a bruchid species and supports RNAi as a potential future method of control.

Keywords: RNAi; Bruchidae; Bruchinae; reference gene; RT-qPCR; RNA interference; dicer; argonaute; RISC

2.1 Introduction

Bruchids (also commonly named "seed beetles") are severe pests of leguminous seeds causing important economic losses. These pests correspond to small Coleoptera belonging to the family of Chrysomelidae and to the subfamily Bruchinae, which en-compass 1700 recorded species, out of which 30 species are of economic concern and nine species are distributed worldwide (including genera *Acanthoscelides*, *Bruchus*, *Callosobruchus*, *Caryedon*, and *Zabrotes*) (Kingsolver 2004; Kergoat *et al.* 2007; Segers *et al.* 2021). *Callosobruchus maculatus* (Fabricius 1775), commonly named "the cowpea weevil", is a multivoltine species that infests seeds of Cowpea crops (*Vigna unguiculata* ((L.)Walp.—Fabaceae). This pest is particularly harmful in West Africa where 20 to 90% of beans are ravaged while constituting an essential food resource for local populations (Caswell 1977). Damage is caused by seminovorous larvae which entirely develop inside seeds (Credland and Dick 1987). Females are able to oviposit on ripening pods in the field (*i.e.*, primary infestation) and also on stored seeds (*i.e.*, secondary infestation). Howe and Currie (1964) reported a maximal fecundity of 97.2 eggs per female on average and the shortest development period lasts 23 days at 35 °C. The thermal development of this pest is 526.3 degree-days with a developmental threshold of 10.4 °C (Mobarakian *et al.* 2014). Such characteristics are highly detrimental to the stored commodities because only two percent of primary infestation in the field leads to the total destruction of crop products after 6 months of storage (Alebeek 1996).

The current management of *C. maculatus* in storage mostly relies on the use of chemical insecticides by fumigation, but technical and financial locks impede this technique which also presents well-known impacts on the environment, human health, non-target organisms (NTOs), and is faced with the emergence of insect resistances (Karaağaç 2012; Tiroesele *et al.* 2015; Naqqash *et al.* 2016; P. Vivekanandhan *et al.* 2021). Alternatives such as essential oils and plant extracts were widely investigated but most studies demonstrated that these treatments were impacting beneficial insects such as chalcidoid parasitoids. In addition, their use was effective for a short duration which required repeated applications (Aziz and Abbass 2010; Esther Ojebode and Ojo Olaiya 2016; Nattudurai *et al.* 2017; Idoko and Ileke 2020). Innovative and selective methods of control must be investigated to find effective management strategies to minimize collateral effects on NTOs.

RNA interference (RNAi) is an emerging pest control method in integrated pest management (IPM) (Rodrigues and Figueira 2016). This biotechnological method relies on the introduction of exogenous double-stranded RNA (dsRNA) in insects that will decrease the expression level of a target gene via three possible pathways including the microRNA (miRNA), the piwiRNA (piRNA), and the small interfering RNA (siRNA) (Zhao *et al.* 2015; Rodrigues and Figueira 2016). In the siRNA pathway, dsRNAs enter in insect cells (**Figure 42**) and are cleaved into siRNAs duplexes of 21–24 nucleotides (nt) by the enzyme *dicer-2* (*dcr-2*) (Zamore *et al.* 2000; Kim *et al.* 2006). SiRNAs are then incorporated into a multi-protein complex named the *RNA-induced silencing complex* (*RISC*) via the RNA-binding protein called *R2D2* (Liu *et al.* 2003; Meister and Tuschl 2004). One strand is degraded and the other strand guides the complex for homological recognition with the target gene transcript, *i.e.*, the messenger RNA (mRNA) (Kyre *et al.* 2020). *Argonaute-2* (*Ago-2*) proteins, the core catalytic components of *RISC*, then degrade the mRNA preventing the translation into protein (Hammond *et al.* 2000; Hammond 2005; Sen and Blau 2006). The prevention enzymes or protein production is expected to cause lethal effects of the targeted pest.

RNAi technology has been widely applied to insects (Baum and Roberts 2014). The principal asset of this biotechnological control tool is the absence of collateral effects in NTOs as at least 19 nucleotide homology is required for activation of the RNAi pathway (Whyard *et al.* 2009; Chen *et al.* 2021). However, its success varies considerably among pests (Baum and Roberts 2014) and depends on intraspecific factors such as the presence of RNAi core machinery, *i.e.*, *dicer-2*, *argonaute-2*, and *R2D2* (Zhao *et al.* 2015), the length of dsRNAs (Li *et al.* 2015), the presence of transmembrane proteins that allow entry of dsRNAs into cells (Cappelle *et al.* 2016), the targeted stage in insect life cycle (Huvenne and Smagghe 2010; Guo *et al.* 2015), the targeted gene (Kola *et al.* 2015), and the method of dsRNA delivery (Yu *et al.* 2013). Molecular mechanisms driving cellular uptake and systemic spread of silencing are the most impacting components on RNAi success (Joga *et al.* 2016).

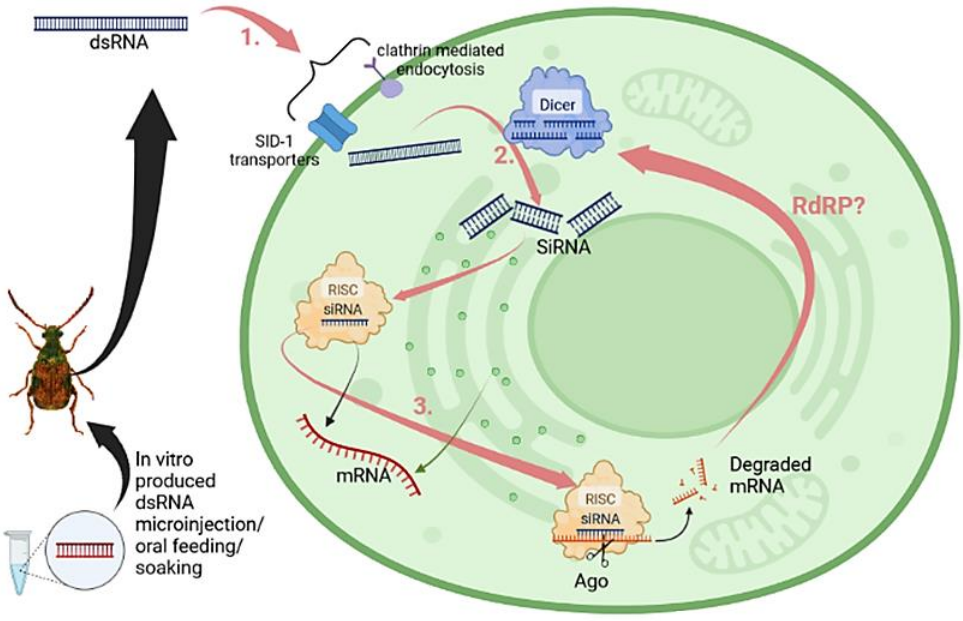

Figure 42 : RNAi mechanism by siRNA pathway in insect cells and proteins involved in the degra-dation of double-stranded RNA into small interfering RNAs. The proteins involved in this pathway include *dicer-2* proteins that cleave exogenous dsRNAs, and the *RNA induced silencing complex* (*RISC*) containing *argonaute-2* and *R2D2* proteins for the homological degradation of the messenger RNA (mRNA). In some insects, the presence of *RNA-dependent RNA polymerases* (*RdRp*) is reported to retroactively amplify the siRNA mechanism. © A. Segers (created with biorender).

Many herbivorous beetles have already been subject to RNAi studies including species from Chrysomelidae, Tenebrionidae, Coccinelidae, Curculionidae, Nitidulidae, Buprestidae, and Cerambycidae families (Willow and Veromann 2021). Studies led on leaf beetles (Chrysomelidae) such as *Diabrotica virgifera* (J. L. LeConte 1868), *Leptinotarsa decemlineata* (Say 1824), *Phaedon cochleariae* (Fabricius 1792), or *Plagiodera versicolora* (Laicharting 1781) showed high RNAi efficiency via feeding on transgenic plants, diets containing naked dsRNA or bacterially expressed dsRNA (Baum *et al.* 2007; Vélez *et al.* 2020). However, no study has yet broached RNAi with bruchid species while the cancellation of key proteins and enzyme production in bruchids with RNAi could provide a pledging alternative facing limitations or current methods of control. Recently, Zhang *et al.* (2018) demonstrated that *laccase 1* (*lac 1*) was a polyphenol oxidase involved in the detoxification process of phenolic compounds in *Sitobion avenae* (Fabricius 1775) (Hemiptera: Aphididae). In the case of cowpea weevils, tannin content is also a potential biochemical defense against feeding larvae (Lattanzio *et al.* 2005). Avoiding the production of enzymes involved in bruchid polyphenol detoxification mechanisms, such as *lac 1*, would provide a new tool supporting a new biotechnological method of control.

In this study, the RNAi mechanism and the gene silencing of *lac 1* were investigated as a potential new biotechnological tool of bruchid's control. As the RNAi systemic mechanism could not be conserved across organisms (Rodrigues and Figueira 2016), three principal objectives were followed including (i) the identification of all RNAi machinery core components and a key protein to silence in the C. maculatus genome (*cf.*, *dicer-2*, *argonaute-2*, *R2D2*, and *lac 1*), (ii) the identification of suitable reference gene for RT-qPCR analyses, and (iii) the micro-injection of *lac 1* dsRNA to adults of *C. maculatus* to assess gene expressions levels by RT-qPCR and potentially related mortalities. Results provided identifications and descriptions of all investigated proteins or protein complexes. A new reference gene for RT-qPCR analyses in adults of *C. maculatus* was highlighted (*i.e.*, *alpha-tubuline 1*) and micro-injection experiments demonstrated that RNAi decreased

significantly in the *lac 1* expression. This study is the first report of RNAi in a bruchid species which provides new insight into a potential method of control of these pests.

2.2 Material and methods

2.2.1 Identification of lac 1, dcr-2, ago-2, and R2D2 in C. maculatus

Sequences of *Dcr-2*, *Ago-2*, *R2D2*, and *lac 1* were identified in *C. maculatus* following a quite similar methodology as Zhao *et al.* (2015). First, protein queries in *Tribolium castaneum* (Herbst 1797) (Coleoptera: Tenebrionidae), a reference model of beetle, were performed and followed by translated basic local alignment search with tBLASTn on transcriptomic information of *C. maculatus* provided by Sayadi *et al.* (2016) (E-value threshold: 1×10^{-10}). The sequence *Tcas-Dcr-2* (NP_001107840.1), *Tcas-Ago-2a* (NP_001107842.1), *Tcas-R2D2* (NP_001128425.1), and *Tcas-Lac-1* (NP 001034514.1) were searched against the Transcriptome Shotgun Assembly (TSA) database limited by *C. maculatus* (taxid: 64391). The RNA sequences obtained were identified according to the E-value and the percentage identity and were subsequently assigned as *Dcr-2* (GEUD01209535.1), *Ago-2* (GEUH01006697.1), *R2D2* (GEUD01188481.1), and *lac 1* (GJDX01063393.1). These sequences were downloaded for manual trimming and truncation with Serial Cloner software (version 2.6.1). On the basis of these RNA sequences, corresponding *C. maculatus* proteins were generated.

Then, protein sequences of *C. maculatus* were submitted for the detection of domain architecture using the ScanProsite tool (de Castro *et al.* 2006). The proteins involved in the RNAi mechanism (*cf.*, *Dcr-2*, *Ago-2*, and *R2D2*) were tested with the amino acid sequences from *T. castaneum*, *Agrilus planipennis* Fairmaire 1888 (Coleoptera: Buprestidae), and *Drosophila melanogaster* Macquart 1843 (Diptera: Drosophilidae). As there may be several forms of *dicer* (two forms) or *argonaute* proteins (three forms) (Zhao *et al.* 2015), all possible forms were considered and phylogenetic analyses were performed to confirm that the sequence corresponded to *Dcr-2* or *Ago-2* following the

clustering. Amino acid sequences used in these analyses are provided in **Table 19.**

Table 19: Accession number of amino acid sequences used for the analyses of the protein domain architecture concerning *Dcr-2*, *Ago-2*, and *R2D2* in *C. maculatus*.

	Callosobruchus maculatus		
	Cmac-Dcr-2 ***GEUD01209535.1***	***Cmac-Ago-2*** **GEUH01006697.1**	***Cmac-R2D2*** **GEUD01188481.1**
Tribolium castaneum	*Tcas-Dcr-1*, XP_968993.2 *Tcas-Dcr-2*, NP_001107840.1	*Tcas-Ago-1*, EFA09197.2 *Tcas-Ago-2a*, NP_001107842.1 *Tcas-Ago-2b*, NP_001107828.1 *Tcas-Ago-3*, XP_968053.2	*Tcas-R2D2*, NP_001128425.1
Argillus plannipennis	*Apla-Dcr-1*, AJF15702.1 *Apla-Dcr-2*, AJF15703.1	*Apla-Ago-1*, AJF15704.1 *Apla-Ago-2*, AJF15705.1 *Apla-Ago-3*, AJF15706.1	*Apla-R2D2*, AJF15707.1
Drosophila melanogaster	*Dmel-Dcr-1*, AAF56056.1 *Dmel-Dcr-2*, NP_523778.2	*Dmel-Ago-1*, AAF58314.1 *Dmel-Ago-2*, AGB94575.1 *Dmel-Ago-3*, NP_001163498.1	*Dmel-R2D2*, NP_609152.1

Concerning the identification of *lac 1*, the two forms of insect laccases (*laccase 1* and *laccase 2*) were considered in seven species for which sequences have been previously listed. A phylogenetic analysis was performed to confirm whether the putative sequence considered in *C. maculatus* fitted with *laccase 1* (Janusz *et al.* 2020). The sequence *Cmac-Lac1* (GJDX01063393.1) was tested with *T. castaneum* (*Tcas-Lac1*, NP 001034514.1; *Tcas-Lac-2*; NP 001034487.2), *Absolus verrucosus* (LeConte 1852) (Coleoptera: Tenebrionidae) (*Aver-Lac1*, RZB39173.1; *Aver-Lac-2*, RZC35935.1), *Monochamus alternatus* Hope 1843 (Coleoptera: Cerambycidae) (*Malt-Lac1*, ATI08981.1; *Malt-Lac-2*, ABU68466.1), *Apis mellifera* L. 1758 (Hymenoptera: Apidae) (*Amel-Lac1*, XP 026295929.1; *Amel-Lac-2*, BAJ06133.1), *Manduca sexta* L. 1763 (Lepidoptera: Sphingidae) (*Msex-Lac1*, AAN17506.1; *Msex-Lac-2*, AAN17507.1), *Nephotettix cincticeps* Uhler 1896 (Hemiptera: Cicadellidae) (*Ncin-Lac1*, BAJ06132.1; *Ncin-Lac-2*, BAJ06133.1) and *Anopheles sinensis* Wiedemann 1828 (Diptera: Culicidae) (*Asin-Lac1*, KFB43437.1; *Asin-Lac-2*, ARG47519.1). After the identification of *lac1*, pairs of primers were designed for the amplification of the gene from the cDNA of *C. maculatus* (**Table 20**). Electrophoresis gel migrations and a Sanger sequencing

were performed on PCR products by Eurofins Genomics (Germany) and confirmed the *lac 1* gene identity (data not shown).

Table 20 : Primers used for *laccase 1* and *gfp* gene amplification.

Gene	Primers	Sequence (5′ to 3′)	Amplicon Size (bp)
lac 1	Lac1-CM-F	ATT CCT GTT TTA AAT AAT TTG ATG ACA TG	2433
	Lac1-CM-R	TTG ATG TGT CAC TGT GTT TCT	
T7—*lac 1*	Lac1-CM-T7-F′	TAA TAC GAC TCA CTA TAG GG TGT CTT TGC TTC CGT TCC C	588
	Lac1-CM-T7-R′	TAA TAC GAC TCA CTA TAG GG CGT GAT GCT CTA TTG CTT TCC	
T7—*gfp*	GFP-T7-F′	TAA TAC GAC TCA CTA TAG GG GCC AAC CTT AGT CAC TAC TTT C	542
	GFP-T7-R′	TAA TAC GAC TCA CTA TAG GG TGG GTA ATA CCA GCA GCA G	

The ORF of *C. maculatus lac 1* was deduced with the *ORF finder* (https://www.ncbi.nlm.nih.gov/orffinder/, accessed on 24 November 2022) tool. The deduced protein sequence of amino acids was analyzed with the *Protein Molecular Weight* (https://www.bioinformatics.org/sms/prot_mw.html, accessed on 24 November 2022) tool and the Cu-oxidase Pfam domains were predicted with the *SMART* (http://smart.embl-heidelberg.de/, accessed on 24 November 2022) tool.

Phylogenetic analyses were performed using MEGA *version X* and MUSCLE alignment (Edgar 2004; Kumar *et al.* 2018). The appropriate model was determined for each of the sequence sets prior to the tree construction and were « LG+G+F » for *Dcr-2*, « LG+G+I » for *Ago-2*, and « LG+G » for *lac 1*. The Maximum Likelihood (ML) method with partial deletion was used to construct phylogenetic trees. The Bootstrap value was fixed at 1000 replicates.

2.2.2 DsRNA synthesis

Two types of dsRNA were produced for micro-injection experiments. The DsRNA coding for the *C. maculatus laccase 1* was synthesized to induce the decrease in *lac 1* expression and dsRNA coding for the *green fluorescent protein* (*gfp*), was synthesized to ensure the specificity of the RNAi mechanism on the target (*i.e.*, cellular mRNA of *lac 1*) that should not impact the expression level of *lac 1*. *Green fluorescent protein* amplicons of 542 nt containing a T7 promoter sequence were obtained by PCR using star plasmids (see primers in **Table 20**) and the kit Q5® High-Fidelity PCR (New England Biolabs, Inc). *Laccase 1*

dsRNAs were produced by extracting total RNA from adults of *C. maculatus* with the kit RNeasy® (Qiagen, Chats-worth, CA, USA). Total RNA was checked for quality and quantity with a Nanodrop spectrophotometer. Retrotranscription was then performed with the kit High-Capacity cDNA reverse Transcription (Applied Biosystems, CA, USA) to obtain total cDNA. *Laccase 1* amplicons of 588 nt containing a T7 promoter sequence were finally obtained by PCR using a Q5® High-Fidelity PCR kit (see primers in **Table 20**). PCRs were performed with an initial denaturation cycle of 30 s at 98 °C, followed by 35 cycles of denaturation at 98 °C for 10 s, annealing for 30 s at 54 °C (amplification of *gfp*) or 64° (amplification of *lac 1*) and extension at 72 °C for 30 s. The final extension was maintained at 72 °C for 2 min. The size and the quality of the amplicons were checked by electrophoresis gel migration (1% agar), then purified with the Nucleospin® kit (MACHEREY-NAGEL GmbH & Co. KG, Valencia). The synthesis of dsRNAs was carried out from purified PCR products with the MEGASCRIPT® RNAi kit (Invitrogen, Waltham, MD). Around 1 µg of template DNA was used in 20 µL of the in-vitro transcription mix that was then incubated for 6h at 37 °C, before being treated for 15 min with DNAse/RNase and purified. DsRNAs were stored in an elution buffer (10 mM Tris-HCl pH 7, and 1 mM EDTA). The quality, size, and quantity of dsRNAs were also checked by NanoDrop spectrophotometer, then by electrophoresis gel migration (1% agar), and stored at −20 °C until micro-injection.

2.2.3 Micro-injection experiments

Adults of *C. maculatus* that were subjected to dsRNA micro-injections were reared on *V. unguiculata* seeds at 27 ± 2 °C and 60 ± 10% of relative humidity in total darkness. These insects came from Lincoln University (UK). Emerging adults were immediately collected from the rearing and were distributed in 10 × 10 × 5 cm boxes for micro-injection experiments.

Micro-injections were performed under a dissecting stereomicroscope (Euromex DZ series, Euromex microscope bv, Netherlands) using capillaries with a 10 mm long tip and 500 µm outer diameter designed from 1.0 mm × 0.50 mm BF100-50-10 silica tubes (model P-97 Flaming/Brown Micropipette

Puller, Sutter Instrument Company; program 0: heat = 555, pull = 150, time = 250, pressure = 500). These capillaries were mounted on a micropump (Nanoliter 2010, World Precision Instruments, Inc.) that was connected to a flow controller (Micro4™, World Precision Instruments, Inc.). Insects were microinjected at the pygidium with 400 nL of dsRNA solution at a rate of 200 nL/s and a concentration of 1000 ng/µL, *i.e.*, 400 ng of dsRNA were administered per insect. These insects were anesthetized for 30 s with CO_2 and then maintained on an adhesive surface cooled on crushed ice to maintain them immobile during micro-injections (**Figure 43**). Three treatments were performed on 50 insects: dsRNA of *laccase 1*, dsRNA of *gfp*, and a control consisting of micro-injection of the elution buffer (10 mM Tris-HCl pH 7, and 1 mM EDTA) to correct mortalities induced by micro-injections and to compare the gene expression profiles of *lac 1*. Three additional batches of 20 insects were subjected to the same micro-injection treatments to assess mortalities. Dead adults were recorded every day of post-injection for seven days.

Micro-injected insects were maintained at 23 ± 0.1 °C during the entire experimental period. Samples of three adults were polled as one biological replicate every 24 h after the injection (*i.e.*, day post-injection, dpi). Three biological replicates were sampled in each treatment per dpi. Sampled insects were flash-frozen in liquid nitrogen and stored at −80 °C for RNA extraction and retrotranscription. A summary diagram of the methodology used in the micro-injection experiments and the statistical analyses performed is presented in **Appendix G** of the supplementary material.

Figure 43 : Micro-injection dispositive for the administration of 400 nl of dsRNA/elution buffer at the pygidium of *C. maculatus*.

2.2.4 Reference gene and RT-qPCR analyses

Gene expression analyses were performed from reverse transcripted RNA by RT-qPCR to examine the gene expression profiles of *lac 1*. First, validation of reference genes, also named endogenous control or housekeeping gene (HKG), was necessary to normalize gene expression across samples that may present different levels of mRNAs due to methodological variations instead of biological causes (Shakeel *et al.* 2018). These reference genes are generally related to cellular functions such that their expression level is constant and independent of biotic variations such as the sex, the developmental stage, or the age of individuals and independent of abiotic factors such as temperature, photoperiod, or dsRNA treatments (Wallace and Rieske 2021).

As no reference genes were already identified in *C. maculatus* at the setting up of experiments, the stability of three reference genes in adults was investigated: *arginine-kinase* (*arg-K;* GEUF01011058.1), *alpha-tubulin1* (*tuba1*; GEUH01049608.1), and *beta-actin* (*bactin*; GEUH01052590.1). Their validation followed MIQE guidelines and a stepwise process for producing quality and reproducible data (Bustin *et al.* 2009; Taylor *et al.* 2019). Principal criteria necessary for the primer validation of HKGs and the target gene were (i) the relative efficiency of the primers (determined according to a regression line conducted on serial dilutions) must be between 90 and 110% (Pfaffl 2001), (ii) the absence of primer dimer in the melting curves to confirm the specificity of amplification. The validation of HKGs was then performed after stability tests by comparing the gene expression in seven samples that differed from age (*cf.,* biotic condition) and from dsRNA exposure (*cf.,* abiotic condition). The sample for stability test according to the age consisted in the pooling of three adults of the same age, from one to seven days old. Samples for stability test according to dsRNA exposure were three pooled adults from different dsRNA exposures (no dsRNA, 100 ng, 200 ng, 400 ng of microinjected *lac 1* dsRNA; 100 ng, 200 ng, 400 ng of microinjected *gfp* dsRNA). The GeNorm algorithm was used to characterize the stability of HKG expression (Vandesompele *et al.* 2002). Brar *et al.* (2022) completed the ongoing research in the selection of

reference genes by suggesting *gluthiatone S-transferase* (*gst*) and *tata binding protein* (*tbp*) as stable HKG in adults of *C. maculatus*.

Quantitative PCRs were performed with TB Green® Premix Ex Taq™ kit (Takara Bio Inc.) and were analyzed with the cfxMaestro™ software of the Bio Rad C1000 touch™ thermocycler, with two technical replicates. One RT-qPCR reaction contained 12.5 µL of TB Green Premix Ex Taq, 0.75 µL of each primer (10 µM), 2 µL of 25 ng/µL retro-transcripted cDNA, and 9 µL of nuclease-free ddH_2O. Thermal cycles consisted of initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 30 s, elongation at 72 °C for 30 s, and finalized at 95 °C for 30 s before generating melting curve to check the presence of non-specific products or primer dimers in *No Template Controls* (60 °C for 5 s to 95 °C with an increment of 0.5 °C/s).

2.2.5 Statistical Analyses

Relative gene expression analyses were based on the method of Vandesompele *et al.* (2002) using the geometric mean of two HGK Cq values for normalization. For statistical analyses, differences in the *lac 1* expressions (from *gfp* and *lac 1* treatments) were assessed with an unpaired t-test relative to the control group (*p*-value of 0.05). This test was based on the log2 converted expressions that were tested for normality with a Shapiro–Wilk Normality test.

Mortalities of each treatment were assessed with median lethal times (LT_{50}) that were estimated with a non-parametric method (Kaplan–Meier) attributing survival probability from observed survival time (Kaplan and Meier 1958). Survival curves obtained were then compared with a non-parametric equality test approximately distributed as a Chi-squared test (log-rank test), to check for significant differences between treatments and control. All these statistical treatments were performed with RStudio software *version 1.3.959*, using packages "*survival*" and "*survminer*".

2.3 Results

2.3.1 Identification and Description of RNAi Core Machinery (dcr-2, ago-2, and R2D2) in C. maculatus

Putative sequences of RNAi proteins were identified in *C. maculatus* after tBLASTn of *dcr-2*, *ago-2,* and *R2D2* proteins with *T. castaneum* followed by phylogenetic analyses considering all potential forms of these proteins (i.e., homolog sequences) in *A. plannipennis*, *D. melanogaster,* and *T. castaneum* to confirm that the putative sequences match to the RNAi proteins.

Phylogenetical trees showing similarities of the putative *C. maculatus* sequence with *dcr-2* and *ago-2* proteins of other insect species are presented in **Figure 44**. These trees highlight that the predicted *C. maculatus dicer* protein sequence is clustered with the *dcr-2* protein of *T. castaneum*, *A. planipennis,* and *D. melanogaster* with the maximal bootstrap value support, confirming the *dcr-2* homology (Cmac-Dcr-2). Concerning *ago-2,* the sequence Cmac-Ago-2 is distantly related to the homolog sequences of *ago-1* and *ago-3*. Moreover, it forms a subclade with the *ago-2* sequences from the Coleoptera species. These phylogenetic analyses are in line with the tBLASTn search results.

Alignment and architecture analyses of protein domains performed with the scan Prosite tool are presented in **Figure 45**. Domain architecture of the putative *R2D2* highlighted a protein sequence of 321 amino acids including two dsRNA binding domains (DSRB). The prosite profile hit score and the positions of DSRB domains are more similar to Coleoptera species rather than *D. melanogaster*. Concerning the protein complex *dcr-2*, a complex of 1597 amino acids was identified which includes two amino-terminal helicase domains, a *dicer* dsRNA binding fold domain, two carboxy-terminal RNAseIII, and a *PAZ* domain. This architecture is also like other Coleoptera species and differs from *D. melanogaster* which includes an additional domain of carboxy-terminal dsRNA binding. The *ago-2* protein complex identification corresponded to a sequence of 803 amino acids including a *PAZ* domain and a *PIWI* domain.

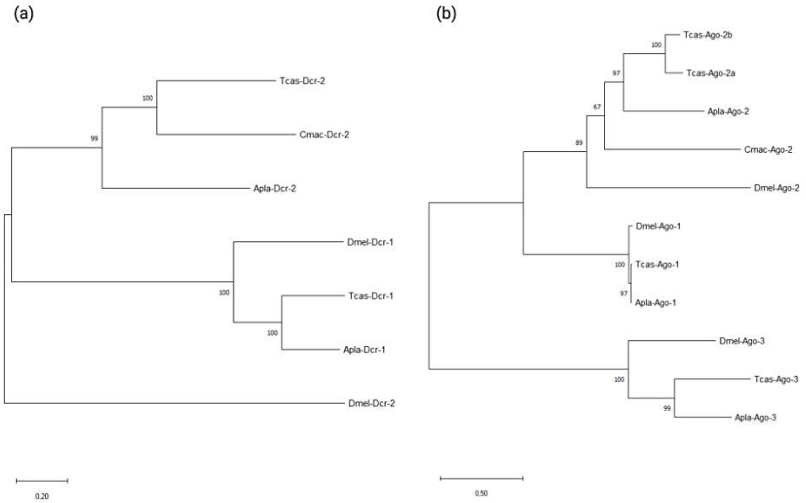

Figure 44: Maximal likelihood inferred phylogeny of *dicer* proteins (a) and *argonaute* proteins (b) and clustering of putative sequences of Cmac-Ago-2 (accession n° GEUH01006697.1) and Cmac-Dcr-2 (accession n° GEUD01209535.1) with RNAi protein complex of other insects.

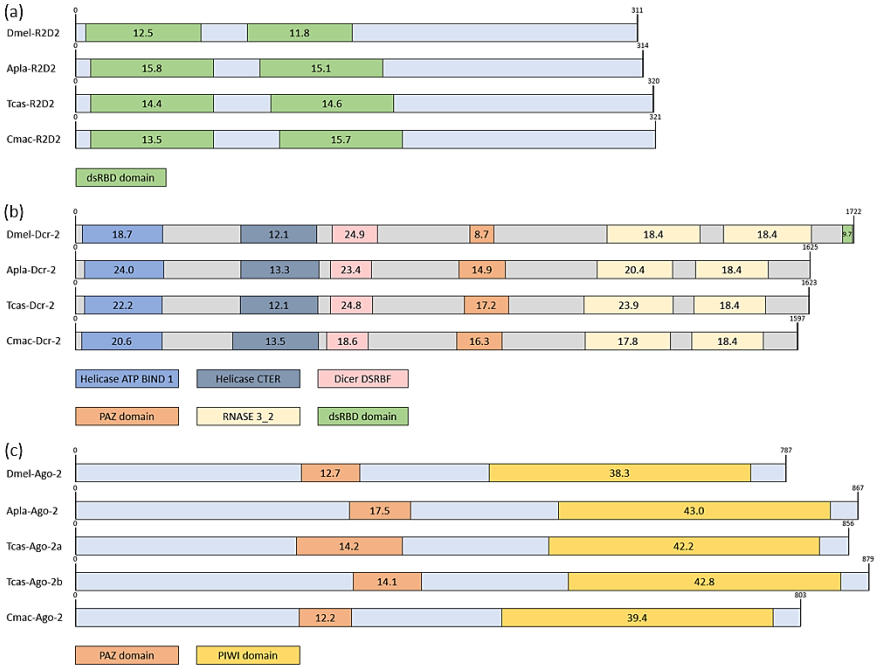

Figure 45: Domain architecture analyses of RNAi core components according to the putative proteins complex identified in *C. maculatus* and alignments performed with *D. melanogaster*, *A. plannipennis*, and *T. castaneum*. (a) *R2D2* proteins with localization of *dsRBD* domains; (b) *dicer-2* protein complex including multiple proteic domains of *helicase ATP Bind1, helicase CTER, DSRBF, PAZ, RNAse, and dsRBD*; (c) *argonaute-2* protein complex including domains *PAZ* and *PIWI*.

2.3.2 Identification and Description of the laccase 1 Protein in C. maculatus

The complete cDNA sequence of the *lac 1* gene identified in *C. maculatus* (accession number GJDX01063393.1) corresponded to a gene of 2803 *bp* including a coding sequence of 2064 *bp* which corresponds to a protein of 688 amino acids with a predicted weight of 79.01kDa (**Figure 46).**

Phylogenetic analyses following the maximum likelihood method led on this sequence (**Figure 46 a**) confirmed that it corresponded to *lac 1* with the discrimination of two clades corresponding to the two forms of laccases as expected. In Coleoptera, Tcas-Lac-2 and Aver-Lac-2 form a subclade corresponding to the Tenebrionidae family while Malt-Lac-2 form another subclade. The same pattern is observed concerning the clade of *laccase 1*. Cmac-Lac-1 and Malt-Lac-1 are clustered together with the maximal bootstrap value.

The full length of the deduced amino acid sequence and domain architecture are provided in **Figure 46 b.** The architecture of protein domain analyses performed on the *C. maculatus lac 1* revealed that three typical Cu-oxidase domains were present, including a type 1 (T1), a type 2 (T2), and a type 3 (T3) copper domain of respectively 158 amino acids (from 219 to 376), 152 amino acids (from 485 to 636) and 118 amino acids (from 89 to 276). The predicted N–terminal signal peptide identified included 16 amino-acid residues. No transmembrane domain was found.

2.3.3 Validation of Primers and Reference Gene for qPCR Analyses

Several sets of primers were evaluated during the validation tests to select gene for RT-qPCR analyses, including the target gene (*lac 1),* the potential HKG (*tuba1*, *arg-K,* and *bactin*) and the two HKG identified by Brar *et al.* (2022) (*tbp* and *gst*). Validated primers and relative efficiency results are presented in **Table 21**. Melting curves highlighting their specificity are presented in **Figure 47**. Validated HKGs primers corresponded to genes of *tuba1*, *arg-K,* and *gst*. The primers of *tbp* presented primer dimer in melting curves and a relative efficiency out of the acceptance range (RE > 110%). None of the primers tested

for *bactin* were validated because of primer dimer and/or relative efficiencies out of the acceptable range.

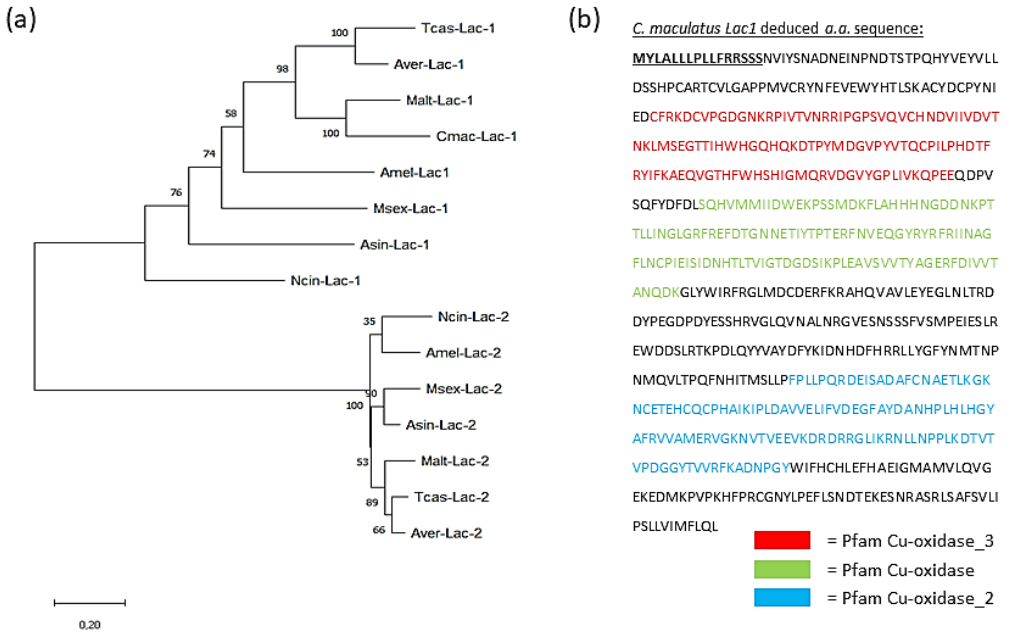

Figure 46: Maximal likelihood inferred phylogeny of *lac 1* (a); and *lac 1* amino-acids sequence (b) deduced from GJDX01063393.1 highlighting three copper domains TI (*Pfam Cu-oxidase* in green), T2 (*Pfam Cu-oxidase_2* in blue), and T3 (*Pfam Cu-oxidase_3* in red) and the putative signal peptide predicted (bold and underlined).

Based on the two identified HKGs (*cf.*, *tbp* and *gst*) and validated primers of *tuba1* and *arg-K*, further tests were performed to assess the stability of their expression in samples of different ages and different dsRNA treatments. **Figure 48** provides the mean coefficient of stability (M-value) based on the cq-values of each tested sample (n = 7). Genes of *tbp*, *gst*, and *tuba1* presented ideal stability with an M coefficient <0.5 which means that these genes of reference present a minimal variation across tested samples (in both biotic and abiotic variations) and may therefore be selected for RT-qPCR analyses. The *arg-K* gene was less stable (0.5 > M-value > 1) and presented moderate variations in the tested samples. Following these results of primer validation and of stability assessment, two reference genes were selected for RT-qPCR analyses, including the *tuba1* gene, a new HKG in *C. maculatus*, and the *gst* gene identified by Brar *et al.* (2022).

Table 21: Description of validated primers for the target gene (*lac 1*) and for the reference gene (*tuba1*, *arg-K*, *tbp*, and *gst*). Parameters of the standard curve generated from amplifications on serial dilutions are presented. RE = relative efficiency; R^2 = determination coefficient of the linear regression.

Gene Name	Gene Symbol	Accession Number	Primers	Sequence (5′ to 3′)	Amplicon Size (bp)	Melt. Temp.	RE	R^2	Slope	y Intercept	Primer Dimer
Alpha-tubulin1	*tuba1*	GEUH01049608.1	Tuba1 F1 Tuba1 R1	TGC ATC ACT AGC TTT TCT GAA CAA TTC CCA GCA GGC ATT AC	149	80.5 °C	97.8%	0.997	−3.375	22.147	No
Arginin-kinase	*arg-K*	GEUF01011058.1	ArgK F23 ArgK R2	ATT TGA CCT TCT GCC CGA CC CCT GCA AGT TGA ACT GTC CC	123	84 °C	108.5%	0.993	−3.113	30.981	No
Tata binding protein	*tbp*	GEUH01047165.1	TBP F1 TBP R1	TTG CTC ACA ACG CAA GTA GG TCG CCT GCA AGT CTT TCA TA	103	83 °C	**117.6%**	0.991	−2.962	39.977	Yes
Gluthiatone-S-transferase	*gst*	GEUE01064616.1	GST F1 GST R1	CAG TCC CTG TCA AGA GCA CA TGC ATG GAG TGC AAT TCC TA	120	82 °C	108.7%	0.999	−3.129	40.815	No
laccase 1	*lac 1*	GJDX01063393.1	Lacc F3 Lacc R3	ACA CAA GCA CCC CTC AAC AT GAA GCT GTA CCG ACA CAC CA	110	84.5 °C	107.5%	0.998	−3.154	43.238	No

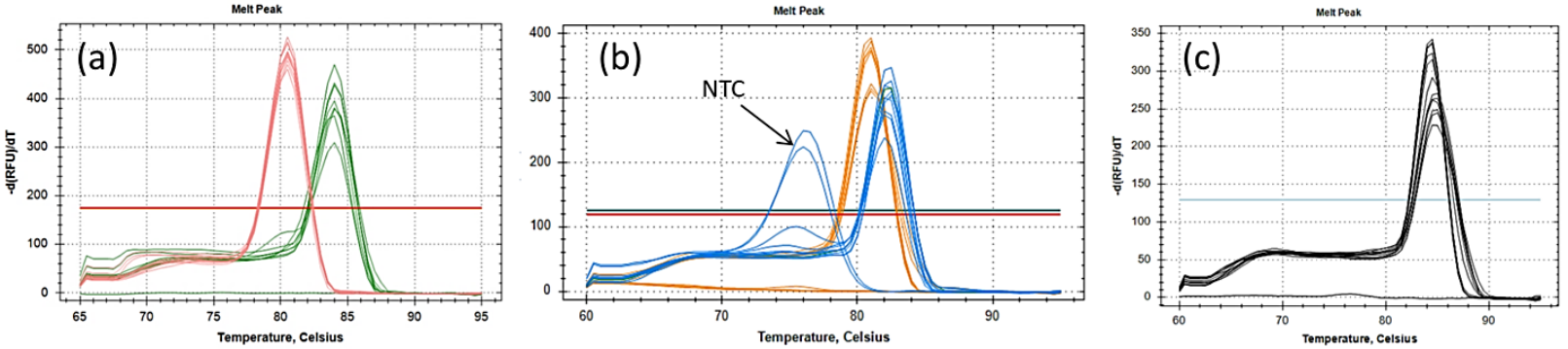

Figure 47: Melting curves associated with the amplifications performed in the serial dilutions during the primer validation showed amplification in *No Template Control* (NTC). (a) Melting curves of the gene *tuba1* (pink) and *arg-K* (green); (b) melting curves of the gene *tbp* (blue) and *gst* (orange); and (c) melting curves of the gene *lac 1.*

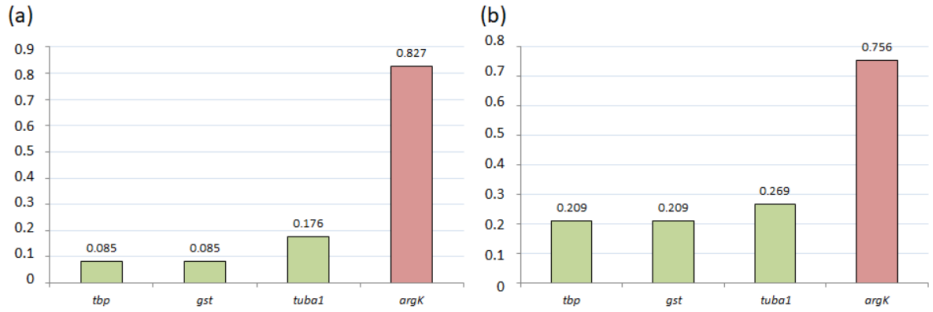

Figure 48: Average M values computed from stability tests (algorithm GeNorm) according to the age (a) and according to dsRNA treatments. (b) Ideal reference genes (green) are *tbp*, *gst*, and *tuba1*. The acceptable reference gene (red) is *arg-K*.

2.3.4 *Gene expression analyses and survival curves*

To test the RNAi-mediated gene silencing of *lac 1* in *C. maculatus*, 400 ng of dsRNA (400 nl at 1µg/µL of dsRNA) coding for a fragment of 588 bp of *lac 1* were micro-injected in freshly emerged adults. The same amount of dsRNA coding for a fragment of 542 bp of *gfp* was injected in parallel to check the specificity of the gene silencing mechanism as this dsRNA treatment would not impact the expression of *lac 1*. The effect of micro-injections on mortalities as well as the consideration of a reference group for the quantification of the *lac 1* expressions (*cf.*, *lac 1*, and *gfp* dsRNA treatments) was assessed via the micro-injection of 400 nl of elution buffer without dsRNA (*i.e.*, the control group). The expression profiles of these three treatments and the statistical test for the assessment of differences in *lac 1* expression are respectively presented in **Figure 49** and in **Table 22**.

A decrease in the *lac 1* expression is observed in both *gfp* and *lac 1* dsRNA treatments when compared with the control group during the three days of post-injection (dpi). Significant differences are observed in *lac 1* dsRNA treatment at two dpi (p-value = 0.022) and at three dpi (p-value < 10^{-6}). The expression of *lac 1* in *gfp* dsRNA treatment is also statistically different from the control group at three dpi (p-value = 0.004), where the expression of *lac 1* is decreased by around 40%. However, the *lac 1* expression in the *gfp* dsRNA treatment increases at five dpi (115%) while the expression in the *lac 1* dsRNA treatments remains stably decreased at around 80% during the rest of the experiment. This highlights a stable and specific decrease in the *lac 1*

expression, which was induced by micro-injected dsRNA on adults of *C. maculatus*.

Table 22: Relative expression of *laccase 1* in different treatments (*cf.*, biological groups) and statistical comparison with the control group ("*" indicates significance levels).

Dpi	Target Gene	Biological Group	N Samples	Expression	Lower Error Bar	Upper Error Bar	*p*-Value (*t*-Test)
1 day	*lac 1*	Control	3	1.00	0.85	1.18	
1 day	*lac 1*	GFP	3	0.78	0.69	0.89	0.109
1 day	*lac 1*	Laccase	3	0.85	0.59	1.23	0.534
2 days	*lac 1*	Control	3	1.00	0.68	1.47	
2 days	*lac 1*	GFP	3	0.67	0.56	0.79	0.245
2 days	*lac 1*	Laccase	3	0.35	0.22	0.56	0.022 *
3 days	*lac 1*	Control	3	1.00	0.98	1.02	
3 days	*lac 1*	GFP	3	0.67	0.59	0.75	0.004 **
3 days	*lac 1*	Laccase	3	0.24	0.23	0.25	$<10^{-6}$ ***
4 days	*lac 1*	Control	3	1.00	0.64	1.56	
4 days	*lac 1*	GFP	3	0.60	0.47	0.77	0.163
4 days	*lac 1*	Laccase	3	0.21	0.19	0.23	0.004 **
5 days	*lac 1*	Control	3	1.00	0.77	1.30	
5 days	*lac 1*	GFP	3	1.15	1.04	1.27	0.646
5 days	*lac 1*	Laccase	3	0.27	0.23	0.31	0.012 *

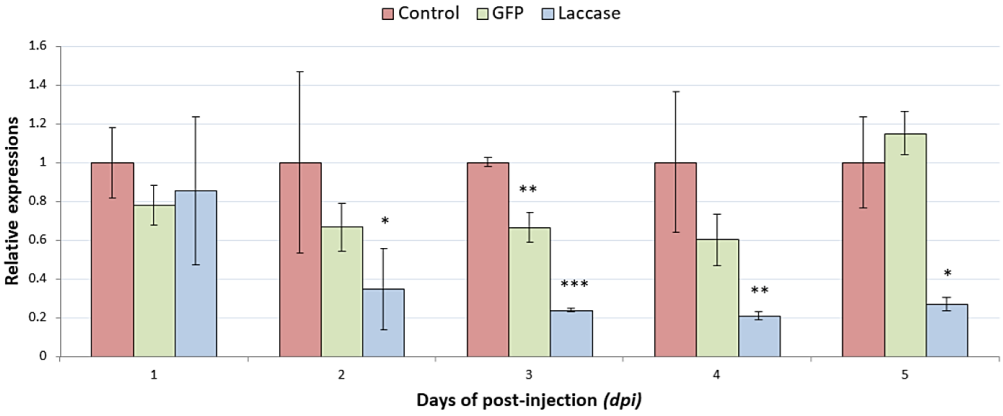

Figure 49: Evolution of the *lac 1* expression in different treatments of the micro-injection experiment. Red = control group; green = *gfp* dsRNA treatment; blue = *lac 1* dsRNA treatment.

No significant mortality was observed in *lac 1* treatments despite the decreasing *laccase* expression (**Figure 50**). The LT50s were not reached in all treatments, and survival curves led to Kaplan–Meier estimations of survival probabilities that did not differ statistically according to the log-rank test (Pval = 0.44).

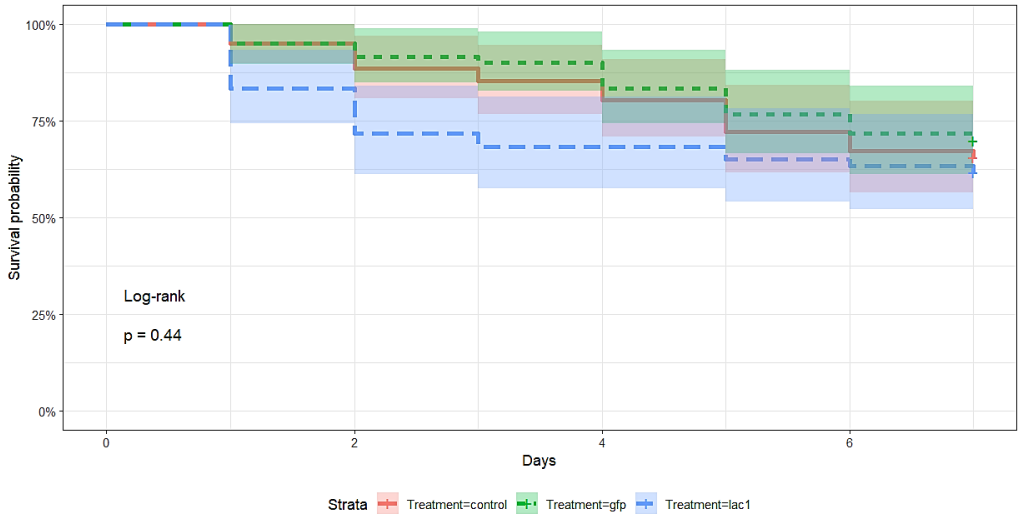

Figure 50: Survival curves highlighting survival probabilities (%) with days of post injection (*dpi*) of *C. maculatus* and log-rank test computed from mortalities recorded in the control (red), *gfp* dsRNA treatment (green) and the *lac 1* dsRNA treatment (blue).

2.4 Discussion

2.4.1 Identification of RNAi machinery core components and lac 1 in C. maculatus

Since the discovery of the ability of dsRNA to silence gene expression in *Caenorhabditis elegans* thirty years ago (Fire *et al.* 1991, 1998), RNAi mechanisms were described in several species and were widely used to knock down genes and analyze their functions (Schmitt-Engel *et al.* 2015). RNAi-based control of pests relies on the introduction of dsRNA into insect bodies to silence a gene of interest via the siRNA pathway that depends on key protein complexes named *dicer-2* and *RISC* including *argonaute-2 and R2D2*. In this study, RNAi core components were identified in *C. maculatus* using

phylogenetical analyses and tBLASTn followed by protein architecture description with prosite tool. Phylogeny inference followed the Maximum Likelihood (ML) method which is reported to be more robust than the neighbor-joining (NJ) methods when appropriate models of nucleotide substitution are used (Hasegawa and Fujiwara 1993; Ota and Li 2000; Zhao *et al.* 2015; Yang *et al.* 2017; Zhang *et al.* 2018). The RNAi genes identified in *C. maculatus* presented similarities with other insect species, supporting that bruchids would have conserved the same cellular mechanism. Phylogenetic clustering gathered Coleoptera species together, more precisely Chrysomelidae species that are reported to be highly sensitive to RNAi (Tomoyasu *et al.* 2008; Willow and Veromann 2021). These identifications provided a first indication that the RNAi mechanism via the siRNA pathway would be functional in *C. maculatus* and should be further assessed by exposing insects to specific dsRNAs for confirmation.

The identification of *laccase 1* in *C. maculatus* was based on the two types of *laccases* identified in insects, *lac 1* and *lac 2* (Dittmer *et al.* 2004). These enzymes are multicopper oxidases (MCOs) able to catalyze the oxidation of numerous phenolic and non-phenolic compounds (Dwivedi *et al.* 2011). *Laccase 1* is playing a protective role against a plant-based diet while *lac 2* is involved in the tanning of the insects' cuticle (sclerotization and pigmentation) (Thomas *et al.* 1989; Tomoyasu and Denell 2004). In *C. maculatus*, the *lac 1* inferred phylogeny showed that sequences are similar among different orders suggesting that the *lac 1* protein is evolutionarily conserved. Molecular architecture descriptions identified three copper domains fitting with orthologous *lac 1* architecture described in Hemipteran insects such as *Bemisia tabaci* (Gennadius,1889) (Hemiptera: Aleyrodidae), *Nephotettix cincticeps* Uhler 1896 (Hemiptera: Cicadellidae), *Acyrtosiphon pisum* (Hemiptera: aphididae), and *S. avenae* (Hattori *et al.* 2005; Liang 2006; Yang *et al.* 2017; Zhang *et al.* 2018). These cupredoxin-like domains were reported to include four copper atoms and were named type-1 or blue copper center, type 2 or normal copper center, and the type 3 coupled binuclear copper centers. They allow the enzyme to reduce oxygen in the water without producing harmful byproducts (Janusz *et al.* 2020). Such architectural indications suggest that *lac 1* in *C.*

maculatus has also oxidative activity. The whole enzyme size as well as the locations/sizes of copper domains in *C. maculatus* was similar to the *lac 1* of *S. avenae* (Zhang *et al.* 2018). However, the absence of a transmembrane domain suggests that *lac 1* is a secreted enzyme in *C. maculatus* as it was reported with *B. tabaci* (Yang *et al.* 2017). No indication about the site of the enzyme production can be highlighted in the present study.

2.4.2 Identification of reference genes for RT-qPCR

The prior identification of at least two reference genes is essential to perform viable gene expression studies as they serve for the normalization in relative gene quantification (Bustin *et al.* 2005). The validation of these genes should follow an appropriate stepwise approach to get reliable results in RT-qPCR analyses (Vandesompele *et al.* 2002; Xiao *et al.* 2015; Taylor *et al.* 2019). In this work, it was investigated whether *arg-K, tuba 1* and *bactin* genes could constitute new reference genes. Following primer validation, these genes were tested for stability under biotic and abiotic variations that are likely to interfere with the expression patterns among samples of subsequent micro-injection experiments (*cf.*, age and exposure to dsRNA). *Alpha-tubulin 1* emerged as the most promising candidate that presented all necessary criteria for validation (Taylor *et al.* 2019). Primers of the *Arg-K* gene presented a moderate stability coefficient which was not optimal for subsequent experiments. The results of Brar *et al.* (2022) provided two new reference genes (*gst* and *tbp*) that were tested for validation in parallel with *arg-K* and *tuba1*. Primers of the *gst* gene presented a relative efficiency of 108.7% and ideal stability while the relative efficiency of *tbp* primers was out of the validation range (117.6%). These results are consistent with the study of Brar *et al.* (2022) that highlighted respective efficiency of 102.25% and 118.9% for *gst* and *tbp*. The high relative efficiency of *tbp* primers was probably explained by unspecific amplification and a fluorescence emission that is not directly linked to the target gene. Non-specific elements such as primer dimers may also induce non-specific fluorescence as suggested by melting curves (*cf.*, Figure 6b) but no melting curve was provided in the study of Brar *et al.* (2022). Consequently, the consideration of *tbp* gene as reliable reference gene was not emphasized in this study that used *tuba1* and *gst* genes in RT-qPCR analyses.

Alpha-tubuline 1 is involved in the cytoarchitecture of cells and is stably expressed in several organisms (Keeling and Doolittle 1996; Bustin 2002). The *gst* gene codes for an enzyme involved in the detoxification of endogenous/exogenous compounds and is involved in the intracellular transport and biosynthesis of hormones (Enayati *et al.* 2005; Brar *et al.* 2022). This work is the first study suggesting *tuba 1* as a new reference gene in *C. maculatus* but it should be relevant to further test the stability of the gene expression in more biotic and abiotic variations, such as different developmental stages of *C. maculatus*, different temperatures, or different photoperiods. Other algorithms could also be used in the calculation of the gene expressions stability such as Normfinder (Andersen *et al.* 2004), bestkeeper (Pfaffl *et al.* 2004), delta-cq (Silver *et al.* 2006), or RefFinder (a web-based tool combining the latter algorithms).

2.4.3 Gene knockdown and mortalities

Micro-injection experiments and RT-qPCR analyses highlighted a stable and significant decrease in the *lac 1* gene expression in the *lac 1* treatment after two *dpi* that was stabilized until the end of the experiment at about 20% (*i.e.*, an expression decrease in about 80%). The *lac 1* gene expression in the *gfp* treatment did not differ statistically from the control, except at three *dpi.* The *lac 1* expression in the *gfp* treatment stabilized at around 65% and became higher in the control group (115%) at the end of the experiment. This observation completes the previous identification of RNAi core machinery and supports that RNAi via siRNA pathway would be specifically triggered by dsRNA exposure in *C. maculatus*. The extent of gene knockdown ranging at ~80% following the micro-injection of 400 nL of dsRNA also suggests that *C. maculatus* would be a sensitive species to RNAi, such as other chrysomelid species (Joga *et al.* 2016). Moreover, the RNAi would be expected to be systemic in *C. maculatus* as the RNAi affected the whole insect body (*cf.*, the pooling of three complete adults in RNA extractions) (Tomoyasu and Denell 2004). The stability of the *lac 1* expression decrease also suggests that no compensation mechanism would be observed to rebalance the decrease in *lac 1* expression as it could be observed with *T. castaneum* (Perkin *et al.* 2017). All these observations contribute to the first report of gene silencing induced by dsRNA in *C. maculatus* and support that RNA interference via the siRNA pathway would provide a pledging alternative method of control against *C. maculatus.* However, no subsequent mortalities were observed with the *lac 1*

gene knockdown which differs from results obtained in *S. avenae* (Zhang *et al.* 2018). This was probably due to the low feeding of *C. maculatus* adults on water or nectar (De Loecker 1982). Further studies should focus on other target genes or should focus on larval life stages that are feeding on seeds and that are more susceptible to the intake of anti-nutritional factors such as tannins or antitrypsic factors for which a decrease in *lac 1* expression should induce mortalities (De Loecker 1982; Lattanzio *et al.* 2005).

2.4.4 Perspectives for future research in the development of RNAi pesticide against bruchids

The systematic identification of suitable RNAi target genes that lead to dead phenotype insects is a challenge in the development of efficient RNAi-based control methods because some insects are difficult to rear in controlled conditions and because the whole genomic information is often lacking (Ulrich *et al.* 2015). In this study, the first demonstration of systemic RNAi in *C. maculatus* was mainly limited by the difficulty of reaching larvae that develop inside seeds, which restrained micro-injection experiments on emerging adults. Further bioassay should test other delivery methods of dsRNA such as feeding bioassay led on larvae and should assess if the systemic RNAi could be transmitted to the next generation (Bucher *et al.* 2002).

The identification of new genes that would present a combined lethal effect with gene silencing is also needed to develop new selective pest management tools for *C. maculatus* (Kola *et al.* 2015). Such genes could already be identified in *T. castaneum,* including eleven genes (NCBI referred gene symbols *cact, srp54k, rop, alpha snap, shi, pp1alpha-96a, inr-a, hsc70-3, rpn7, gw,rpt3,copi coatomer, vATPased, vATPase a*)(Ulrich *et al.* 2015). Other research led on larvae of *P. cochleariae* has identified that five of these genes were highly lethal when sprayed at very low doses (300 ng/leaves), *srp54k, rop, alpha snap, rpn7, and rpt3* (Mehlhorn *et al.* 2021). These genes could be pledging genes to efficiently induce mortality in *C. maculatus*.

The use of insecticidal dsRNA products in large-scale pest management is always a challenge. Most recently suggested approaches include (i) dsRNA encapsulation into nanoparticles (Kontogiannatos *et al.* 2021), (ii) host-induced gene silencing (HIGS) technologies via the use of transgenic cultivars

containing RNAi traits, (iii) spray induced gene silencing (Willow and Veromann 2021), or (iv) bacterial expressing RNAi traits ingestion (Zhu *et al.* 2011). If foliar spraying of dsRNA could be efficiently developed for the control of leaves chrysomelid pests such as *L. decemlineata* or *D. virgifera* (Petek *et al.* 2020; Vélez *et al.* 2020), the consideration of endophytic larval development of bruchids would restrict possibilities of RNAi pesticides applications to HIGS through transgenic cultivars or endophytic bacterial deliveries.

2.5 Conclusions

This study provided a complete description of the necessary protein involved in the RNAi mechanism via the siRNA pathway and also described the architecture and the deduced function of *laccase 1* in *C. maculatus*. The administration of dsRNA coding for this protein confirmed a systemic and constant gene knockdown after two dpi. This evidence of gene silencing in *C. maculatus* offers a new perspective for a specific control. Although no lethal effect could be demonstrated, future studies should focus on other promising proteins to develop an effective control method with high specificity as has been done in many species of beetles of the Chrysomelidae family.

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Institutional Review Board Statement: Not applicable

Data Availability Statement: Data about these experiments are available under demand at the laboratory of Functionnal and Evolutionnary Entomology, Gembloux Agro-Bio Tech—University of Liège. Passage des Déportés, 2, 5030, Gembloux, Belgium (see corresponding author).

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Chapter VII

General discussion

1 IPM paradigm for the management of *B. rufimanus*

Faba bean crops are expected to gain interest given their multiple benefits to agroecosystems, their supply of sustainable plant proteins to meet the EU's growing deficit, the trend towards lower inputs in farming systems (*cf.*, the EU Sustainable Use Directive (SUD 2009/128/EC) and the promotion of agri-environmental policies (Voisin *et al.* 2014; Renard and Tilman 2019). The sustainable promotion of faba bean seeds for human uses should cope with the high interannual variability in terms of quality and quantities of the harvested seeds (Cernay *et al.* 2015) and notably provide growers with management methods of key insect pests such as BBWs or aphids that considerably affect the yields and the qualities of faba bean seeds (Kaniuczak 2004; Stoddard *et al.* 2010).

This thesis addressed the biocontrol-based management of *B. rufimanus* which is becoming increasingly prevalent in Europe and is expected to intensify if the expansion of faba bean cultivation is not supported by effective pest control measures. The alternatives to the use of insecticides were broached following a multidisciplinary approach that took into consideration the plant physiology, the pest ecology, and the broader crop ecosystemic services with five distinct research axes. Given the complexity of this plant-insect interaction, it has proven challenging to identify a single, effective short-term control method. Nevertheless, by combining multiple approaches or research directions, as demonstrated in this thesis, progress can be made towards finding potential solutions in this regard.

It seems that the most appropriate strategy for the reduction of *B. rufimanus* infestations would consist in collective and homogeneous measures implemented at the scale of a production region aiming at the gradual decrease of pest populations from years to years. In this context, the outcomes from the five research axes have provided insights on the most appropriate control levers into conservative biological control measures related to the identification of parasitoid species in the Wallonia region, the adoption of suitable agricultural practices based on the pest's thermal development, the

identificaiton of most optimal protein-producing varieties with minimal infestation rates, and the potential development of biopesticides utilizing HEFs, semiochemicals and/or dsRNA for curative purposes.

Based on the literature review from the Chapter I and on the research outcomes from all thesis Chapters, a general outline of *B. rufimanus* IPM is presented in **Figure 51**, graduating all potential measures for the progressive reduction of *B. rufimanus*. These graduated practices are adapted from IPM paradigm concerning other insect pests (Gwynn *et al.* 2021). The paradigm is cleaved into two main domains, the faba bean crops and the storage facilities, given the association of *B. rufimanus* all along the production and storage of faba bean seeds (see Chapter 1).

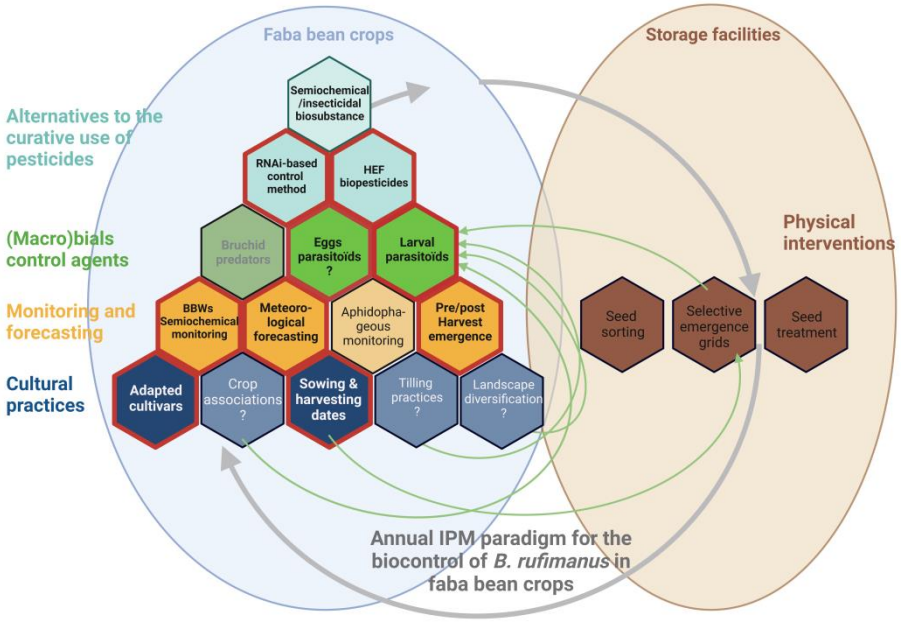

Figure 51: Annual IPM paradigm for year to year decrease of the population level of *B. rufimanus*. Red outlined cases correspond to the investigated aspects in this thesis; green arrows correspond to conservative practices for the enhancement of biological control

IPM paradigms generally present the chemical pesticides at the top of the pyramid (Stenberg 2017), but the rare active substances authorized across Europe definitively became obsolete as as it has been evidenced by the

abandonment of the bruchidcast program developed by PRGO and Syngenta in the UK in 2021. As a consequence, curative alternatives were presented instead.

A proposal of a faba bean cultivation itinerary for minimizing the impact of BBWs is discussed in the light of preliminary thesis results obtained. In this sense, it is worth noting that the conclusions of the thesis must be repeated over several years in order to provide reliable indications to the growers, especially regarding the varietal control of BBWs. Other control levers presented in **Figure 51,** which have not been addressed in this thesis but could still provide interesting tools, are presented as thesis perspectives. All these proposals are contextualized in the Walloon legislative framework. The future research needed to establish functional control tools, other key pest species that should be co-targeted such as aphids, and the potential synergy that can improve their efficacy within the most appropriate cropping itineraries against *B. rufimanus* are also broached in the last section of the discussion.

2 Cropping itinerary proposal for minimizing *B. rufimanus* impacts

In France and Belgium, numerous annual editions of agricultural manuals have been published, providing guidance on optimizing faba bean cultivation in organic and conventional farming systems (Abras *et al.* 2015; Biarnès *et al.* 2022). However, strategic interventions to mitigate bruchid infestation in the field remain scarce. To conclude this thesis, the results obtained in each chapter pave the way for the formulation of propositions. However, these suggestions do not qualify as recommendations, as research efforts would need to be extended beyond the current two-year span to solidify certain findings.

- **Adapted cultivar and sowing period**: Early sowing is likely to avoid periods of summer drought/heat stress and delay seed maturation with the bruchid cycle as suggested in Chapters III and IV. In this regard, winter varieties (sown at 35 seeds/m² and 7 to 10 cm depth)

appear more favorable than spring varieties as long as the autumnal plant development does not exceed the 5 to 6 leaf stage, which would pose a risk of frost or foliar disease development (Le Gall *et al.* 2021). Winter varieties seem to offer better yields, favoring winter varieties in pure crops (Chapter IV). To a lesser extent, these crops have the advantage of covering the soil during the winter period and are also sown at lower densities than spring varieties. However, no winter faba bean variety with low levels of antinutritional factors (T-V-C) could be developped. Considering this factor, spring varieties with early maturation and a suitable sowing date (between mid-February and mid-March, depending on the bioclimatic areas) would be recommandable. According to the varietal ranking established in the Chapter IV, most recommendable cultivars would be Fanfare (density 50 seeds/m²) that exhibited the most high and stable protein production with minimal bruchids infestation under increasing drought environment. Concerning the winter cultivars, Augusta and Tundra provided similar performance as Fanfare.

- **Chemical weeding after sowing**: This aspect was not broached by the thesis, multiple recommendation are provided in the litterature for the adventices management such as the alternance of winter faba bean and spring faba bean, tilling practices, diversified long rotations, or mechanical weeding (Le Gall *et al.* 2021; Biarnès *et al.* 2022). During the field trials performed in this thesis, chemical weeding was carried out after sowing and provided an appropriate management of adventices.
- **Pest monitoring with white traps and IPS lures**: In Chapters II and V, BBWs infestations were demonstrated to start at the beginning of pods setting and the most effective semiochemical trap, considering appropriate adaptations to decrease the bycatches of beneficials, was demonstrated to be the white trap lured with IPS flower kairomones. The monitoring of BBWs would enable the detection of BBWs in faba bean crops, which in turn would indicate the need to monitor first ovipositions and to plan potential curative methods. The thermal

conditions recorded from the date of first oviposition would anticipate the period of BBWs emergence and potentially break the emergence phase of the pest life cycle.

- **Fungicide application**: Fungal diseases are important factors in faba bean crop losses, especially in pure crops. In Belgium, botrytis has a particularly strong impact in wet years, while rust is more favorable in warmer periods (Christine Cartrysse, personal communication, August 2023). The preventive management chosen in this thesis consisted in the application of fungicides during the flowering phase of the faba bean crop and, if necessary, additional applications were made after a period of ten days. However, according to potential curative methods against bruchids, such as HEF spraying, the use of fungicide would not be suitable. Alternatives to the use of fungicides could also be investigated, such as MBCAs, as they could be developed for other crops(Coninck *et al*. 2020). Such research could provide specific disease antagonists that would not affect the virulence of HEFs against bruchids.
- **Biobased-curative intervention with HEF or RNAi**: Chapter VI provided insight into the potential development of biopesticides based on HEFs or RNAi. More research is needed for their development and for their potential efficiency in large-scale application in combination with semiochemical monitoring of BBWs. This topic will be discussed in the following sections.
- **Harvesting period and storage management of BBWs infestations**: The appropriate timing of faba bean harvest should respect the appropriate seed relative humidity (up to 16%) and ideally be done before BBWs emergence (*i.e.*, from 550 degree-days after first oviposition). Harvesting the seeds at 17-18% followed by hot air drying (between 50 and 70°C) is suitable both to kill the bruchids and to reduce the relative humidity of the seeds to the standard of 14%, but this would represent a cost of about 19 euros per ton (Le Gall *et al.* 2021; Fegra and Synagra 2023).

3 Perspectives

3.1 Cultural practices: varietal choice, and genetic improvement

Varietal choice is the first level of agronomic management techniques in IPM strategy aiming the reduction of the pest population and the host plant susceptibility (Gray *et al.* 2008). In the case of BBWs, the breeding of resistant cultivars is suggested to be the most adequate approach to decrease pest injury levels (Dell'Aglio and Tayeh 2023). In this thesis, main finding of the varietal control study (Chapter IV) were as follows: (i) notable variations in BBWs injury levels among different cultivars, (ii) impact of the varietal selection and the sowing dates on the protein production capacity and adaptability to variable climatic conditions, and (iii) development of a comprehensive analytical framework for ranking cultivars based on multiple variables relevant to the food industry. Other aspects of cultural practices such as the timing of harvest were also investigated in regards of the thermal development of *B. rufimanus* in the Chapter III.

These varietal recommendation discussed so far for pure crops destined to food industries did not considered the antinutritionnal factors of faba bean seeds, *i.e.*, T-V-C that present impacts on protein digestibility and on the occurrence of favism (Crépon *et al.* 2010). Currently, no quantitative threshold of T-V-C contents can be determined in the developing sector of food industry regarding their potential health concerns. The health effects of tannins are a subject of debate due to the fact that they offer health benefits such as anti-cancer properties, despite some potential drawbacks like the decrease of protein digestibility. Vicine and convicine are also reported as least concern for human health at this stage of the industrial sector development because there are numerous gaps of knowledge about the favism occurrence such as the proportion of humans showing this genetic degenerescence, the thresholds in terms of quantity to trigger the erythrocytes degradation mechanism, and the fact that they may be easily denatured during the transformation process. However, the food industry tends to promote cultivars containing minimal

amounts of these antinutritionnal factors (L. Dumoulin, personal communication June 2023).

The Tiffany cultivar, which had low vicine and convicine contents, did not exhibit the highest protein yield among the cultivars tested. It seems that a tradeoff between protein production and antinutritionnal factors should therefore be established when choosing varieties for human consumption. Another cultivars named Fevita, that was not tested during this thesis, present simultaneous characteristics of low T-V-C contents but this cultivar remain still low productive (Le Gall *et al.* 2021). Genetics could bring improvements to face high productive protein production and low antinutritionnal factors. The recent completion of whole genome sequencing and assembly for faba bean opens up new opportunities for comparing it with other legume crops. This advancement also allows for the potential integration of individual genetic trait selection findings, such as the VC1 gene responsible for V-C content control, the WD40 transcription factor associated with the zero tannin1 locus, and the gene governing seed size. By combining these genetic traits, it is possible to enhance field yields to achieve optimal levels (Webb *et al.* 2016; Björnsdotter *et al.* 2021; Jayakodi *et al.* 2023). In parallel, this would offer new opportunities for balancing the amino-acids contents, reducing seed phytate and protease inhibitors contents at appropriate levels for human food with more sulphur amino acids such as methionine and cysteine (Duc *et al.* 2015; Dumoulin *et al.* 2021; Jayakodi *et al.* 2023).

These potential genetic improvements of seed quality should however not compromise the pest resistances. Further research is needed for a better understanding of the resistance mechanisms against bruchids in order to identify the potential involved genes (Carrillo-Perdomo *et al.* 2019; Segers *et al.* 2022). In this sense, the concept of "*taxonomic conservatism in host plant use*" evocated in the chapter 1, would offer an essential framework for comparative genetic studies between leguminous plants. Indeed, the complex chemistry observed among leguminous plants and their specific bruchid species is quite diversified. Genetic comparison between plants showing resistance

mechanisms and the faba bean genome would potentially allow the identification of similar genes. A restricted literature broached the antagonistic effect of secondary metabolites such as toxic compounds in seeds content or some repellents compounds in epicuticular waxes towards ovipositing females (Kergoat 2011). Most of the studies that assessed the toxic effects of secondary metabolites contained in seeds against feeding bruchid larvae pointed the role of antinutritionnal factors such as T-V-C, phytic acid and trypsin inhibitors that are also toxic for humans and would therefore not be recommendable (Gatehouse and Boulter 1983; Gatehouse *et al.* 1990; Desroches *et al.* 1995; Dhole and Reddy 2016). It would be preferable to focus on the epicuticular waxes for their repellent effects towards ovipositing females. Currently, the only study that reported oviposition preference of *B. rufimanus* for some faba bean cultivars pointed that this preference was positively related to the seed size (Dell'Aglio and Tayeh 2023). To our opinion, the most plausible interpretation of such oviposition preferences would probably be linked to the greater emission of attractive VOCs, rather than anti-apetant epicuticular compounds. Further analyses of epicuticular waxes, suh as performed by Zhao *et al.* (2019), would be interesting deeply investigate potential oviposition preferences mechanisms.

3.2 Cultural practices: crop associations, tilling and landscape diversification

Mixed cultivation of crop species in the same field, *i.e.*, crop association, has been reported in numerous studies to enhance ecological mechanisms for weed suppression (Liebman and Dyck 1993), for increasing total crop yield compared to pure crops (Bedoussac *et al.* 2015; Li *et al.* 2020), and for improving pest and disease control (Trenbath 1993; Lopes *et al.* 2016). During this thesis, field trials were conducted to compare bruchid infestation rates between pure and associated faba bean (wheat x faba bean) in 14 m² plots (unpublished results). The results of the study did not indicate any significant patterns in reducing infestation rates through crop associations. These results are also supported by the comparisons between associated crops (faba bean x oat) in the Chapter II, where no improvements in terms of BBWs infestation

rates were observed. This phenomenon could be explained by several biological traits of BBWs, such as their high mobility, their fecundity and the dispersal behavior of their eggs, and their semiochemical sensitivity to different host plant organs (Chapter I). Consequently, the crop associations tested with cereals were not sufficient to disrupt the mating and oviposition behavior of BBWs. However, other agronomic benefits of crop associations have been demonstrated in the literature, such as reduction of *A. fabae* populations, the yield stability and increase in total yield (Yahuza 2011; Sammama *et al.* 2023) and remain interesting to promote.

Other cultural practices, such as tilling practices and wildflower strips (WFS), could already be highlighted for their supportive effects on the abundance of biocontrol agents that can naturally control pests of faba bean, including aphid predators and parasitoids.

Regarding tilling practices, no study has yet addressed the impact of different tilling practices in faba bean crops on the abundance of the natural bruchid enemies identified in Chapter II, which remain biologically poorly studied. Further studies could be carried out on other crop pests such as *Brassicogethes aeneus* (Fabricius, 1775) in oilseed crops. The tillage practices were reported by Nilsson (2010) as influencing the abundance of their parasitoids, *i.e.*, *Tersilochus* spp. (Hymenoptera: Tersilochidae). Their results supported that post-harvest plowing can reduce the population of *Tersilochus* spp. by 50% compared to plots where reduced and non-invasive tilling methods were used. At present, it seems difficult to imagine such extrapolations for faba bean crops. On the one hand, tillage promotes root and nodule establishment (Le Gal *et al.*, 2021), and on the other hand, known parasitism rates for the parasitoids identified in Chapter II do not exceed 10% in Mediterranean agrosystems(Boughdad 1994; Medjdoub-Bensaad *et al.* 2015). Nevertheless, a better understanding of the bioecology of these parasitoids could guide optimal conservation strategies. At this stage of the thesis, larval parasitoids cannot yet be proposed as a viable biocontrol solution.

Regarding the amenagment of WSFs, no study broached their effect on natural enemies of bruchids, *i.e.*, larval parasitoids. This last decade, agri-environmental practices such as the implantation of wild flower strips have been proposed to increase the functional biodiversity among cropping system and enhance notably the presence biocontrol agents (Albrecht *et al.* 2020). These strips are supposed to provide suitable habitat for natural enemies to overwinter and serve as a feeding source of pollen and nectar, which significantly increases their lifespan and fecundity (Wäckers *et al.* 2005). Subsequently, these beneficial insects migrate to adjacent crops and contribute to pest control efforts (Hatt *et al.* 2018). However, the recent studies have shown that the biocontrol services observed in the crop in the crop are relatively spatially limited (Bernard Bodson, personal communication, August 2023). Moreover, in certain situations, it seems difficult to maintain flowering plants beneficial to pollinators and beneficial insects in the plant communities of these strips in the long term, as they can be overtaken by a weed flora that is less interesting in terms of ecosystem services. Nevertheless, it would be worthwhile to evaluate the effectiveness of such measures in the case of B. *rufimanus*. These studies would have to take into account that adults and larval parasitoids of *B. rufimanus* share the same ecological niche, and that the implementation of WSF could also favor BBW populations, as observed in the case of *B. aeneus* (Rusch 2010). Given this ecological reality, appropriate practices related to the potential benefits of WSF would have to be studied for their effect on the balance of parasitoid populations over bruchid populations (see next sections), such as (i) implementing appropriate timing of harvest, specifically at seed maturity before the cumulative degree-days needed for total BBWs development (Chapter III), to retain BBWs inside the seeds; and (ii) using physical interventions in storage facilities, such as selective emergence grids, that allow parasitoids to emerge while preventing bruchids from leaving the storage facilities. These practices would favor the presence of parasitoids in the WSF or seed storage areas, but further studies are needed to confirm these theoretical concepts.

3.3 Monitoring and forecasting: economic thresholds and thermal development

In the IPM strategy, pest monotoring is essential because it provides growers and surveillance institutions with the baseline information for choosing between the most appropriate management options (Binns *et al.* 2001). The conceptual background of pest monitoring and related decision support models rely on concepts of economic injury levels (EIL) and economic thresholds (ET). Economic injury level is defined as *the level of pest density at which the reduction in revenue from the crop, due to pest injury is equal to the cost of controlling the pest* (Painter 1951; Stern *et al.* 1959; Pedigo *et al.* 1986). Mathematical equation for EIL assessment includes therefore the cost of control ($/ha), the proportion of the pest population controlled by the crop intervention (%), the proportion of the crop damaged per unit of pest density, and the value of harvested products ($/ha) (Binns *et al.* 2001). The EIL concept is often criticized because it supposes perfect knowledge on its different componants, but in reality, some of them may greatly vary over time, such as the crop value or the input costs depending on climatic conditions, energy prices and market instabilities. Other components like the pest population and their damage caused on crops or the effects of intervention on pest populations are, for some pests, completely unknown (Ramsden *et al.* 2017). Moreover, this concept completely neglect the effects of crop intervention on natural enemies as well as the regulation services they provide in term of economic value (Binns *et al.* 2001). Such ecosystemic services should not be neglected, especially in greatly attractive crops towards beneficials like faba bean. Another level of intervention is commonly used, the economic threshold (ET). The ET is defined as *the pest population density that will cause EIL if no control measure is implemented* (Painter 1951; Stern *et al.* 1959; Pedigo *et al.* 1986). This concept is similar to the EIL but it focuses on the pest population density rather tan on economic costs. It is anticipating the EIL by considering pest population evolution without intervention and determine the moment anticipating the timeshift between the pest population level at the moment of

intervention and the effect of the intervention on the pest population (Binns *et al.* 2001).

The ET of *B. rufimanus* was defined in the Chapter I as two consecutive days of maximal temperature reaching 20°C when green pods are forming and bruchids are detected in parcels (Ward and Smart 2011). However, this ET is not yet applicable because it mainly relies on insecticide spraying that are now inefficient due to climatic reasons (see Chapter I), as it has been evidenced by the abandonment of the bruchidcast program by PRGO and Syngenta in the UK in 2021[1]: "*This decision has been made due to it's inability to act as a stewardship tool to reduce spray events. From experiences in the last couple of seasons, increasing temperatures have caused prolonged periods of spray events, rather than few targeted events. As such the tool was not promoting good practice, nor was it helping to achieve higher efficacy and therefore better quality*." Additionally, it was observed that this ET was not correlated with pest densities and was influenced by limitations related to uncertainties in crop values caused by the considerable year-to-year fluctuations in faba bean yields and the incomplete understanding of the impact of pests on seed quality. New reliable ET should be defined for the application of IPM paradigm presented in **Figure 51**. In the context of this thesis, the determination of this threshold should primarily focus on exploring potential alternative methods to pesticides that, although not currently operational, offer the only feasible interventions for reducing infestation levels in monoculture crops in the short term.

In the Chapter II, two methods of bruchid monitoring were conducted: manual capture (active method) and semiochemical monitoring (passive method) (Eymann *et al.* 2010). Semiochemical monitoring offers a more standardized approach to monitoring insect populations, as it eliminates biases caused by operators and by weather conditions during monitoring sessions. However, it should be noted that the attractants used in the semiochemical monitoring compete with faba bean crops as they emit similar odors (*cf.*, Chapter V). In the context of integrated bruchid management,

[1] https://www.syngenta.co.uk/bruchidcast (accessed 20 may 2023)

semiochemical traps would offer practical, standardized and cost effective tool for both pests and beneficial monitoring, and their integration into reliable ET. Most effective traps for BBWs and beneficials captures were identified in Chapter V, *i.e.*, white traps from AgriOdor with IPS floral kairomones. Further investigation is warranted to explore bruchid density through the use of semiochemical trapping and to assess the extent of damage caused on seeds of the most vulnerable varieties. Gathering data on spring BBWs densities (number of adults per hectare) and subsequent crop damage (number of damaged seeds per hectare) will provide the necessary information to establish a robust quantitative ET. Additionally, it is essential to incorporate the presence of aphidophageous beneficial insects into management strategies targeting aphids. By considering these beneficial organisms much comprehensive and integrated pest management practices can be developed on the base of these traps.

Connected detection devices offer real-time information on pest populations to growers, streamlining trap application and overcoming logistical hurdles (Bordes 2017). Additionally, the widespread availability of connected meteorological stations enhances accessibility for growers. By integrating connected detection devices with meteorological data, growers can make early estimations of bruchid emergence when seeds reach maturity. This approach can determine the ideal harvest timing by considering the growing degree days required for the total development of BBWs. Ideally, the harvest should take place when the seed moisture levels reach 14% and before the cumulative thermal development of 550 degree-days for BBWs (with a base temperature of 12°C). By harvesting the seeds at this optimal time and implementing post-harvest pest management measures, significant reductions in populations can be achieved in the subsequent year (Mihiretu and Wale 2013).

In conclusion, the monitoring and forecasting stage of the IPM paradigm against *B. rufimanus* presented in **Figure 48** relates future research topics to improve the monitoring methods and determine the specific ET for bruchid management. This involves conducting comprehensive surveys of insect

population densities, as well as monitoring aphidiphagous beneficials and weather conditions. By integrating these data, alternative interventions to crop treatments for short-term BBW control and optimized harvest periods for long-term BBW control can be identified. These findings would contribute to the development of a decision support model, similar to the one employed for managing *Sitodiplosis mosellana* (Gehin, 1857), as documented in previous studies (Ellis *et al.* 2009; Ramsden *et al.* 2017; Gahukar and Reddy 2018).

3.4 Promotion of macrobiological control agents

Natural enemies of *B. rufimanus* include parasitoids, which can be divided into three groups, namely larvaphageous endoparasitoids, larvaphageous ectoparasitoids and oophagous endoparasitoids. In this thesis, the first identifications of natural enemies of BBWs in Wallonia were made in Chapter II. Among the 11 species identified in the literature, only four species of larvaphagous parasitoids have been identified from harvested seeds. From a biocontrol point of view, these parasitoids do not yet represent a recommended control lever to reduce *B. rufimanus* infestations, as more research should be carried out on (i) possible conservative measures that would increase the abundance of larval parasitoids or (ii) innovative strategies based on oophagous parasitoids identified in the literature but not yet reported in Wallonia.

In this sense, oophagous parasitoids would stand out as the most interesting biocontrol agents as they prevent egg hatchings. The genus *Uscana* is particularly noteworthy because these species are specifically associated with Bruchinae (Van Huis *et al.* 1990). This genus includes 32 species in Europe, with the most common ones being *Uscana senex* (Grese, 1923), *Uscana olgae* Fursov, 1987, and *Uscana inflaticornis* (Novicki, 1936) (Dr. Fursov, personal communication, March 2020).

Considering the demonstrated effectiveness of *U. senex* as a control agent against *Bruchus pisorum* L. (Hormazabal and Gerding 1998), it would be valuable to investigate the presence of such specimens in Wallonia and determine whether species, such as *U. semifumipennis* (Bellifa and Chapelin-Viscardi 2021a), are associated with *B. rufimanus*. Indeed, these micro-hymenopterans could potentially be used in an innundative control strategy, similar to the approach used against *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) in maize. The biocontrol technique consisted in the release of capsules containing *Trichogramma ostriniae* nymphs with multirotor unmanned aerial vehicles (UAVs) (Zhan *et al.* 2021; Martel *et al.* 2021). A noteworthy illustration of the potential of this biocontrol strategy can be seen in the recent

introduction of bio-logic® biopesticides by De Sangosse SAS (Marquion, France). These capsules are packed with approximately 1,250 Trichogramma sp. females that emerge over a span of three weeks.

Releasing the capsules via UAVs offers the advantage of positioning the emerging natural enemies in close proximity to the *B. rufimanus* ovipositions at the base of the plant. However, it should be noted that none of these parasitoids have been observed in Wallonia, and there is currently no established mass rearing method for them. The only available information on rearing such parasitoids comes from Alebeek (1996), which provides some methodological insights regarding *U. lariophaga*, a specific egg parasitoid of *C. maculatus*.

Other parasitoids, including larval parasitoids, may also be interesting biocontrol agents. However, until the biology of these organisms is better understood and conservative approaches are evaluated (see previous section), they cannot yet be considered as viable biocontrol agents.

3.5 Alternative curative methods

3.5.1 Hypocrealean entomopathogenic fungi (HEF)

In this thesis, five HEF strains were tested against two bruchid species and showed interesting lethal and sublethal effects (Chapter VI – part. 1). The most interesting HEF species was *B. bassiana* (strain GHA) which is formulated in the commercialized biopesticide BotaniGard®, while other strains such as *M. brunneum* strains V275 and USDA 4556 also presented interesting LT 50 and sublethal effects but are not yet formulated in some commercial biopesticides.

The timeframe related to the development of HEF-based biopesticide requires at least six years up to the commercialization of the products in Europe (**Figure 52)**. This timeframe is differently organized according to the researcher approach and the commercializing firm approach. The researcher approach aims to identify effective microbials that could be further assessed for their upscaling abilities (Gwynn *et al.* 2021). This approach includes steps

of (i) bioprospection study or isolates acquisition (up to 18 months), (ii) *in vitro* screening for lethal activity (up to six months), (iii) small scale tests for dose influence assessments, (iv) small scale tests for environmental influence assessments. Then, when potential microbial candidates have been identified, upscaling procedures are undertaken for the effective production of the biopesticide. This includes steps of (i) selection of the most productive, fast-growing and cost-effective candidates, (ii) developing appropriate formulation which ensures the viability of the biopesticide and the feasibility for large scale applications in field, and (iii) the commercial development of the product according to multiple legislative approving procedures. These three last steps may last up to four years and the cost of the overall activities related to the development of a new biopesticide acount from 30 to 71 millions of dollars according to Gwynn *et al.* (2021). The legislative framework concerning the placing of active substance and plant protection products on the market is presented in the Regulation (EC) No 1107/2009 in Europe and in the Royal Decree February 28, 1994 at the Belgian level.

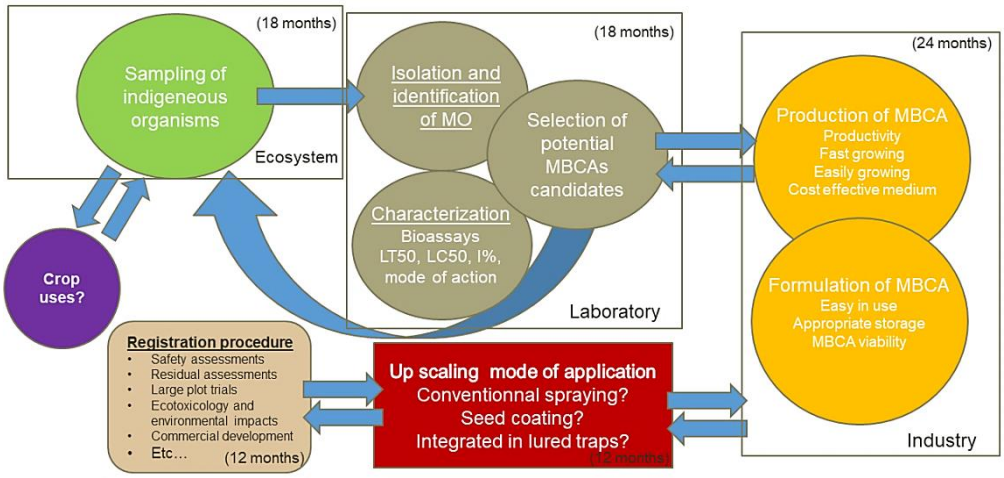

Figure 52: Methodological background and timeframe for the production and commercialization of microbial biopesticides - adapted from Coninck *et al.* (2020) and Gwynn *et al.* (2021)

Given the timeframe required and the total cost of biopesticide development, it could be questioned whether it would not be preferable to test available biopesticide in the commerce against BBWs. Indeed, as faba bean is a minor

crop in Belgium, applying for the extension of authorization of an already marketed product would be faster compared to a first authorization procedure (also governed by the Regulation (EC) No 1107/2009 and the Royal Decree February 28, 1994).

In this sense, results obtained in the Chapter VI (part. 1) would suggest that the use of Botanigard® could provide an effective curative strategy of biocontrol that can be applicable in large scale, as it could be highlighted on other pest species such as *L. decemlineata* or *Hypothenemus hampei* Wood & Bright, 1992 (Coleoptera: Curculionidae) (Wraight and Ramos 2002; Wraight *et al.* 2021). Innundative stragegies consisting in spraying of conidial suspensions on the crop would be interesting as it could provide similar application methods as PPPs and are therefore adapted to farmers' infrastructures and equipments. However, given the egg laying behavior of BBWs and the dense canopy of faba bean crops, conventional spraying would be physically limited by spatial obstruction, preventing the conidial suspensions to reach their target (*i.e.*, the pods at the base of the plants). Moreover, conidia sprayed at the top of the plant would be exposed to detrimental climatic conditions for their viability. The most appropriate mode of application would be the Dropleg^{UL} technique that directly sprays at the pods level via 90-cm-long legs hanging the nozzle carriers for guidance under the canopy (Lechler 2017). Recent studies could highlight the potential of the dropleg technique against insect pests in oilseed crops (Hausmann *et al.* 2019), but no information is available to our knowledge concerning BBWs in faba bean.

3.5.2 RNAi-based control strategy

Since the first conception of RNAi-plant-mediated pest control in 2007 (Baum *et al.* 2007; Mao *et al.* 2007), many improvements were performed and led to development of high specific RNA based biotechnologies for plant protection, mainly based on HIGS and SIGS strategies. Two pratical examples underline the potential of such biotechnologies that are (i) the implementation in the USA of open-field RNAi based management of *D. virgifera* via HIGS with

maize cultivars SmartStax PRO[2] (Bayer, Germany) (Head *et al.* 2017), and the SIGS strategy against *L. decemlineata* developed by the company GreenLight biosciences[3] (Willow and Veromann 2021).

The main challenges for successful RNAi-based control of pests are the identification of the effective gene to silence with lethal effects and the optimal delivery method for dsRNA ingestion and cell delivery which should be systemically spread (Rodrigues and Figueira 2016; Xue *et al.* 2023). Concerning the subfamily of Bruchinae, none studies were carried out for the examination of RNA interference. This was successfully performed on *C. maculatus* following micro-injectisons experiments presented in the Chapter VI (part. 2). This suggests that the mechanisms of RNAi could serve for the future new biopesticide development for bruchid control, but more research is required for the development of this new generation RNAi-biopesticide that is not yet marketed in the EU.

Firstly, the identification of highly lethal genes to be silenced could not be performed on adults of *C. maculatus*. Thus, further studies should focus on genes that were already reported for their lethal effects in other Coleopteran pests like genes of *srp54k, rop, alpha snap, rpn7, and rpt3* (Mehlhorn *et al.* 2021). Then, the biology of bruchids would present a significant obstacle when it comes to implementing a large-scale strategy for optimal administration of dsRNA in open field conditions. This challenge arises from the endophytic development of their larvae that would guide potential strategies toward HIGS or endophytic bacteria producing dsRNA. Indeed, the SIGS strategy seems quite complicated for targeting hatching larvae at the base of the plants with substantial dsRNA quantities.

HIGS would be the most appropriate strategy regarding the BBWs biology. The modifications of the faba bean genome should aim the integration of

[2] https://traits.bayer.com/corn/Pages/SmartStax-PRO.aspx (accessed on 16 June 2023)

[3] https://www.greenlightbiosciences.com/ (accessed on 16 June 2023)

RNAi traits in the pod's teguments, so that emerging larvae woud directly ingest these dsRNA and die before reaching the seeds. as this technique would be considered as a genetic modification and would be subjected to the Directive 2001/18/EC requiring a strict risk assessment and a fixed-term authorization (European Commission 2001). Recent regulatory updates by the European Commission are designed to encourage the integration of *new genomic technologies*[4] (NGT).

Based on the high sensitivity of Chrysomelidae species to RNAi (Joga *et al.* 2016; Shukla *et al.* 2016), it can be presumed that the RNAi mechanism observed in *C. maculatus* is also likely to be conserved in *B. rufimanus*. Unfortunately, the absence of a reference genome for this species has hindered further investigations into the RNAi mechanism. Sequencing the pest genome should be performed prior to the validation of the reference gene by RT-qPCR analyses on *B. rufimanus*. The genome sequencing could be carried out via new sequencing technologies such as Oxford Nanopore that provides high-quality insect genome and is more used for insect genome assembly (Li *et al.* 2019). Then, the identification and validation of the reference gene for RT-qPCR analyses on this pest should be assessed (see Chapter VI – part 2). These research activities can be estimated at one to two years to obtain the prerequisite for RNAi assessments on *B. rufimanus.*

Then the identification of the lethal gene inducing dead phenotypes genes should be carried out. The *in vitro* production of dsRNA and their topical administration via micro-injection into the insect offer a suitable methodology for screening the different lethal genes. Once achieved, the optimal strategy for delivering dsRNA to the feeding stage of the pest should be performed. These experiments would be facilitated by the development of artificial diets for larvae or adults as proposed in the Chapter III. Uptaking experiments of dsRNA-formulated in adult's artificial diets (*i.e.*, 10% sucrose solutions) would be the most appropriate method for RNAi assessment in *B. rufimanus.*

[4] https://ec.europa.eu/commission/presscorner/detail/en/qanda_23_3568

Through these laboratory research activities, the aim is to identify the most promising candidates for the production of RNAi-based pesticides and determine the most suitable method for their large-scale applications., *i.e.*, bacterial delivery via endosymbiotic bacteria (Whitten *et al.* 2016). The achievement of RNAi-based pesticide for large scale crop application would then require the cost-effective production and formulation of substantial quantities of dsRNA. The dsRNA amounts that are required in RNAi-based field strategies were reported to vary from 2 to 10 g per hectare (Zotti *et al.* 2018). The cost of such dsRNA production represents the most significant challenge in RNAi-based control strategies (Rodrigues *et al.* 2021). In this sense, the laboratory approach for dsRNA production including steps of total insect RNA extraction, retrotranscription, amplification of the target gene, transcription into dsRNA and purification is too expensive for agricultural pest control applications. Faced with this challenge, new methods have recently emerged for improving the commercial reliability of dsRNA production, such as proposed by Nwokeoji *et al.* (2022). This method is based on microbial fermentation (*i.e.*, *E. coli* strain harboring plasmids of dsRNA) followed by dsRNA purification process that would be in line with economic reliability and most appropriated application methods for BBWs control.

3.5.3 Semiochemicals

The evaluation of semiochemical traps in Chapter V revealed certain limitations for biocontrol of BBWs through mass trapping strategies, such as competition with host plant VOCs and potential impacts on beneficial communities. However, when combined with other strategies, these traps may offer other valuable opportunities for biocontrol (as discussed in the sections above). As emphasized in Chapter I, the main constraint of semiochemical-based control methods is the limited understanding of pheromonal communication modes in *B. rufimanus*. So far, only one molecule has been identified as a sex pheromone, but it does not effectively attract the pests over long distances in field conditions (Bruce *et al.* 2011).

An interesting aspect of pheromonal communication discussed in Chapter I, which was not formally addressed in this thesis, is the study of epidictic pheromones. It has already been observed in many insects that females release a marking pheromone with their eggs (Prokopy 1972). These pheromones, known as epidictic pheromones or Oviposition-Deterring Pheromones (ODP) or Host-Marking Pheromones (HMP), are emitted by accessory glands located at the tip of the abdomen (Behan and Schoonhoven 1978), mandibular secretions, or larval feces (Klein *et al.* 1990; Li and Ishikawa 2004). They have the effect of reducing or even preventing egg layings.

The regulation of population through oviposition is believed to be an evolutionary process that enhances the fitness of offspring by limiting the number of eggs on a limited substrate. The reduction in larval density decreases the risk of intraspecific competition when they become too numerous. Several biological characteristics suggest this mode of oviposition regulation, such as: (i) the success of offspring is strongly linked to larval density given their confinement within a limited food resource (Prokopy *et al.* 1984); (ii) cannibalism behavior among larvae is common (Thiéry 1991); (iii) regular spacing of egg laying (Guoqing *et al.* 2001), with an equal distribution of eggs based on the available substrate quantity (Messina *et al.* 1987).

Several studies were conducted some decades ago on these semiochemical regulation phenomena of oviposition behavior in several bruchid species. Oshima *et al.* (1973) demonstrated this phenomenon in *C. chinensis*, and Szentesi (1981) observed it in *A. obtectus*. However, these studies did not identify the composition and chemical nature of these epidictic pheromones. The initial studies were conducted by Yamamoto (1976), who revealed the lipid nature of the pheromonal compounds involved in *C. chinensis* ovipositions.

Later, Tanaka *et al.* (1981), Mori *et al.* (1983), Yamamoto (1990), and Parr *et al.* (1998) deepened the understanding of oviposition and mating behaviors in *C. chinensis* and *C. maculatus* and were able to characterize the chemical compounds influencing these behaviors. The main findings were that the egg-

laying behaviors of *C. maculatus* and *C. chinensis* are characterized by an equitable distribution of eggs among available seeds. This phenomenon is regulated by both the presence of eggs and compounds emitted by the insects, up to a certain threshold before the ovipositions become randomly distributed. These compounds that are emitted by the insects during prospecting and egg-laying were referred to as *Biological Conditioning Substances* (BCS). The activity of BCS was determined by Sakai *et al.* (1984), and although they do not have an inhibitory effect on egg laying, they act as markers for egg laying and guide females towards seeds with fewer of these substances. The chemical identification of BCS was carried out by Yamamoto (1990) and revealed a mixture of lipids consisting mainly of hydrocarbons, triglycerides, and fatty acids. These findings align with the work of Parr *et al.* (1998), who concluded that non-volatile compounds present in epicuticular waxes of seeds influence the oviposition preferences of *C. maculatus*.

BCS are not the only factors involved in oviposition preference, as eggs also play a role. The quantification of these two factors was conducted by Honda *et al.* (1989) in *C. chinensis*: the behavior of equitable egg distribution is observed until the number of eggs per seed reaches 3 to 4, along with 20µg of BCS per seed. Beyond this threshold, eggs are distributed randomly. Another interesting finding regarding BCS is their ovicidal effects. As the number of adults per seed increases, the number of laid eggs also increases, but the number of hatched eggs and emerging adults remains constant. Ovicidal activity occurs beyond 75 µg of BCS per seed. A pair of *C. maculatus* emits approximately 58 µg during their lifetime.

This ovicidal effect of BCS, particularly those containing fatty acids, led Yamamoto (1990) to study the effects of edible oils as a means of protection against bruchids. They tested 16 oils, among which rapeseed oil showed no *C. maculatus* hatching. Effectively, rapeseed oil is already known and marketed as an insecticide, but did not showed any significant efficiency against *B. rufimanus* during field test performed in France (Riquet *et al.* 2021).

Regarding *B. rufimanus* biology, several indications from female oviposition behavior suggest the potential presence of epideictic pheromones. Oviposition on green pods in the field never exceeds 10 eggs per pod (considering that each pod of its host plant contains approximately 5 seeds). Furthermore, larval overcrowding leads to a risk of cannibalism (Pölitz and Reike 2019). Unpublished oviposition experiments conducted during this thesis have successfully obtained *B. rufimanus* egg layings on glass tubes. It would be highly interesting to perform liquid extraction with hexane and to identify the compounds accompanying these egg layings via gas chromatography before considering potential similar bioassays than those presented on other bruchid species.

3.6 Physical interventions in storage facilities

In traditional Integrated Pest Management (IPM) programs, physical or mechanical interventions are typically implemented in the field following monitoring activities (Gwynn *et al.* 2021). However, due to the endophytic development of *B. rufimanus* larvae, these interventions need to be postponed at the end of the growing season when the seeds are harvested and stored. Three primary physical intervention measures have been identified, one of which focuses on enhancing the value of infested lots to meet quality standards, while the other two aim to impact the emerging pest population that will contribute to the next year's BBWs population.

The detection of infestation rates on harvested seeds is crucial for marketization of seeds (see Chapter I). Standards for the evaluation of infested seeds are internationally defined in France by the standard NF ISO 605 (AFNOR 1991) and more specifically for faba beans by the standard NF V03-944 (AFNOR 2021). These definitions are based on visual detections. However, visual detection of bruchid infestations is only possible when the adults have emerged (*i.e.*, observation of emergence holes). In situations where the adults are still inside the seed, detection is very difficult. The methodological adaptation for the detection technique of infested seeds is currently being revised within the *Cap protéine* project to take into account these limitations

(Jauvion 2023). Several detection methods are envisaged, including insect activity detection (CO_2 measurement, acoustic detection and emergence induction), imaging detection (near-infrared spectroscopy, hyperspectral imaging, infrared thermography) and X-ray-based methods (radiography and tomography). The results will provide a standardized and reliable quantification of seed damage. These informations would be very interesting to associate to adults field monitoring in order to relate the pest pressure with the subsequent seed damages and build reliable ET (*cf.*, previous section). Finally, these methods are also essential to assess whether seed sorting methods could be considered to eventually recover the non-infested parts for human consumption.

During seed storage, two measures can be taken to regulate emergence in a biocontrol friendly manner prior to applying seed treatments to kill insects in storage. The results of Chapter II showed that larval parasitoids also emerge at the harvest time and the results of Chapter III defined the total thermal development of BBWs. Based on these conclusions, if the indeterminate growth of *Vicia faba* allows seed maturation before BBWs emergences, insuring a maximal amounts of insects inside seeds, including BBWs and their parasitoids, selective grids based on the difference of the parasitoids/BBWs size should enable the parasitoids to emerge and keep BBWs in the storage facilities. In this way, parasitoid populations would be favored near storage sites.

Many faba bean growers keep harvested seeds in open sheds, allowing significant numbers of bruchids to fly away (personal observation during surveys). This is likely to stop the gradual reduction of pest populations. It would therefore be worthwhile to propose the use of selective netting or the installation of appropriate grids at silo level in the IPM specifications. This should be a faba bean-specific regulatory requirement which must be integrated into the Measure 1.5 *Preventing the spread of pests through hygiene measures* of the first principle "Good Agricultural practices" in the regional

IPM specifications described in the current Wallon Ministerial Decree October 13, 2022.

Finally, to manage potential late emergence strategies of BBW, harvested seeds can be treated with various physical methods to kill non-emerging bruchids, such as chilling, CO_2 saturation, or temperature elevation (Schmidt 2000; Wong-Corral *et al.* 2013; Gad *et al.* 2022). Harvesting the seeds at 17-18% followed by hot air drying (between 50 and 70°C) is suitable to both kill the bruchids and reduce the relative humidity of the seeds to the standard of 14%, but this would represent a cost of around 19 euros per ton. (Le Gall *et al.* 2021; Fegra and Synagra 2023).

4 Thesis conclusion

In conclusion, the broad bean weevil (BBW) is a pest with a complex biology that requires multiple, complementary and integrated management measures to progressively reduce its annual populations from one year to year. Given the current lack of effective control methods, several levers were evaluated in this thesis and an IPM paradigm is proposed based on these results. The most optimal cultivars were identified, as well as the natural enemies of this pest in Wallonia. Thermal development modeling was used to recommend cultivation practices in terms of sowing and harvesting dates in order to limit the annual cycle as much as possible. Possible conservative measures regarding indigenous parasitoids should be further studied to improve reliable biocontrol of BBWs, and appropriate storage measures must be implemented to prevent post-harvest emergence. The most interesting semiochemical traps have been identified and should be used to set reliable economic intervention thresholds linking observed populations to damage caused, for which detection standards are currently being revised. This thesis has also shed light on new biotechnological tools to control BBWs, including HEF biopesticides and RNAi-based pesticides, which hold promise as future control methods in the short term, complementing the current preventive measures. There are several avenues for further research, such as inundative biocontrol methods based on potential oophagous parasitoids or oviposition

pheromones. Certain combinations, such as the HEF approach combined with semiochemicals, would be very interesting to study further. Faba bean cultivation is promoted by the CAP (CAP 2023-2027). It would therefore be crucial to continue on the development of effective alternatives to pesticides and to see how pest management evolves with the development of these hitherto minor crops, in order to contribute as much as possible to the resilience and independence of European agro-ecosystems.

Chapter VIII

Scientific publications and communication

1 Scientific publications in peer reviewed journal

Segers A., Caparros Megido R., Lognay G., Francis F. (2020). *Overview of Bruchus rufimanus Boheman 1833 (Coleoptera: Chrysomelidae): biology, chemical ecology and semiochemical opportunities in integrated pest management programs*. Crop Protection 140(3):105411. DOI: 10.1016/j.cropro.2020.105411

Segers, A.*, Dumoulin, L.*, Megido, R. C., Jacquet, N., Cartrysse, C., Kamba, P. M.,Pierreux, J., Richel, A., Blecker, C., & Francis, F. (2021). *Varietal and environmental effects on the production of faba bean (Vicia faba L.) seeds for the food industry by confrontation of agricultural and nutritional traits with resistance against Bruchus spp. (Coleoptera: Chrysomelidae, Bruchinae).* Agriculture, Ecosystems & Environment, 327, 107831. DOI: 10.1016/j.agee.2021.107831. *Authors contributed equally to the publication

Zhang J., Li H., Zhong X., Tian J., Segers A., Xia L., Francis F. (2022). *RNA-Interference-Mediated Aphid Control in Crop Plants: A Review*. Agriculture, 12, 2108. https://doi.org/10.3390/agriculture1212210

Zhang J., Li H., Zhong X., Tian J., Segers A., Xia L., Francis F. (2023). *Silencing an aphid-specific gene SmDSR33 for aphid control through plant-mediated RNAi in wheat.* Frontiers in Plant Science, 13. DOI: 10.3389/fpls.2022.1100394

Segers, A., Carpentier, J., Francis, F., & Caparros Megido, R. (2023). *Gene Silencing of laccase 1 Induced by Double-Stranded RNA in Callosobruchus maculatus (Fabricius 1775) (Coleoptera: Chrysomelidae) Suggests RNAi as a Potential New Biotechnological Tool for Bruchid's Control.* Agriculture, 13(2), Article 2. DOI: 10.3390/agriculture13020412

Segers, A., Noël, G., Delanglez, L., Caparros Megido, R., & Francis, F. (2023). *Impacts of Semiochemical Traps Designed for Bruchus rufimanus Boheman 1833 (Coleoptera:Chrysomelidae) on Nontarget Beneficial Entomofauna in Field Bean Crops.* Insects, 14(2), Article 2. DOI: 10.3390/insects14020153

2 Oral presentations at international/national conferences

Segers A. , Caparros Megido R., Francis F. (30th of January 2020). *La bruche en féverole, un insect étudié de près dans le projet de recherche FEVERPRO.* Lutte intégrée en colza et en Protéagineux (A.P.P.O., Gembloux, Belgium)

Segers A., Caparros Megido R., Francis F. (31st of January 2022).*La bruche en féverole – Présentation des résultats de recherche (FEVERPRO – WP1) et avancées dans les connaissances*. Réunion Protéagineux (virtual conference - CePiCOP, Gembloux,Belgium).

Segers A., Caparros Megido R., Francis F. (8th of Devember 2022). *FEVERPRO : problématique de la bruche en culture de féverole et lutte biologique intégrée.* L'autonomie protéique en France et en Belgique : quelle place pour la féverole bio ? (Colloque de clotûre de projet Interreg SymBIOse, Lille, France).

Segers A., Caparros Megido R., Francis F. (30th of January 2023). La problématique de la bruche en culture de féverole et lutte biologique intégrée au sein du projet FEVERPRO. Réunion d'information sur les protéagineux et le colza (CePiCOP, Gembloux, Belgium).

3 Posters presented at international/national conferences

Segers A., Caparros Megido R., Lognay G., Francis F. (2020). *FEVERPRO Project Development of biological control methods in alternative to pesticides to manage Bruchus rufimanus Boheman 1833 in field bean crops.* Poster session presented at Conference 25th National Symposium for Applied Biological Sciences (Gembloux, Belgium). https://hdl.handle.net/2268/253754

Segers A., Caparros Megido R., De Clerck C., Francis F. (2021). *Susceptibility of Bruchus rufimanus Boheman 1833 (Coleoptera: Chrysomelidae) to three entomopathogenic fungi: Limits of conidia suspension sprayings and pledging alternatives in integrated pest management strategy.* Poster session presented at 2021 International Congress on Invertebrate Pathology and Microbial Control & 53rd Annual Meeting of the Society for Invertebrate Pathology (Virtual meeting, France-Mexico). DOI: 10.13140/RG.2.2.23290.06086

4 Popularization articles

Vandegoor J. (2020) *De nouveaux moyens de lutte contre la bruche pour booster la culture de la féverole*. Restitution from oral presentation of Segers *et al.* (2020). Le sillon Belge. https://www.sillonbelge.be/5535/article/2020-02-13/de-nouveaux-moyens-de-lutte-contre-la-bruche-pour-booster-la-culture-de-la

Segers A. Lugendo R., Caparros Megido R., Francis F. (April 2023). *Bruche de la fève, quells impacts en Wallonie ? Les ennemis naturels peuvent-ils être une solution ?* Itinéraire bio n°69. https://www.biowallonie.com/wp-content/uploads/2023/03/Brochure_A4_Itineraires-BIO_69_Web.pdf

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Apprendix

Appendix A: Table of all sampled species collected from faba bean seeds in 2021 and 2022 in different bioclimatic areas

Site	Emergence date	Family	Subfamily	Species	Ab.
Assesse	14/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**81**
Assesse	17/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**28**
Assesse	21/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**10**
Beho	17/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**1**
Beho	21/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**14**
Beho	24/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**10**
Beho	29/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**7**
Beho	01/10/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**1**
Bierwart	09/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**33**
Bierwart	14/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**40**
Bierwart	17/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**5**
Bierwart	21/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**5**
Ciney	06/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**4**
Ciney	08/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**12**
Ciney	09/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**23**
Ciney	14/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**34**
Ciney	17/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**6**
Ciney	21/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**6**
Battice	07/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**3**
Battice	09/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**1**
Battice	14/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**1**
Isnes	08/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**76**
Isnes	09/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**52**
Isnes	14/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**39**
Isnes	17/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**11**
Isnes	21/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**12**
Isnes	24/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**3**
Rebecq	06/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**6**
Rebecq	08/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**3**

Rebecq	09/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**2**
Rebecq	14/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**1**
Verdenne	09/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**1**
Verdenne	14/09/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**2**
Assesse	25/08/2021	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**1**
Assesse	12/08/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**127**
Assesse	16/08/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**96**
Assesse	17/08/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**22**
Assesse	18/08/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**40**
Assesse	21/08/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**5**
Assesse	24/08/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**40**
Assesse	25/08/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**22**
Assesse	29/08/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**12**
Assesse	01/09/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**25**
Erezée	05/08/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**1**
Erezée	11/08/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**8**
Erezée	16/08/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**6**
Erezée	17/08/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**12**
Erezée	21/08/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**7**
Erezée	24/08/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**4**
Ferrière	11/08/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**17**
Ferrière	16/08/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**9**
Ferrière	17/08/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**6**
Ferrière	21/08/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**9**
Ferrière	24/08/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**8**
Ferrière	25/08/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**3**
Ferrière	01/09/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**2**
Itre	11/08/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**5**
Itre	17/08/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**12**
Itre	21/08/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**2**
Itre	24/08/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**1**
Rotheux	12/08/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**109**
Rotheux	16/08/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**62**
Rotheux	17/08/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**158**
Rotheux	21/08/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**107**

Rotheux	24/08/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**37**
Rotheux	25/08/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**11**
Rotheux	01/09/2022	Chrysomelidae	Bruchinae	*Bruchus rufimanus* Boheman 1833	**7**
Assesse	14/09/2021	Braconidae	Brachistinae	*Triaspis luteipes* (Thomson, 1874)	**2**
Assesse	12/08/2022	Pteromalidae	Pteromalinae	*Pteromalus sp.*	**1**
Assesse	16/08/2022	Pteromalidae	Pteromalinae	*Pteromalus sp.*	**1**
Assesse	16/08/2022	Braconidae	Brachistinae	*Triaspis sp.*	**4**
Assesse	17/08/2022	Pteromalidae	Pteromalinae	*Pteromalus sp.*	**1**
Assesse	18/08/2022	Pteromalidae	Pteromalinae	*Pteromalus sp.*	**1**
Assesse	21/08/2022	Pteromalidae	Pteromalinae	*Pteromalus sp.*	**5**
Assesse	21/08/2022	Braconidae	Brachistinae	*Triaspis sp.*	**7**
Assesse	24/08/2022	Braconidae	Brachistinae	*Triaspis sp.*	**2**
Assesse	25/08/2022	Braconidae	Brachistinae	*Triaspis sp.*	**4**
Bierwart	09/09/2021	Braconidae	Brachistinae	*Triaspis luteipes* (Thomson, 1874)	**1**
Bierwart	17/09/2021	Pteromalidae	Pteromalinae	*Pteromalus sequester* Walker, 1835	**1**
Bierwart	21/09/2021	Braconidae	Brachistinae	*Triaspis luteipes* (Thomson, 1874)	**1**
Ciney	06/09/2021	Braconidae	Brachistinae	*Triaspis luteipes* (Thomson, 1874)	**24**
Ciney	08/09/2021	Braconidae	Brachistinae	*Triaspis luteipes* (Thomson, 1874)	**4**
Ciney	08/09/2021	Braconidae	Brachistinae	*Triaspis thoracica* (Curtis, 1860)	**5**
Ciney	09/09/2021	Braconidae	Brachistinae	*Triaspis thoracica* (Curtis, 1860)	**7**
Erezee	11/08/2022	Pteromalidae	Pteromalinae	*Pteromalus sp.*	**1**
Erezee	11/08/2022	Braconidae	Brachistinae	*Triaspis sp.*	**5**
Erezee	16/08/2022	Braconidae	Brachistinae	*Triaspis sp.*	**1**
Erezee	24/08/2022	Braconidae	Brachistinae	*Triaspis sp.*	**1**
Ferrière	11/08/2022	Braconidae	Brachistinae	*Triaspis sp.*	**1**
Ferrière	16/08/2022	Braconidae	Brachistinae	*Triaspis sp.*	**2**
Ferrière	24/08/2022	Braconidae	Brachistinae	*Triaspis sp.*	**1**
Isnes	15/09/2019	Pteromalidae	Pteromalinae	*Pteromalus sequester* Walker, 1835	**2**
Isnes	15/09/2019	Braconidae	Brachistinae	*Triaspis luteipes* (Thomson, 1874)	**2**
Isnes	03/08/2020	Braconidae	Brachistinae	*Triaspis luteipes* (Thomson, 1874)	**22**
Isnes	05/08/2020	Braconidae	Brachistinae	*Triaspis luteipes* (Thomson, 1874)	**2**
Isnes	05/08/2020	Braconidae	Brachistinae	*Triaspis thoracica* (Curtis, 1860)	**1**
Isnes	07/08/2020	Braconidae	Brachistinae	*Triaspis thoracica* (Curtis, 1860)	**3**
Isnes	10/08/2020	Braconidae	Brachistinae	*Triaspis thoracica* (Curtis, 1860)	**11**
Isnes	11/08/2020	Braconidae	Brachistinae	*Triaspis thoracica* (Curtis, 1860)	**16**

Isnes	13/08/2020	Braconidae	Brachistinae	*Triaspis luteipes* (Thomson, 1874)	**1**
Isnes	13/08/2020	Braconidae	Brachistinae	*Triaspis thoracica* (Curtis, 1860)	**6**
Isnes	17/08/2020	Braconidae	Brachistinae	*Triaspis thoracica* (Curtis, 1860)	**4**
Isnes	24/08/2020	Braconidae	Brachistinae	*Triaspis thoracica* (Curtis, 1860)	**1**
Isnes	10/09/2020	Pteromalidae	Pteromalinae	*Pteromalus fasciatus* (Thomson, 1878)	**1**
Isnes	10/09/2020	Braconidae	Brachistinae	*Triaspis luteipes* (Thomson, 1874)	**3**
Isnes	10/09/2020	Braconidae	Brachistinae	*Triaspis thoracica* (Curtis, 1860)	**1**
Isnes	08/09/2021	Braconidae	Brachistinae	*Triaspis luteipes* (Thomson, 1874)	**6**
Isnes	09/09/2021	Pteromalidae	Pteromalinae	*Pteromalus sequester* Walker, 1835	**1**
Isnes	10/09/2021	Braconidae	Brachistinae	*Triaspis luteipes* (Thomson, 1874)	**21**
Isnes	14/09/2021	Braconidae	Brachistinae	*Triaspis luteipes* (Thomson, 1874)	**1**
Isnes	20/09/2021	Pteromalidae	Pteromalinae	*Pteromalus sequester* Walker, 1835	**1**
Isnes	29/09/2021	Pteromalidae	Pteromalinae	*Pteromalus sequester* Walker, 1835	**1**
Isnes	10/08/2022	Braconidae	Brachistinae	*Triaspis sp.*	**5**
Isnes	12/08/2022	Braconidae	Brachistinae	*Triaspis sp.*	**2**
Isnes	25/08/2022	Braconidae	Brachistinae	*Triaspis sp.*	**3**
Itre	05/08/2022	Pteromalidae	Pteromalinae	*Pteromalus sp.*	**1**
Itre	11/08/2022	Braconidae	Brachistinae	*Triaspis sp.*	**1**
Mazy	26/08/2020	Braconidae	Brachistinae	*Triaspis luteipes* (Thomson, 1874)	**10**
Rebecq	06/09/2021	Braconidae	Brachistinae	*Triaspis luteipes* (Thomson, 1874)	**1**
Rebecq	08/09/2021	Braconidae	Brachistinae	*Triaspis thoracica* (Curtis, 1860)	**1**
Rotheux	17/08/2022	Braconidae	Brachistinae	*Triaspis sp.*	**7**
Rotheux	21/08/2022	Braconidae	Brachistinae	*Triaspis sp.*	**2**

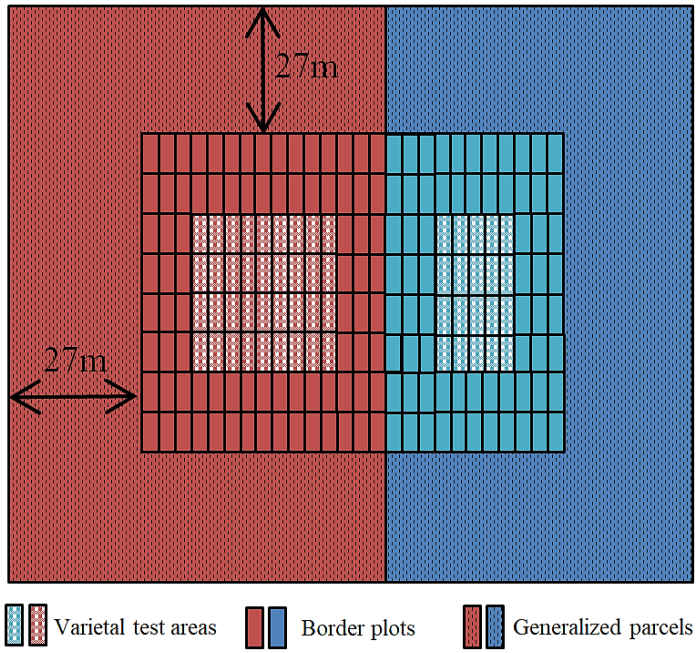
Appendix B: Rearing results obtained in each cages

temp. (°C)*	cage	Devpmt_tot_avg ± sd (day)	Ovip. delay	Emerging bruchids	sex ratio	% Emergence
14.04 ± 1.81	A1	-	-	-		-
	A2	-	-	-		
	A3	-	-	-		
17.47 ± 0.97	B1	-	-	-		-
	B2	-	-	-		
	B3	-	-	-		
22.02 ± 1.98	C1	67.65 ± 5.11	17	24 (17M,7F)	0.41	67.5% (79 out - 38in)
	C2		18	26 (14M, 12F)	0.86	
	C3		19	12 (4M, 8F)	2.00	
	C4		19	17 (10M, 7F)	0.70	
21.05 ± 0.61	D1	65.95 ± 9.76	9	13 (4M, 9F)	2.25	78.6% (48 out - 13in)
	D2		9	17 (8M, 9F)	1.13	
	D3		9	18 (7M, 11F)	1.57	
21.10 ± 0.68	E2	66.37 ± 8.83	18	15 (10M, 5F)	0.5	58.5% (38 out - 27 in)
	E3		18	26 (15M, 11F)	0.73	
23.57 ± 1.49	F3	64.16 ± 6.46	19	8 (5M, 3F)	0.60	48.6% (18 out - 19in)
	F4		25	10 (4M, 6F)	1.50	
26.75 ± 1.45	G1	46.65 ± 3.89	7	14 (7M, 7F)	1	58% (49 out - 34 in)
	G2		7	20 (10M, 10F)	1	
	G3		7	15 (7M,8F)	1.14	
28.06 ± 0.80	H1	34.57 ± 5.78	-	15 (ND)	ND	62% (49 out - 30 in)
	H2		-	14 (ND)	ND	
	H3		-	20 (ND)	ND	

Appendix C: General description of the 14 faba bean varieties tested in field trials (2019–2020) according to technical information provided by owners.

Name	Owner (subscribing year)	Type	Flower	TSW (g)	Protein content (%)	Height (cm)	Start flowering	Stop flowering
Axel	Sem Partners (2014)	Winter	Coloured	535	28.2	97	15/04	24/05
Irena	Agri Obtention (2001)	Winter	Coloured	495	29.6	85	13/04	22/05
Honey	Agri Obtention (2011)	Winter	Coloured	566	28.5	89	25/04	30/05
Nebraska	Agri Obtention (2015)	Winter	Coloured	470	28.0	102	21/04	21/05
Bumble	Limagrain Europe (2014)	Winter	Coloured	551	28.1	104	22/04	31/05
Bering	NPZ Lembke (2017)	Winter	Coloured	487	27.8	113	21/04	28/05
Diva	Agri Obtention (2001)	Winter	Coloured	449	28.1	105	17/04	24/05
Augusta	NPZ (2018)	Winter	Coloured	ND	ND	ND	ND	ND
Tundra	Limagrain (2013)	Winter	Coloured	600	28%	105.3	26/04	01/06
Bobas	DANKO HODOWLA ROSLIN (2002)	Spring	Coloured	ND	ND	ND	ND	ND
Fanfare	RAGT (2013)	Spring	Coloured	448	30.1	94	24/05	14/06
Julia	SAATZUCHT GLEISDORF (2007)	Spring	Coloured	406	ND	ND	ND	ND
LG cartouche	Limagrain Europe (2017)	Spring	Coloured	527	29.1	ND	ND	ND
Tiffany	RAGT (2014)	Spring	Coloured	423	30.4	95	25/05	14/06

Appendix D: Representative scheme of varietal field test dispositive



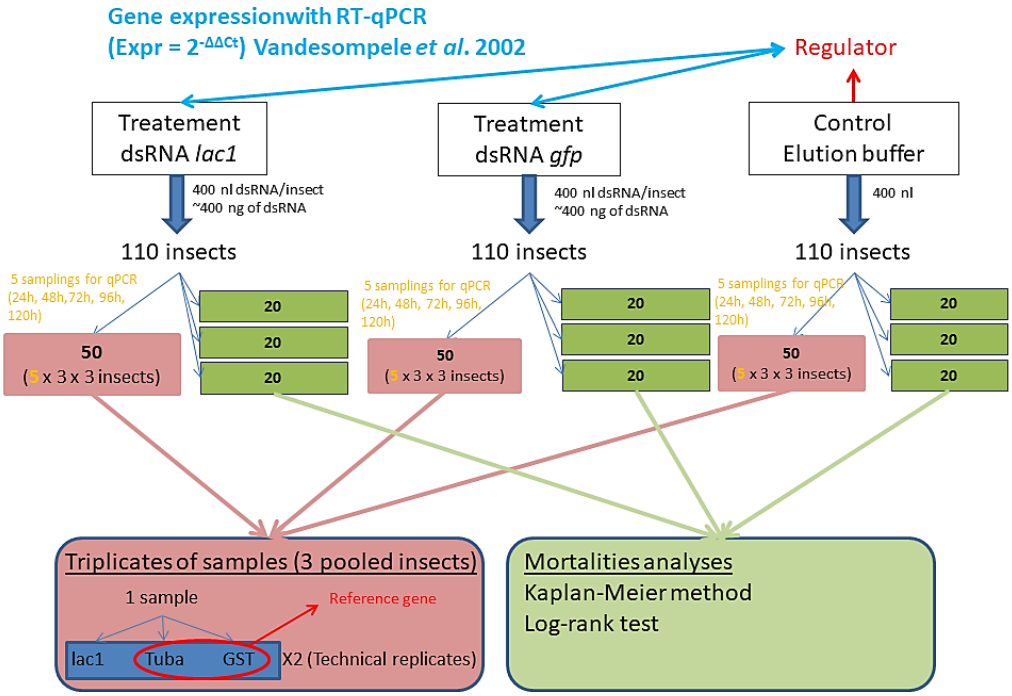
Appendix E: Varietal phenology of each tested variety during the two growing seasons (2018–2019) and (2019–2020). DFF: day of first flowering; DLF: day of last flowering; DFP: day of first pods; DF: duration of flowering.

		2019				2020				Owner information		
Name	**Type**	**DFF**	**DLF**	**DFP**	**DF**	**DFF**	**DLF**	**DFP**	**DF**	**DFF**	**DLF**	**DF**
Diva	**Winter**					20/04/2020	04/06/2020	09/05/20	45	17/04/20	24/05/20	37
Irena	**Winter**					11/04/2020	29/05/2020	05/05/21	48	13/04/20	22/05/20	39
Honey	**Winter**					19/04/2020	04/06/2020	10/05/20	46	25/04/20	30/05/20	35
Axel	**Winter**					13/04/2020	03/06/2020	04/05/20	51	15/04/20	24/05/20	39
Bumble	**Winter**					16/04/2020	04/06/2020	10/05/20	49	22/04/20	01/06/20	40
Nebraska	**Winter**	06/05/2019	17/06/2019	20/05/2019	42	19/04/2020	02/06/2020	10/05/20	44	21/04/20	21/05/20	30
Bering	**Winter**					25/04/2020	08/06/2020	11/05/20	44	ND	ND	ND
Augusta	**Winter**					16/04/2020	08/06/2020	09/05/20	53	ND	ND	ND
Bobas	**Spring**					02/06/2020	02/07/2020	12/06/20	30	ND	ND	ND
Julia	**Spring**					01/06/2020	28/06/2020	11/06/21	27	ND	ND	ND
Fanfare	**Spring**	31/05/2019	30/06/2019	21/06/2019	30	30/05/2020	28/06/2020	12/06/20	29	24/05/21	14/06/21	21
Tiffany	**Spring**					01/06/2020	27/06/2020	11/06/20	26	25/05/21	14/06/21	20
LG cartouche	**Spring**					02/06/2020	29/06/2020	11/06/20	27	ND	ND	ND

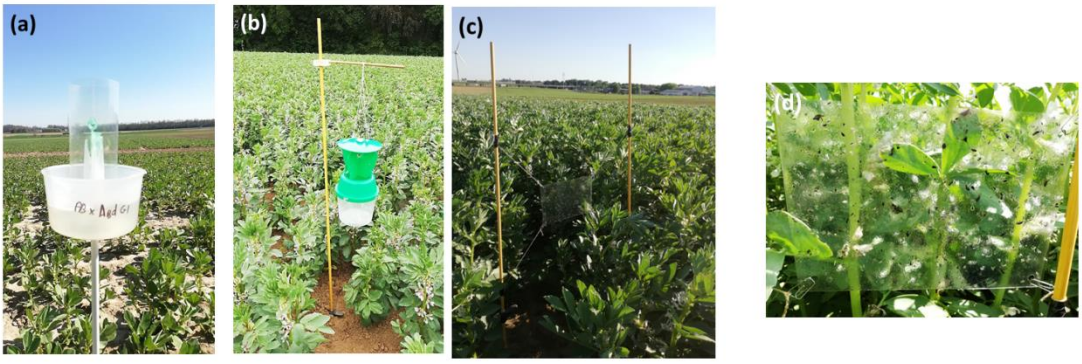
Appendix F: PCA supplementary data

		PC1	PC2	PC3	PC4	PC5
Factor contributions	**FY**	24.249	0.210	8.699	9.330	5.636
	TSW	21.340	2.534	0.597	1.465	70.315
	CP	6.835	2.292	54.126	1.321	9.362
	PP	26.952	0.034	1.354	9.444	8.820
	AC	9.318	6.389	13.365	41.923	4.124
	CL	10.914	7.602	10.355	36.400	0.395
	HS	0.338	41.569	3.160	0.002	0.172
	EH	0.055	39.371	8.344	0.116	1.177
Eigenvalue		3.244	2.231	1.206	0.809	0.322
Proportion		40.544	27.889	15.069	10.240	4.025
Cumulated		40.544	68.433	83.503	93.742	97.767

Appendix G: Summary diagram of the methodology and statistical analyses used in the micro-injection experiments for the assessment of gene expression by RT-qPCR and survival analyses.



Appendix H: Traps illustration. (a) White pan traps with transparent cylinder-PB (AgriOdor, Rennes, France), (b) Green funnel pan traps with barrier cross bar-PV (Pherobank B.V., Wijk bij Duurstede, The Netherlands), (c,d) Sticky control traps © A. Segers.



Appendix I: Climatic conditions recorded during the experimental period. Avg(T) = weekly average of day temperature. Avg(Tmax) = weekly average of maximal day temperatures (°C). Avg(prec.) = weekly average of the weekly precipitations.

N° Week	Avg (T) (°C)	Avg (Tmax) (°C)	Avg (Pcum) (mm)	Notes
14	9.2	15.0	0	
15	4.2	9.1	30.5	
16	4.2	9.9	2	
17	8.5	15.3	0	Trap installation in WFB
18	7.9	14.5	3.9	
19	7.8	13.8	6.2	
20	13.0	18.9	5.4	
21	11.2	15.4	30.2	
22	11.7	16.7	20.8	
23	17.4	23.9	11.6	Trap installation in SFB
24	17.9	24.2	0	
25	21.1	28.0	25	
26	16.4	20.2	30	
27	17.3	22.0	35.5	
28	17.5	22.0	22	
29	17.1	20.3	117	
30	19.1	25.2	6	
31	18.1	22.0	10.7	Traps removal in WFB
32	17.1	21.2	56.5	Traps removal in SFB

Appendix J: List of the 1424 captured beneficials including 67 species from eight different families.

Taxonomy	Number of specimens
Coleoptera	**291**
Coccinellidae	**291**
Adalia bipunctata (Linnaeus, 1758)	2
Coccinella septempunctata Linnaeus, 1758	191
Harmonia axyridis (Pallas, 1773)	71
Hippodamia variegata (Goeze, 1777)	7
Propylea quatuordecimpunctata (Linnaeus, 1758)	17
Psyllobora vigintiduopunctata (Linnaeus, 1758)	3
Diptera	**316**
Syrphidae	**316**
Epistrophe diaphana (Zetterstedt, 1843)	1
Epistrophe euchroma (Kowarz, 1885)	1
Epistrophe nitidicollis (Meigen, 1822)	1
Episyrphus balteatus De Geer, 1776	129
Eristalis tenax (Linnaeus, 1758)	5
Melanostoma mellina (Linnaeus, 1758)	45
Metasyrphus corollae (Fabricius, 1794)	31
Metasyrphus luniger (Meigen, 1822)	7
Scaeva pyrastri (Linnaeus, 1758)	3
Sphaerophoria scripta (Linnaeus, 1758)	84
Syrphus vitripennis Meigen, 1822	5
Xylota lenta Meigen, 1822	1
Hymenoptera	**817**
Andrenidae	**75**
Andrena barbilabris (Kirby, 1802)	1
Andrena bicolor Fabricius, 1775	5
Andrena carantonica Pérez, 1902	1
Andrena cineraria (Linnaeus, 1758)	9
Andrena dorsata (Kirby, 1802)	4
Andrena flavipes Panzer, 1799	2
Andrena fulva (Müller, 1776)	6
Andrena gravida Imhoff, 1832	1
Andrena haemorrhoa (Fabricius, 1781)	3
Andrena minutula (Kirby, 1802)	13

Andrena nigroaenea (Kirby, 1802)	12
Andrena nitida (Müller, 1776)	3
Andrena ovatula (Kirby, 1802)	6
Andrena subopaca Nylander, 1848	8
Andrena wilkella (Kirby, 1802)	1
Apidae	**674**
Apis mellifera Linnaeus, 1758	321
Bombus hortorum Linnaeus, 1761	33
Bombus hypnorum (Linnaeus, 1758)	1
Bombus lapidarius (Linnaeus, 1758)	20
Bombus pascuorum (Scopoli, 1763)	15
Bombus pratorum (Linnaeus, 1761)	5
Bombus terrestris (Linnaeus, 1758)	275
Bombus vestalis (Geoffroy, 1785)	1
Nomada flavoguttata (Kirby, 1802)	1
Nomada fuscicornis Nylander, 1848	1
Nomata signata Jurine, 1807	1
Colletidae	**3**
Hylaeus communis Nylander, 1852	2
Hylaeus hyalinatus Smith, 1842	1
Halictidae	**56**
Halictus scabiosae (Rossi, 1790)	2
Lasioglossum calceatum (Scopoli, 1763)	5
Lasioglossum laticeps (Schenck, 1870)	9
Lasioglossum lativentre (Schenck, 1853)	8
Lasioglossum leucozonium (Schrank, 1781)	2
Lasioglossum minutissimum (Kirby, 1802)	5
Lasioglossum minutulum (Schenck, 1853)	1
Lasioglossum morio (Fabricius, 1793)	2
Lasioglossum parvulum (Schenck, 1853)	1
Lasioglossum pauxillum (Schenck, 1853)	14
Lasioglossum punctatissimum (Schenck, 1853)	1
Lasioglossum sp.	3
Seladonia tumulorum (Linnaeus, 1758)	4
Sphecodes ephippius (Linnaeus, 1767)	1
Sphecodes puncticeps Thomson, 1870	1
Megachilidae	**8**
Chelostoma campanularum (Kirby, 1802)	1
Megachile ericetorum Lepeletier, 1841	2
Osmia bicornis (Linnaeus, 1758)	3
Osmia caerulescens (Linnaeus, 1758)	1

Stelis signata (Latreille, 1809)	1
Melittidae	**1**
Melitta leporina (Panzer, 1799)	1