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Advancing the biological control of a global wetland invader, and a case study on ecosystem recovery

Gianmarco Minuti

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Vrije Universiteit Brussel (VUB)
Faculty of Sciences &
Bio-Engineering
Department of Biology

Rhodes University (RU)
Faculty of Science
Department of Zoology &
Entomology

Supervisor:
Prof. Dr. Iris Stiers

Supervisor:
Prof. Dr. Julie A. Coetzee



Iris pseudacorus L.

Illustration by Pierre Joseph Redouté (1759-1840)

Members of the Examination Committee

Prof. Dr. Farid Dahdouh-Guebas (Chair),

Department of Biology, Vrije Universiteit Brussel

Department of Organism Biology, Université Libre de Bruxelles

Prof. Dr. Iain Paterson,

Department of Zoology & Entomology, Rhodes University

Prof. Dr. Jonas Schoelynck,

Department of Biology, University of Antwerp

Prof. Dr. Emily Strange,

Department of Environmental Biology, Universiteit Leiden



Azolla filiculoides Lam.

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Some say life is the sum of all our choices, branching out like a convoluted tree. I always preferred to think of it as a disordered stew of moving particles. Each of us thrown into the world just to bump into each other, striving to follow our route, adjusting the course at every collision. There's beauty in this chaos, I believe.

Studying natural sciences was not my first choice, nor the second, for that matter. And had I made the cut into the university I had originally planned to go, I would have never ended up studying in Milan. This is pivotal to the story, as it was a dear friend from my bachelor who, years later, asked me as a favor to visit the VUB in search for information. This was also the first time I met Iris, my promotor and mentor of all these years. Jump forward a couple years and I am looking for a thesis to complete my MSc in Biology at the VUB. The story goes that around that time Iris met Julie at a conference and that, jokingly, she suggested they should work together on *Iris pseudacorus*, an incipient weed in South Africa. That's it, a coincidence. A pun based on homonymy decided the next years of my life. And what years have they been.

I guess what I am getting at, if a bit clumsily, is that it was not only my choices that brought me here today. I was lucky enough to be at the right place at the right time, guided and encouraged by many wonderful people. Some built the path I had the chance to walk on, others accompanied me along that path, making sure I was safe, and some joined just for a moment to nudge me in the right direction. Words cannot even start to express my gratitude for all these people, but words are all I got, so I hope I will do them justice.

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greenhouse watering plants, or identifying critters through the lenses of a microscope. I learned so much from each of you, and I hope I was able to teach you something in return. I am very proud to have been your supervisor and wish you all the best in your future endeavors.

If I learned one thing during this PhD is that the happiness of accomplishing something great does not compare to that of spending time with the people you love. Thus, these next few paragraphs are dedicated to my friends and family who, just by virtue of being there, have made not only my work, but my life, fundamentally better.

Fede, you know I wouldn't even be here if it weren't for you. You are the kindest and most supportive person I know, and I am so lucky to be your friend. **Giorgio**, I could not have asked for a better first mate to accompany me through these rough waters. The three musketeers, we went through so much together that we could probably write a book, or maybe a videogame, or open a food truck, who knows. I hope the future will have some more adventures in store for us.

To **Elisa**, my lighthouse, my port in the storm. You always stood beside me, through thick and thin, ever encouraging, without criticism, judgement or reproach. You are the best thing that has ever happened to me, and I look forward to spend the rest of my life trying to make you happy. I love you.

Hestia, our cat, kept me from losing my mind through the uncountable hours spent writing, reading, deleting and re-writing each part of this thesis. I firmly believe she should get a co-authorship in all of my manuscripts.

Finally, and most importantly, I would like to dedicate this thesis to **my family**. **Dad**, you infused me with your passion for nature and travelling, turning my life in a never-ending adventure. **Mom**, your wit, curiosity and thirst for knowledge are what brought me here and made me who I am today.

And to my **grandparents**, who taught me to walk, to talk, to eat, to laugh and to love. Having to be away from you has been, by far, the hardest part of this journey. Your unconditional love and endless support is what made all of this possible, I will never thank you enough.



“There is no such thing as a ‘*self-made man*’.
We are made up of thousands of others.
Everyone who has ever done a kind deed for us,
or spoken one word of encouragement,
has entered into the make-up of our character
and our thoughts, as well as our success.”

– George Matthew Adams

TABLE OF CONTENTS

Summary/Samenvatting	i
Symbols and Abbreviations	vii
General Introduction	1
Model Species	26
Thesis Outline and Objectives	38
Chapter 1 – Selection and prioritization of natural enemies for the biological control of the invasive macrophyte <i>Iris pseudacorus</i> L. (Iridaceae)	43
Chapter 2 – Predicting the effect of climate change on the invasion risk and biocontrol potential of the invasive macrophyte <i>Iris pseudacorus</i> L.	71
Chapter 3 – Evaluating the host-range of <i>Aphthona nonstriata</i> Goeze (Coleoptera: Chrysomelidae), a candidate biocontrol agent for <i>Iris pseudacorus</i> L.	101
Chapter 4 – Raising winter temperatures will favour the germination of <i>Iris pseudacorus</i> L. (Iridaceae) in Argentina - A flea beetle might hold the key to control seedling recruitment	135
Chapter 5 – From honeymoon to debt: the intricacies of evaluating ecosystem recovery - A case study on the biological control of water fern in Belgium	169
General Discussion	181
Reference List	207
Biographical Sketch	255

SUMMARY

Global change is accelerating habitat degradation and extinction rates, disproportionately affecting freshwater ecosystems. Biological invasions, a core component of global change, have been recognized as a major driver of this phenomenon. Invasive alien aquatic plants (IAAPs) are a prime example, disrupting ecological dynamics and hindering the economic and social activities that depend upon freshwater ecosystems.

The best approach to managing IAAPs is preventing their introduction and taking swift action when infestations are detected. Unfortunately, most IAAPs are only recognized after they have spread, making eradication difficult and requiring expensive, ongoing management through manual, mechanical, or chemical means, which become less effective as infestations grow. In this regard, a sustainable alternative against IAAPs is provided by classical biological control.

This method lays on the assumption that invasive species have been introduced in an environment which is free from their natural enemies, and that such release allowed them to redirect their resources towards growth and reproduction. Accordingly, weed biocontrol involves introducing one such enemy - a so-called *biocontrol agent* - to regulate the population of the plant, ideally reducing its density below a critical threshold, minimizing its impacts.

Biocontrol programs must follow stringent protocols in order to a) ensure the selection of the most appropriate agents; b) guarantee their establishment and effectiveness in controlling their target; c) minimize the risks involved with their release; and d) monitor their impacts after they have been released. This thesis is composed of two parts, focusing on pre-release studies (**Part I, Chapters 1-4**) and post-release evaluations (**Part II, Chapter 5**), respectively.

Part I explores the biological control of *Iris pseudacorus* L. (Iridaceae) – yellow flag – a Eurasian plant that has invaded temperate wetlands worldwide. The first step consisted of conducting field surveys across the native range of the weed, which led to the selection of three candidate biocontrol agents. As a second step, bioclimatic niche modelling was used to forecast the current and future suitability of the weed and its prospective agents. These models helped to identify areas most at risk of invasion and predict how climate change might affect biocontrol efficacy, showing differing impacts in the Northern and Southern Hemispheres.

Further, a scoring system was developed to prioritize non-target plants for host-specificity testing, focusing on species within the family Iridaceae. Most taxa prioritized are native to the Americas, South Africa and eastern Asia, highlighting the importance of collaboration across regions when dealing with global invaders with complex phylogeographic distributions.

Finally, a germination study confirmed that *I. pseudacorus* seeds from Argentina germinate more than Belgian seeds, especially in absence of cold stratification, suggesting increased performances of invasive populations and an adaptation to warmer temperatures. Nonetheless, impact assessments conducted on one of the three biocontrol agents demonstrated that it could cause juvenile mortality, suggesting it may effectively reduce seedling recruitment, though further research on agents targeting reproductive structures is recommended.

Part II of the thesis addresses an often understudied aspect of post-release evaluations: ecosystem recovery following biological control. To do so, a case study is presented, investigating the biological control of water fern, *Azolla filiculoides* Lamb. (Salviniaceae), in Belgium. The results of this study are used to discuss the shortcomings of different experimental designs, the lack of baseline data in invasion ecology, and the intricacies of defining and quantifying ecosystem recovery in light of legacy effects and context-dependency.

SAMENVATTING

Wereldwijde verandering versnelt de degradatie van habitats en de uitstervingscijfers, met een onevenredige impact op zoetwaterecosystemen. Biologische invasies, een kernonderdeel van klimaatverandering, worden erkend als een belangrijke drijvende kracht achter dit fenomeen. Invasieve uitheemse waterplanten (IAAPs) zijn een duidelijk voorbeeld, omdat ze ecologische dynamiek verstoren en economische en sociale activiteiten belemmeren die afhankelijk zijn van zoetwaterecosystemen.

De beste aanpak voor het beheer van IAAPs is het voorkomen van hun introductie en het nemen van snelle maatregelen bij het detecteren van besmettingen. Helaas worden de meeste IAAPs pas herkend nadat ze zich hebben verspreid, wat uitroeiing moeilijk maakt en dure, voortdurende beheersing vereist door handmatige, mechanische of chemische middelen, die minder effectief worden naarmate besmettingen toenemen. In dit opzicht biedt klassieke biologische bestrijding een duurzaam alternatief tegen IAAPs.

Deze methode berust op de veronderstelling dat invasieve soorten zijn geïntroduceerd in een omgeving die vrij is van hun natuurlijke vijanden, waardoor ze hun middelen konden richten op groei en reproductie. Bijgevolg omvat onkruidbestrijding het introduceren van een dergelijke vijand - een zogenaamde biocontrole-agent - om de populatie van de plant te reguleren, idealiter door de dichtheid ervan onder een kritische drempel te verlagen en zo de impact ervan te minimaliseren.

Biocontroleprogramma's moeten strikte protocollen volgen om a) de selectie van de meest geschikte agens te waarborgen; b) hun vestiging en effectiviteit bij het beheersen van hun doel te garanderen; c) de risico's van hun vrijlating te minimaliseren; en d) hun impact na vrijlating te monitoren. Dit proefschrift

bestaat uit twee delen, gericht op respectievelijk pre-release studies (**Deel I, Hoofdstukken 1-4**) en post-release evaluaties (**Deel II, Hoofdstuk 5**).

Deel I onderzoekt de biologische bestrijding van *Iris pseudacorus* L. (Iridaceae) - gele lis - een Euraziatische plant die gematigde moeraslanden wereldwijd heeft gekoloniseerd. De eerste stap bestond uit het uitvoeren van veldonderzoeken in het inheemse verspreidingsgebied van het onkruid, wat leidde tot de selectie van drie kandidaat-biocontroleagenten. Als tweede stap werd bioklimatisch nis-modelleren gebruikt om de huidige en toekomstige geschiktheid van het onkruid en zijn potentiële agens te voorspellen. Deze modellen hielpen gebieden met het grootste risico op invasie te identificeren en voorspelden hoe klimaatverandering de effectiviteit van biocontrole zou kunnen beïnvloeden, met verschillende effecten op het noordelijk en zuidelijk halfrond.

Daarnaast werd een scoresysteem ontwikkeld om niet-doelplanten te prioriteren voor host-specifieke tests, met de nadruk op soorten binnen de familie Iridaceae. De prioritaire taxa zijn inheems in Amerika, Zuid-Afrika en Oost-Azië, wat het belang van samenwerking tussen regio's onderstreept bij het omgaan met mondiale invasieve soorten met complexe fylogeografische verspreiding.

Ten slotte bevestigde een kiemingsstudie dat *I. pseudacorus* zaden uit Argentinië meer kiemen dan Belgische zaden, vooral bij afwezigheid van koude stratificatie, wat wijst op verhoogde prestaties van invasieve populaties en aanpassing aan warmere temperaturen. Nochtans toonden effectbeoordelingen van een van de drie biocontroleagens aan dat deze de mortaliteit van juvenielen kon veroorzaken, wat suggereert dat het effectief kan zijn bij het verminderen van de zaailingrekrutering. Toch wordt verder onderzoek naar agenten die zich richten op reproductieve structuren aanbevolen.

Deel II van het proefschrift behandelt een vaak onderbelicht aspect van post-release evaluaties: ecosysteemherstel na biologische bestrijding. Om dit te realiseren wordt een casestudy aangeboden over de biologische bestrijding van watervaren, *Azolla filiculoides* Lamb. (Salviniaceae), in België. De resultaten van deze studie worden gebruikt voor de bespreking van de tekortkomingen van verschillende experimentele ontwerpen, het gebrek aan basisgegevens in invasie-ecologie en de complexiteit van het definiëren en kwantificeren van ecosysteemherstel in het licht van nalatenschapseffecten en contextafhankelijkheid.

SYMBOLS AND ABBREVIATIONS

ANOVA	Analysis of Variance
AUC	Area Under the receiver operatic characteristic Curve
CABI	Centre for Agriculture and Bioscience International
CBC	Centre for Biological Control
CBI	Continuous Boyce Index
CCI	Chlorophyll Content Index
CECOAL	Centro de Ecología Aplicada del Litoral
Chao2	Incidence-based rare species estimator
Chl- <i>a</i>	Chlorophyll- <i>a</i>
CI	Confidence Interval
CMIP	Coupled Model Intercomparison Project
DAFOR	Dominant Abundant Frequent Occasional Rare
EEA	European Environmental Agency
EICA	Evolution of Increased Competitive Abilities
EPPO	European and Mediterranean Plant Protection Organization
ERH	Enemy Release Hypothesis
EUR	Euros
F _m	Maximum Fluorescence
FUEDEI	Fundación para el Estudio de Especies Invasivas
F _v	Variable Fluorescence
F _v /F _m	Photosynthetic Efficiency
FWO	Fonds voor Wetenschappelijk Onderzoek
GBIF	Global Biodiversity Information Facility
GCMs	Global Circulation Models
HadGEM	Hadley Centre Global Environment Model

IAAPs	Invasive Alien Aquatic Plants
ICE	Incidence-based Coverage Estimator
IPBES	Intergovernmental Platform on Biodiversity and Ecosystem Services
IPSLCM	Institut Pierre-Simon Laplace Climate Model
IUCN	International Union for Conservation of Nature
KG	Köppen-Geiger
MESS	Multivariate Environmental Similarity Surface
MIROC	Model for Interdisciplinary Research on Climate
MM	Michaelis-Menten species estimator
MoD	Map of Dissimilarity
OLS	Olen's Broek
OR	Omission Rate
PCA	Principal Component Analysis
QGIS	Quantum Geographic Information System
RBINS	Royal Belgian Institute of Natural Sciences
ROI	Regions Of Interest
RU	Rhodes University
S(est)	Estimated number of Species
SAC	Species Accumulation Curve
SDMs	Species Distribution Models
SSPs	Shared Socio-Economic Pathways
TPR	True Presence Ratio
USD	US Dollars
VUB	Vrije Universiteit Brussel
WCRP	World Climate Research Program

GENERAL INTRODUCTION

FRESHWATER ECOSYSTEMS

Despite covering only 0.8% of the Earth's surface, freshwater ecosystems are home to ca. 6% of its biodiversity, including as much as 35% of all vertebrate species (Dudgeon et al., 2006; Balian et al., 2008). Furthermore, these ecosystems provide a wide range of irreplaceable services for both nature and society (Kingsford et al., 2016; Lynch et al., 2023). They contribute to habitat connectivity, water purification and flood control, and mitigate the effects of climate change (Kingsford et al., 2016). At the same time, they are pivotal to economic, touristic and recreational activities, as well as cultural and educational significance (Lynch et al., 2023). Alarmingly, freshwaters are increasingly threatened by global change and human activities, among which habitat degradation, water pollution and biological invasions play a major role (Dudgeon, 2019; Reid et al., 2019). As a result, the world is facing a disproportionately accelerated extinction of species in freshwater ecosystems compared to other realms, which scientists labelled as the freshwater biodiversity crisis (Harrison et al., 2018; Albert et al., 2020, Ottoni et al., 2023).

BIOLOGICAL INVASIONS

Biological invasions by non-native species are considered one of the main mechanisms behind global change, leading to species extinction and profound alterations in ecosystem structure and function (Pyšek et al., 2020; IPBES, 2023). Globalisation and the concomitant increase in travel and trade brought with them a rise in the number of alien species introductions to aquatic ecosystems (Early et al., 2016). Although it is estimated that thousands of species have been introduced to regions outside of their native range, only a portion of them are considered invasive (Williamson & Fitter, 1996; Jeschke & Pyšek, 2018;

GENERAL INTRODUCTION

Pfadenhauer & Bradley, 2024). This is because, for a species to become invasive, several *barriers* have to be crossed (Figure I; Richardson et al., 2000; Blackburn et al., 2011; IPBES, 2023).

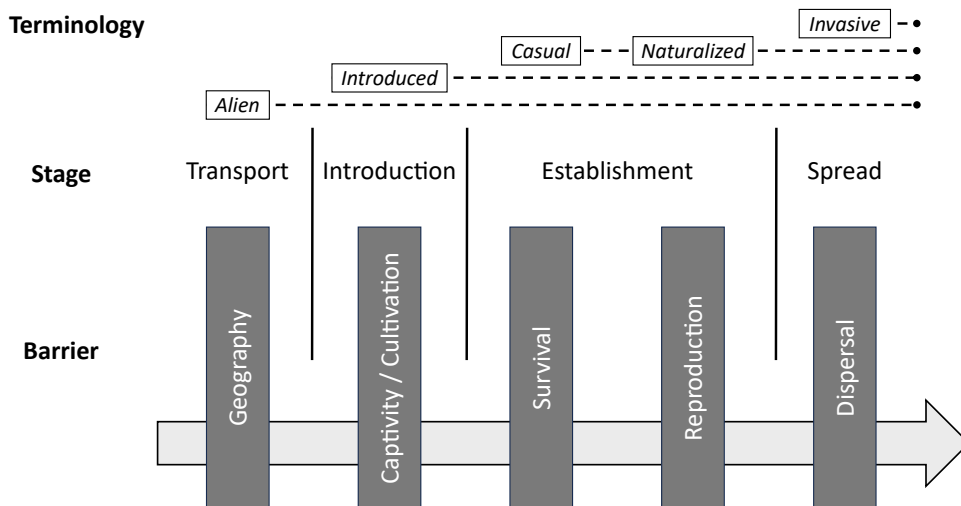


Figure I – Framework for biological invasions. The scheme identifies the terminology used at different stages of the invasion process, and the barriers that need to be overcome for a population to pass on to the next stage (adapted from Blackburn et al., 2011 and IPBES, 2023).

First and foremost, a species – or its propagules (i.e. reproductive unit) – has to be transported and introduced into a new environment, overcoming any *geographical* barrier in the way and, if applicable, escaping captivity or cultivation (Blackburn et al., 2011). Upon introduction into the new environment, the non-native species, now defined as alien, has to survive the local biotic and abiotic conditions (i.e. environmental and reproductive barriers). Many aliens that get introduced into new environments fail to reproduce, and therefore their populations are only considered casual. On the other hand, the few that manage to establish self-sustaining populations are now considered *naturalized*. The next stage of the invasion process consists of the alien, now *naturalized*, being able to cross local *dispersal* barriers and spread into nearby environments. Depending on terminology, an alien capable of spreading independently from

GENERAL INTRODUCTION

the point of first introduction will already be defined as invasive (Richardson et al., 2000; Simberloff et al., 2013). However, according to other frameworks, invasiveness is only recognized once the alien causes detrimental effects to the environment, the economy, or society (Blackburn et al., 2011; IPBES, 2023).

INVASIVE ALIEN AQUATIC PLANTS

Invasive alien aquatic plants (IAAPs) are non-native plant species that have been introduced to freshwater ecosystems, established, spread, and negatively impacted the native environment and/or the socio-economic activities dependent on it. These macrophytes can be rooted or unrooted and are usually categorized based on their growth form as emerged, submerged, floating-leaved, free floating or amphibious (Hussner et al., 2017).

Usually, IAAPs share several characteristic traits that contribute to their success in colonizing and dominating aquatic ecosystems (Brundu, 2015; Hussner et al., 2021). The production of a large number of seeds, vegetative fragments or spores increases their chances of introduction and establishment in a new environment. Upon establishment, their rapid vegetative growth rate helps IAAPs to outcompete native species for essential resources such as space, light, and nutrients (Kolar & Lodge, 2001; Hussner et al., 2021). Furthermore, the ability to adapt to a wide range of environmental conditions (e.g. water depths, temperature levels, pH range, nutrient concentrations, etc.) allows them to exploit various ecological niches and different habitats (Fleming & Dibble, 2014). Finally, invasive macrophytes usually show high resilience to natural and human-induced disturbance events, helping them thrive in dynamic and disturbed ecosystems (Hussner et al., 2021).

Sometimes, IAAPs can show morphological or physiological variability (i.e. *phenotypic plasticity*) in response to changes in the conditions present in the new environment (Gratani, 2014). A common example is the ability to alter their

GENERAL INTRODUCTION

foliar traits (i.e. *heterophylly*) in response to temperature, submersion, light intensity, pH, salinity, oxygen or nutrients concentration. The submerged *Elodea nuttallii* (Planch.) H. St. John (Hydrocharitaceae), for instance, shows accelerated apical elongation and branching in response to eutrophic conditions and changes in light intensity (Szabó et al., 2019). This in turn allows it to reach the water surface sooner, outcompeting other plants for light and nutrients.

Other macrophytes release chemicals into the soil or the water that influence their own growth and that of nearby plants (i.e. *allelopathic activity*), often giving them a competitive advantage (Hierro & Callaway, 2003). For instance, the South American water primroses, *Ludwigia hexapetala* (Hook. and Arn.) and *Ludwigia peploides* (Kunth) P. H. Raven (Onagraceae), produce allelochemicals that negatively affect co-occurring nearby macrophytes while stimulating their own growth and germination (Dandelot et al., 2008; Thiebaut et al., 2018). Altogether, these and other characteristics collectively make invasive aquatic plants more likely to establish, spread, and outcompete native aquatic vegetation. However, plant traits are not the sole predictor of the outcome of a biological invasion, which is also strongly dependent on other factors, such as the conditions of the receiving ecosystem.

Freshwater ecosystems are particularly prone to biological invasions due to their intrinsic dynamics and the strong influence of human activities (Santamaría, 2002). Encompassing a variety of habitats, often intricately interconnected, they provide numerous niches for invasive species to establish and spread easily from one water body to another. As flowing water constitutes a rather homogeneous means of transportation, waterways provide corridors for the movement of invasive species, facilitating their dispersal (Santamaría, 2002).

Humans also play a significant role in the introduction and spread of invasive species in freshwater ecosystems. Activities such as shipping, boating, fishing

GENERAL INTRODUCTION

and aquaculture can unintentionally transport invasive species and their propagules from one location to another (Hulme et al., 2008). At the same time, changes in hydrological patterns due to irrigation, damming, or other water management practices can create favourable conditions for invasive species to settle (Hulme et al., 2008). Similarly, pollution from agricultural and industrial runoff, wastewater discharge, and other sources can alter the water chemistry and nutrient levels, creating an overabundance of resources for new species to exploit (Davis et al., 2000).

Finally, natural disturbance events like floods and wildfires – whose occurrences are exacerbated by climate change – can impact native communities and create temporary vacant ecological niches for invasive species to fill (Bixby et al., 2015; Robinson et al., 2020). Due to these factors, freshwater ecosystems face a high and increasing risk of biological invasions (Moorhouse & Macdonald, 2015).

PATHWAYS OF INTRODUCTION

Globalization has facilitated the movement of goods and people across continents, inadvertently aiding the spread of invasive species (Hulme, 2009). Biological invasions in aquatic ecosystems often occur when non-native species are intentionally or accidentally introduced into these ecosystems, often as a result of human activities. The pathway and rate of introduction (i.e. *propagule pressure*, Catford et al., 2009) play a crucial role in the spread of invasive aquatic plants and their establishment in non-native ecosystems (Hulme et al., 2008).

The horticultural, aquacultural and ornamental plant trades are amongst the most significant pathways of introduction (Padayachee et al., 2017). Invasive aquatic plants may be sold online or at nurseries without proper labelling or awareness of their invasive potential, leading to unintentional introductions (Martin & Coetzee, 2011). In some cases, people purchase alien aquatic plants for aquaria and outdoor ponds and sometimes these plants can *escape* into

GENERAL INTRODUCTION

local water bodies, where they may spread and become invasive (Saul et al., 2017, Pergl et al., 2017). For instance, the submerged macrophyte, *Cabomba caroliniana* A. Gray (Cabombaceae), is a popular aquarium species that has been introduced into natural ecosystems through the disposal of surplus aquarium material (Bickel, 2017). In other cases, such as for the floating macrophyte *Pontederia crassipes* Mart. (Pontederiaceae), aquatic plants are intentionally introduced for ornamental purposes or for water purification, not accounting for their invasive potential (Wu & Ding, 2019).

Finally, the construction and maintenance of water infrastructure, such as dams, reservoirs, and irrigation channels, can facilitate the spread of invasive aquatic plants by creating new aquatic habitats and altering water flow patterns (Hulme et al., 2008). Preventing the introduction and spread of invasive alien aquatic plants requires greater awareness, education, and regulatory measures to reduce the risk of future introductions.

HYPOTHESES IN INVASION ECOLOGY

Countless hypotheses have been put forward to predict the outcome of biological invasions (Catford et al., 2009). Most of them attribute invasion success to characteristics of the invader (i.e. *invasiveness*) or features of the invaded ecosystem (i.e. *invasibility*; Richardson & Pyšek, 2006), with relatively few trying to integrate the two concepts (but see Dai et al., 2020). For instance, the *ideal weed hypothesis* (Elton, 1958) predicts that particular plant traits (see section 3 above for details) of the invading species will facilitate its establishment, spread and competition towards indigenous species (Catford et al., 2009). Theories focusing on invasibility, on the other hand, try to disentangle the components making an ecosystem or community more or less susceptible to invasion (Richardson & Pyšek, 2006). Biotic and abiotic conditions of an ecosystem – such as climate, habitat structure and nutrient availability – can influence its invasibility (Richardson & Pyšek, 2006). The *biotic resistance*

GENERAL INTRODUCTION

hypothesis (Levine et al., 2004), for instance, states that the presence of competitors, herbivores and pathogens in the recipient community will limit colonization, naturalization and persistence of invaders (Catford et al., 2009). Similarly, the *disturbance hypothesis* (Sher & Hyatt, 1999) predicts disturbance events will increase resource availability and vacate some ecological niches, giving invading species an opportunity to colonize the new environment (Catford et al., 2009). Disturbance can be natural but can also come in the form of human activities such as eutrophication, habitat modification and changes in land use (Catford et al., 2009). For instance, aquatic ecosystems that have experienced alterations due to urban development, pollution, or changes in water flow patterns may be more prone to invasion by non-native aquatic plants.

Over time, several lines of research tried to shed some light on what makes a species a good invader or what makes ecosystems more vulnerable to invasion, producing conflicting results (Moles et al., 2012). That is, some hypotheses have faced both support and opposition across different habitats and taxonomic groups (Jeschke et al., 2012; Fleming & Dibble, 2015). On the other hand, theories that consider the interaction between alien invaders and their new environments are better supported by empirical evidence than theories focusing on invasiveness or invasibility alone (Jeschke et al., 2012; Figure II). Examples of such theories include: i) the *invasional meltdown hypothesis*, observing that some invaders can alter ecosystem characteristics facilitating invasion of other non-native species (Simberloff & Holle, 1999); ii) the *novel weapon hypothesis*, recognizing that some invasive species release allelopathic chemicals to which indigenous plants are not adapted, thus inhibiting potential competitors and enhancing their own invasion success (Callaway & Ridenour, 2004); and iii) the *enemy release hypothesis* (ERH), which predicts that plant species are introduced to new environments without their naturally co-evolved natural enemies (i.e. herbivores, parasites and pathogens), allowing them to proliferate unchecked (Keane & Crawley, 2002).

GENERAL INTRODUCTION

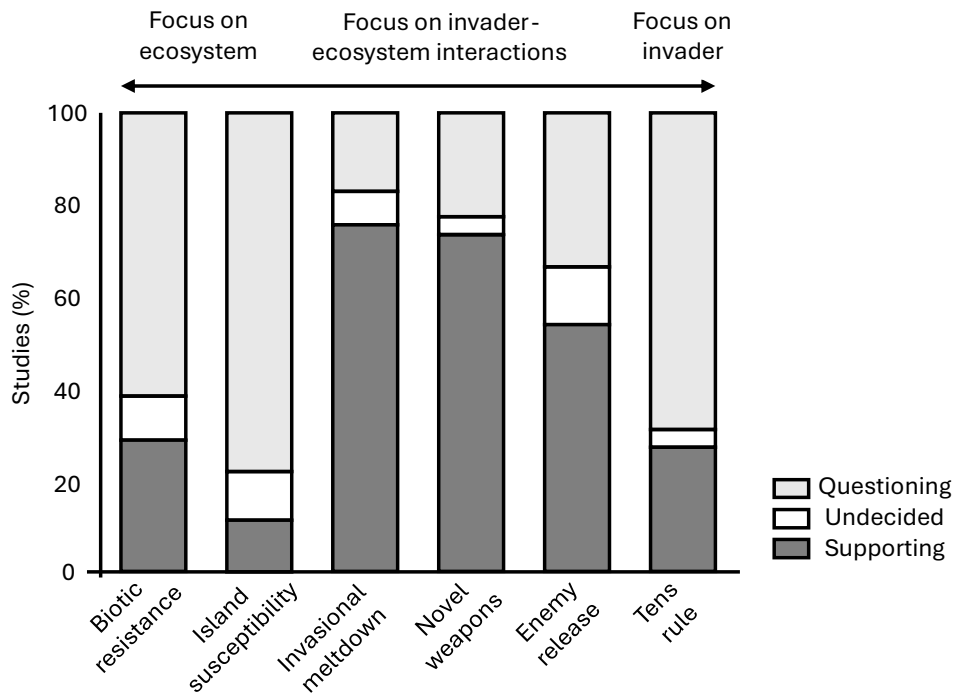


Figure II - Overall level of empirical support for six major hypotheses in invasion biology. Hypotheses are presented on a gradient between those focusing on receiving ecosystem (i.e. *invasibility*) and those focusing on the invader (i.e. *invasiveness*), with hypotheses integrating invader-ecosystem interactions usually receiving more support in the literature (Jeschke et al., 2012).

Enemy release can be *regulatory* – i.e. the species experiences immediate increase in population size due to lack of direct regulation – and/or *compensatory* – i.e. resources that were previously used for defence against natural enemies are now reallocated to growth and reproduction (Catford et al., 2009). Regulatory enemy release is often paired with a competitive release through greater generalist enemy impact on native plants (Keane & Crawley, 2002). This concept, commonly known as accumulation of local pathogens (Eppinga et al., 2006) or *enemy of my enemy* hypothesis (Sabelis et al., 2001), predicts that generalist enemies in the new range will have a stronger regulatory effect on native species than on the invader, resulting in apparent competition (Catford et al., 2009). On the other hand, compensatory enemy release will

GENERAL INTRODUCTION

speed the selection, over longer time periods, of genotypes allocating resources towards vegetative growth and reproduction rather than chemical defences, resulting in the *evolution* of plants with *increased competitive abilities* (EICA; Blossey & Notzold, 1995).

IMPACTS OF IAAPs

Biological invasions in aquatic ecosystems can have significant and wide-ranging impacts, from disrupting ecological dynamics to hindering the economic and social activities that depend on them (Pimentel et al., 2001; Bacher et al., 2018; Rai & Singh, 2020, Diagne et al., 2021, IPBES, 2023). Invasive plants tend to grow quickly into dense monocultures, outcompeting the native vegetation for space and resources (Pyšek et al., 2012). This can in turn lead to habitat homogenization, with cascading effects on other trophic niches (Kumschick et al., 2015; Bellard et al., 2016). The invasion of the emergent macrophyte *Phragmites australis* (Cav.) Trin. ex Steud. (Poaceae) across the Great Lakes of North America, for instance, has been linked with a direct reduction of plant biodiversity and an indirect effect on aquatic invertebrate abundance and community composition (Ward et al., 2023).

Dense stands or mats of aquatic plants can also alter the physical structure (e.g. affecting erosion and sedimentation rates and water flow) and chemical profile (e.g. reducing light penetration and nutrients availability) of freshwater ecosystems (Strayer, 2010). These changes can affect habitat suitability for native aquatic species, excluding them from their preferred feeding or breeding grounds. At the same time, the extensive surface area provided by alien plants offers a new niche for the colonization of epiphytic algae, macro-invertebrates and other organisms, disrupting the existent ecological interactions and triggering a biotic shift in the ecosystem. Water hyacinth, *P. crassipes* is the perfect example of a floating aquatic plant species whose invasion is linked to a variety of multifaceted impacts. Water hyacinth mats decrease water clarity,

GENERAL INTRODUCTION

dissolved oxygen, nitrogen and phosphorous concentrations, with direct negative effects on phytoplankton (Villamagna & Murphy, 2010). These in turn have a negative effect on diversity of macroinvertebrates and planktivorous fish, subsequently affecting higher trophic levels. At the same time, however, the abundance of some specific classes usually increases in response to the habitat heterogeneity and structural complexity provided by the plant itself. This can often lead to alterations of predator-prey dynamics and biotic shifts in the food web (Hill et al., 2021).

Due to their ability to modify the structure and functioning of freshwater ecosystems, some IAAPs are considered *ecosystem engineers* (Table I; Emery-Butcher et al., 2020). As such, they can have significant negative impacts on ecosystem services and human well-being (Vilà et al., 2010; Keller et al., 2018; Rai & Singh, 2020). For instance, dense plant infestations can alter sedimentation rates, nutrient cycling, water availability, flood and fire regimes (Emery-Butcher et al., 2020). Furthermore, dense mats of floating weeds are considered breeding grounds for vectors of infectious diseases such as malaria and schistosomiasis, although further research is needed on the subject (Ofulla et al., 2010; Ogden et al., 2019; Honlah et al., 2019; Rai & Singh, 2020).

IAAPs can also lead to direct and indirect economic losses (Diagne et al., 2021). Dense infestations can damage infrastructure such as irrigation systems, pipelines, dams and water treatment facilities, increasing maintenance costs. The altered ecosystem dynamics can also reduce the productivity of water-dependent industries such as fisheries and agriculture (Strayer, 2010). Infested waterways hinder navigation and access to commercial and recreational activities, degrading the aesthetic and cultural value of aquatic environments and leading to a loss in property values. Finally, the costs associated with managing IAAPs, such as control efforts and damage mitigation, can also be substantial (Hussner et al., 2017). It is estimated that, between 1975 and 2020,

GENERAL INTRODUCTION

the total costs of invasive aquatic plants on the global economy exceeded US\$ 32 billion, of which over US\$ 28.2 billion was direct and indirect damage, and the rest in management costs (Macêdo et al., 2024). Recorded costs were disproportionately high towards freshwater ecosystems, with submerged plants being the most expensive, followed by floating, emergent, and semi-aquatic species (Macêdo et al., 2024).

MANAGEMENT OPTIONS

The management of IAAPs is a priority action for the recovery of freshwater biodiversity (Tickner et al., 2020; Britton et al., 2023). Preventing the introduction of invasive aquatic plants is the most cost-effective approach to limit their impacts (Convention on Biological Diversity, 2002; Simberloff et al., 2013; IPBES, 2023; Figure III). This requires first and foremost the correct identification of primary pathways of introduction and the pre-emptive implementation of sales regulation, interception, quarantine and biosecurity measures (Hulme et al., 2008). Education and outreach also play a key role in prevention: public awareness campaigns can inform people about the risk associated with aquatic invasions and promote responsible behaviour.

Unfortunately, some species bypass these preventive methods and become established in the ecosystem. In such cases, early detection and rapid response measures are pivotal to maximize the chance of successfully eradicating a new invader (Ahmed et al., 2022). However, these are only possible if regular monitoring and surveying efforts are already in place to detect the presence of invasive plants in water bodies, which entail considerable investments (Simberloff, 2021). Often an invader is only recognized as such when its population density has reached the point where it starts generating negative effects on the ecosystem and the economy (i.e. *impact threshold*), and, at this stage, eradication is usually not possible (Ahmed et al., 2022).

GENERAL INTRODUCTION

Table I – Examples of freshwater invasive plants as ecosystem engineers, categorized based on their growth form and their potential ecological impacts (adapted from Hussner et al., 2017 and Emery-Butcher et al., 2020).

Growth form	Effect on ecosystem	Examples
Submerged 	Decreased light penetration Increased sedimentation rates Altered underwater currents Increased habitat heterogeneity Allelopathy	<i>Eleocharis sphacelata</i> <i>Egeria densa</i> <i>Hydrilla verticillata</i> <i>Elodea canadensis</i> <i>Myriophyllum spicatum</i>
Floating 	Decreased light penetration Limited oxygenation Altered nutrients concentration Increased structural complexity Allelopathy	<i>Pontederia crassipes</i> <i>Salvinia molesta</i> <i>Pistia stratioides</i> <i>Trapa natans</i> <i>Azolla filiculoides</i>
Emergent 	Feedback loops Altered erosion and sedimentation Bank destabilisation Affected hydrology Shore habitat provision Allelopathy	<i>Amaranthus retroflexus</i> <i>Typha</i> spp. <i>Glyceria maxima</i> <i>Arundo donax</i> <i>Phragmites australis</i> <i>Alternanthera philoxeroides</i>
Riparian 	Decreased light availability Altered nutrient cycling Seedbank limitation Altered soil biochemistry Impacted shore dynamics and flow regimes	<i>Arundo donax</i> <i>Impatiens glandulifera</i> <i>Tamarix ramosissima</i> <i>Elaeagnus angustifolia</i> <i>Phalaris arundinacea</i> <i>Rubus</i> spp.

GENERAL INTRODUCTION

Once a species has established and spread beyond the point where eradication is considered feasible, management strategies are put into place to minimize its impact on native ecosystems and the economic and recreational value of aquatic environments (i.e. *functional eradication*; Green & Grosholtz, 2021).

Common management strategies consist of physical, chemical and biological control methods (Hussner et al., 2017). Physical control can range from manual removal through hand-pulling, cutting and raking to mechanical harvesting using specialized equipment (Hussner et al., 2017). These methods are most effective in managing small, isolated infestations, as the time and costs tend to increase proportionally with the size of the application (Hussner et al., 2017). For instance, the management costs of *Hydrilla verticillata* (L.f.) Royle (Hydrocharitaceae) were reported to vary significantly between California (\$2.5 million/year) and Florida (\$14.5 million/year) due to the difference in the number and size of infestations (Pimentel et al., 2001). Chemical control makes use of herbicides to target invasive plants and suppress their growth. As a method, it is suited for application at larger scales, with lower labour costs relatively to physical control. However, the most commonly used herbicides (e.g. glyphosate and paraquat) are non-specific and can have deleterious effects on aquatic ecosystems and human health (Myers et al., 2016). Additionally, there is an increasing issue of misuse of herbicides, which can lead to the evolution of resistant weeds (Rodenburg et al., 2019).

Finally, classical biological control involves using natural enemies of the plant (i.e. herbivores, parasites and pathogens) to re-establish the top-down regulation the species was subject to within its native range. Leveraging the ERH and EICA hypotheses, this approach does not aim at directly eradicating the invader, but rather at reducing its increased ability to compete with native plants.

GENERAL INTRODUCTION

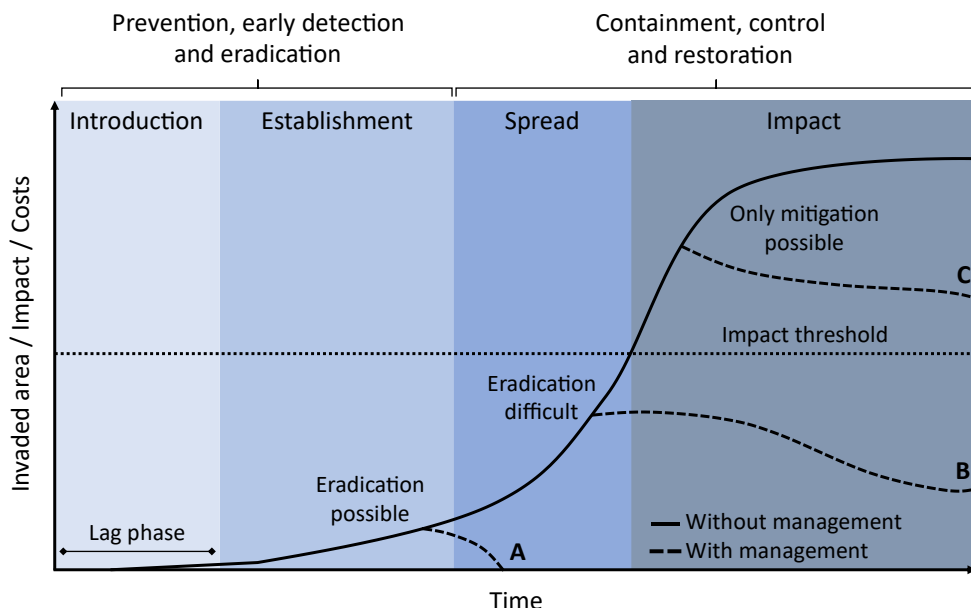


Figure III – Generalized management-invasion continuum in freshwater ecosystems, and the expected changes in trajectory with appropriate management actions (adapted from IPBES, 2023). A) If appropriate management is undertaken upon detection, before the species has had a chance to spread extensively, eradication is still possible. B) Eradicating a spreading invasion is difficult, but if management is set up timely the impacts and costs can be contained. C) Once the invasion is too widespread, eradication becomes unfeasible and only mitigation is possible. A distinction is made between invasions in closed aquatic ecosystems (e.g. lakes, ponds, etc.) and connected water systems (e.g. rivers). In the latter, scenarios B and C are increasingly difficult.

Biological control is considered self-sustaining in that once established, the biocontrol agent should ideally be able to maintain a population and exert control on its host over time (Figure IV). This makes this method suitable for application over larger regions, with relatively lower overall costs and high returns on investment (McFadyen, 2008; Schwarzländer & Mason, 2024).

Due to the high regenerative capacities of invasive aquatic weeds, these control methods often require recurrent application in order to maintain the biomass of the plant under the impact threshold, and effective management is thus a long-term financial commitment.

GENERAL INTRODUCTION

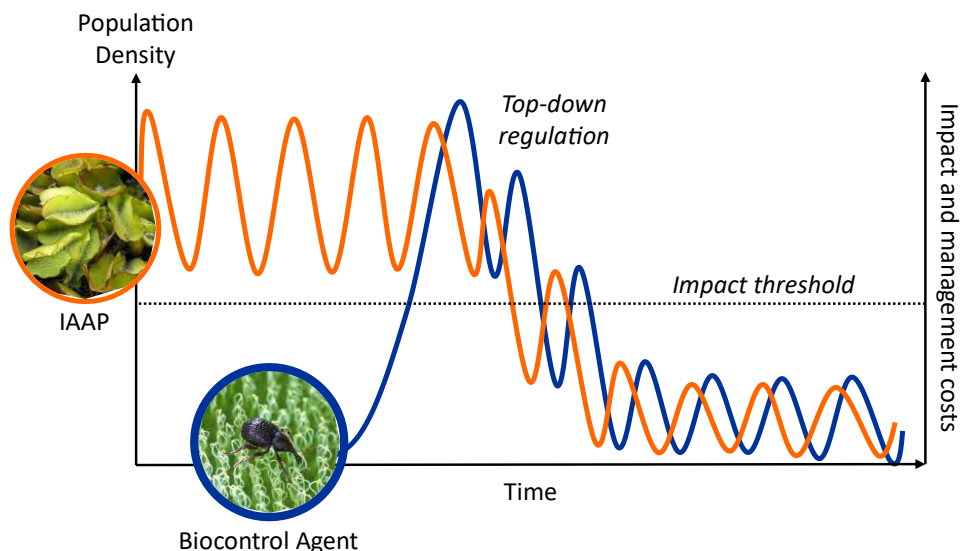


Figure IV – Theoretical framework of classical weed biocontrol. In the absence of its natural enemies, the invasive plant reaches seasonally high densities, causing considerable ecological and economic impacts. Upon introduction, the biocontrol agent builds up in number thanks to the abundance of food, acting as an effective top-down regulator for the plant. In time, this brings the density of the plant below the impact threshold, essentially resulting in its functional eradication. The system reaches a dynamic equilibrium with the agent population thriving at high plant densities and declining upon depletion of its food supply (McEvoy, 2018).

The choice of management strategy or combination of strategies depends on various factors, including the specific invasive plant species, the extent of the infestation, ecological considerations, and the resources available. In many cases, approaches that combine several methods (i.e. *integrated weed management*) are the most effective means of controlling invasive aquatic plants while minimizing environmental impacts (Hussner et al., 2017). The successful management of IAAPs requires ongoing research on the causes of invasion, which can often be a symptom of degraded or eutrophicated habitats, rather than the main driver of ecological change (Van Ginkel, 2011; Coetzee & Hill, 2012). Management must thus work with other restoration strategies to allow the recovery of freshwater ecosystems (Britton et al., 2023).

GENERAL INTRODUCTION

BIOLOGICAL CONTROL

Biological control is defined as “*the actions of parasites, predators and pathogens (i.e. natural enemies) in maintaining an organism’s density at a lower level than it would occur in their absence*” (DeBach, 1964). In classical biological control, these natural enemies are identified within the native range of the invader and, if deemed sufficiently safe and effective (at which point they are called *biocontrol agents*), they are eventually released across the invaded range in order to control its spread and density.

Biological control of weeds holds a long history, with countries such as Australia, Canada, New Zealand, South Africa and the United States leading the way in the discipline since the beginning of the last century (McFadyen, 1998). Over this period, almost 600 biocontrol agents have been released against 250 invasive plants (Winston et al., 2024). Different independent meta-analyses have highlighted the effectiveness of the method, from an ecological as well as an economical perspective (McFadyen, 2000; McFadyen, 2008; Clewley et al., 2012; Schwarzländer et al. 2018). At the turn of this century, success rates – calculated as ratio of species controlled over financed biocontrol programmes – were already in the 50 to 80% range (McFadyen, 2000), and cost-benefit analyses showed a significant return on investment (McFadyen, 2008). A more recent meta-analysis from Schwarzländer et al. (2018) calculated the establishment rate of biocontrol agents at 70.9%, and the control rate of plants targeted by biocontrol at 65.7%, on average. From an ecological perspective, biocontrol agents have been observed to reduce plant size, biomass, flower and seed production by 28 to 56%, with a concomitant increase of 88% in plant diversity following biocontrol (Clewley et al., 2012). Despite certain controversies around the method, research also confirmed the inherent safety of biological control. Of 457 agents released globally up until 2008, over 390 showed no sign of attack on non-target species (Hinz et al., 2019). Of the 67 agents for which non-target

GENERAL INTRODUCTION

attacks were recorded, over 99% would have been predictable, as they occurred on plant species that had not been included in the pre-release screening of the biocontrol agent (Hinz et al., 2020). Furthermore, 73% of the non-target attacks recorded constituted collateral or spillover damage – i.e. temporary, context-dependent feeding – and led to no long-term impacts on the non-target species (Hinz et al., 2019). To highlight the progresses made in the field, the proportion of agents causing sustained non-target damage declined from 12% prior to 1960 to < 1% in the period 1991–2008 (Hinz et al., 2019; 2020).

Weed biological control is an evolving technique, and these figures are only obtained thanks to refined and constantly updated protocols (Hinz et al., 2024). There are several steps involved in a weed biocontrol program, from field surveys to select the most promising biocontrol agents, through host-specificity testing and impact assessment of the selected candidates, to evaluations of the control achieved in the field after release (Müller-Schärer & Schaffner, 2008; Figure V).

TARGET WEED SELECTION AND ECOLOGY

The first step in the implementation of classical biological control is to prioritise which IAAPs should be targeted. Several prioritisation systems have been developed (see Downey et al., 2021; Lesieur et al., 2023), scoring weeds based on their impacts, the costs of current control methods, the efforts envisaged to deliver a biocontrol program, the feasibility and likelihood of success of such a program. Once a plant has been prioritized, bibliographic information is gathered regarding its geographic and genetic origin (Sutton et al., 2017), its ecology and the factors contributing to its invasion (e.g. enemy release hypothesis). This is crucial for understanding the invader and developing an effective biological control program.

GENERAL INTRODUCTION

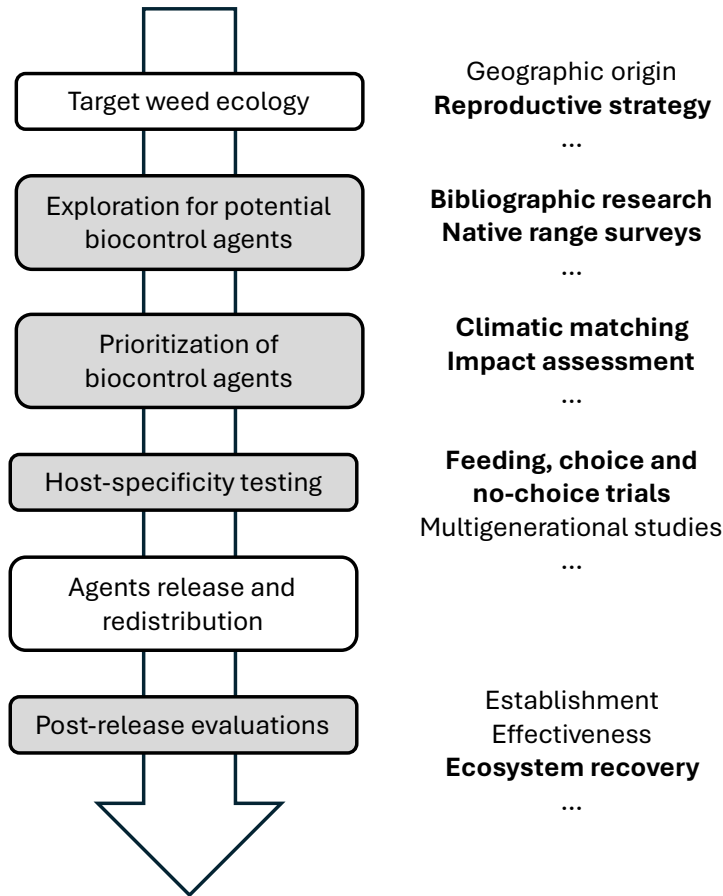


Figure V - Stages in a modern biological control program (topics addressed in this thesis are indicated by shaded boxes and bold text) and some of the methods used in each step (adapted from Müller-Schärer & Schaffner, 2008).

IDENTIFICATION AND PRIORITIZATION OF CANDIDATE BIOCONTROL AGENTS

The identification and selection of the most suitable agent(s) arguably the most important step in a weed biocontrol program. Each agent undergoes extensive testing and preparation before its release, a process that takes a substantial amount of time and resources. For this reason, there is an amount of pressure to make prudent choices when it comes to selecting the right agent(s). Numerous theories and protocols have been put forth to guide the selection of the optimal agent (Harris, 1973; Lawton, 1984; Wapshere, 1985). These take into account a

GENERAL INTRODUCTION

variety of aspects of the biology and life-history of the agents, from geographic distribution and known host-associations, to the type, timing and extent of damage on the target plant, to name just a few. These criteria are currently still employed (e.g. Egli & Olckers, 2020) and are known to be good preliminary indicators of chance of establishment and control (Goolsby et al., 2006). However, time has demonstrated that even agents that look perfect on paper might not be able to achieve the anticipated results once they are released in the field (McFadyen, 1998). That is because the agents' success is influenced, not only by their biological characteristics, but also by interactions with biotic and abiotic factors in the released environment (Harms et al., 2020).

An important aspect to consider when assessing a candidate biocontrol agent is its probability to survive, establish and reproduce in the environment where it will be released. Climatic factors, such as temperature and humidity, play an important role in insect development, and as such they provide perhaps the most important limitation to the success of biocontrol agent (Harms et al., 2020). It is estimated that, of all biocontrol programmes carried until 2005, around 31% were affected by a variation in temperature or precipitation (Harms et al., 2020). Climatic mismatches between source and release regions can hinder the ability of biocontrol agents to establish or exert a significant level of control over their target (Harms et al., 2021). Therefore, it is prudent to source biocontrol agents from areas with similar climatic conditions to those where release is intended, to minimize the time and resources spent on testing and releasing climatically incompatible agents (Sun et al., 2017; Sutton, 2019).

HOST-SPECIFICITY TESTING

Host-specificity testing constitutes a major pillar of weed biocontrol, and as such is the stage of a programme where most time and resources are usually invested. As evolution of phytophagous insects is agreed to have developed from generalists to specialists (Bernays, 2001), host-specificity testing is used to

GENERAL INTRODUCTION

confirm that co-evolved natural enemies have lost the ability to feed and/or reproduce on species other than their host. The aim is to determine the potential host-range of a biocontrol agent and therefore assess the relative risk associated with its release in the field. The process begins with creating a comprehensive list of plants to be tested, including native plants, economically important crops, and species of ecological significance (McFadyen 1998). These plants are then carefully tested starting from species closely related to the target plant and increasing the degree of phylogenetic relatedness (i.e. *Centrifugal Phylogenetic Method*; Wapshere, 1974). This approach – which builds on the co-evolution of host affiliations in phytophagous insects – produces robust predictions of host-specificity while minimizing the time and resources spent on the process (Briese & Walker, 2008; Lesieur et al., 2020).

Once the test plants are selected, a series of tests are conducted where potential biological control agents are exposed to different plants, under controlled conditions, forcing them to make feeding and oviposition choices and thus determining their level of host-specificity (van Klinken, 1999). Due to the different biology of each potential agent, experimental methods have to be adapted for each species to ensure the most accurate results. Decisions about testing include which aspects of the insect's life history to focus on, which morphological and phenological stage of the plant, the experimental designs to use, the order in which tests are conducted, and the balance between laboratory and field trials (van Klinken, 1999). The type and variety of testing procedures has largely progressed over time, now investigating feeding preferences, oviposition cues, reproductive output and multi-generational survival of all life-stages of the candidate biocontrol agents, both in choice and no-choice setups, in controlled laboratory conditions as well as open-field studies in the native range (Clement & Cristofaro, 1995; Heard & van Klinken, 2004; Lesieur et al., 2020).

GENERAL INTRODUCTION

Host-specificity testing for weed biological control is a rigorous process – often taking several years to complete – which has produced robust and reliable results over the last century (Hinz et al., 2020). Unfortunately, no testing procedure can accurately exclude all unsafe agents without also excluding some safe ones (Grevstad et al., 2021). The decision whether to release a biocontrol agent or not is ultimately political, and it must take into account not only the risks of releasing the agent (Blossey, 1995), but also and the impacts associated with alternative control methods, as well as the *do-nothing option* (Simberloff, 2021). In many instances, the impacts of the invader on native ecosystems far exceed the risks associated with the release of a prospective biocontrol agent (Downey & Paterson, 2016). This relative level of risk (i.e. *positive trade-off*) is often deemed acceptable (Cabrera Walsh et al., 2023).

IMPACT ASSESSMENT

Although safety is at the core of biocontrol research and investments, the fundamental goal of classical biological control is to reduce the density and impact of the target plant. This is only achievable if biocontrol agents are effective in controlling their host in the non-native range. Despite this, for the past few decades, selection of weed biocontrol agents focused heavily on host-specificity, and less attention was given to evaluate their predicted efficacy prior to release (McClay & Balciunas, 2005). This reluctance towards pre-release impact assessments can be attributed to three factors: a) the limited resources available, that are better spent on mandatory host-specificity testing; b) a belief, among practitioners, that efficacy in the field is unpredictable because it is affected by too many interacting and unforeseeable factors (McFadyen, 1998); and c) a concern that, because of said unpredictability, false-negatives may cause the rejection of candidates that would, in fact, be successful in controlling the weed if released (McClay & Balciunas, 2005).

GENERAL INTRODUCTION

Several, complex elements are at play in determining the impact of a biocontrol agent on its host, and impact observed in the native range is often not comparable to that realized in the introduced range, which can also be context dependent. Plants in the non-native range can grow bigger and this can sometimes compensate for the top-down regulation caused by biological control agents. At the same time, plants that have had time to evolve in the non-native range might shift their energy deployment from chemical and physical defences towards growth and reproduction (i.e. *evolution of increased competitive abilities*; Blossey & Notzold, 1995). These plants will have fewer defence mechanisms and therefore might be more impacted by their natural enemies. Biotic and abiotic interactions between the agent and the new environment are also difficult to account for. Differences in temperature, humidity, and the presence or absence of predators, competitors and parasites can influence population density and voltinism of biocontrol agents, and therefore the impact on their target plants. Finally, ongoing climate and global change add an additional level of uncertainty, influencing plant-insect interactions in ways that are sometimes unpredictable and idiosyncratic (Sun et al., 2020; Baso et al., 2021; Sun et al., 2022; Paper et al., 2023).

Accordingly, only a portion of biocontrol agents that manage to become established in the new environment contribute substantially to the control of their target plant (Denoth et al., 2002; McFadyen, 2003; Schwarzländer et al., 2018). This can be due to several factors, among which the release of agents feeding on non-essential or unsusceptible plant parts (i.e. *morphological mismatch*) or life-stages (i.e. *phenological mismatch*) of their target (McClay & Balciunas, 2005). An instance of this is given by seed-feeders either not destroying enough seeds or targeting a plant not limited by sexual reproduction (e.g. Impson et al., 1999). In order to minimize the economic and ecological burden of releasing ineffective agents, it has been advocated that greater attention should be paid to selecting agents based on their potential efficacy

GENERAL INTRODUCTION

(Sheppard, 2002; McClay & Balciunas, 2005). Impact assessments, guided by a proper knowledge of the ecology of the target weed, can help prioritize agents whose impacts are best suited for the control of their host. This has led to more research focusing on pre-release evaluations (e.g. Goolsby et al., 2009; Gerber et al., 2008; Madire et al., 2021; Mahlobo et al., 2023).

POST-RELEASE EVALUATION

Evaluation of biocontrol programmes is a necessary – albeit often overlooked – step to correctly assess the effectiveness, safety and economic benefits of biological control. As essential as it may be, evaluation remains a tricky subject, mainly because of equivocation around what constitutes a successful control programme (McFayden, 1998) and a lack of pre-release studies generating appropriate baseline data (Hoffman et al., 2019; Schaffner et al., 2020). Unfortunately, investment needed to keep up with post-release monitoring has not always been proportionate to the funding available, and many programmes have been declared concluded beforehand (Blossey, 1999). In the past, post-release evaluations mainly focused on monitoring the establishment and spread of biocontrol agents, with little investment dedicated to assessing – other than visually – the impact these biocontrol agents exerted on the target weed (McFadyen, 1998). In time, protocols have been developed to experimentally conduct post-release impact studies (Morin et al., 2009), and the importance of long-term monitoring in biological control is now unanimously agreed upon (Hoffman et al., 2019; Schaffner et al., 2020; Faltlhauser et al., 2023). Post-establishment studies are also useful to evaluate indirect effects of biocontrol agents (Denslow & D’Antonio, 2005) and determine the reasons behind their successes or failures (Harms et al., 2020). These ecological insights help improve theories and protocols, making future releases evermore safe and cost-effective.

GENERAL INTRODUCTION

Finally, an often-neglected aspect in biological control, and in particular, aquatic weed management, is that of ecosystem recovery (Prior et al., 2018). For long it was assumed that, upon successful biocontrol of the invader, the ecosystem would shift back into a healthy ecological balance, which might not always be the case (Reid et al., 2009). For instance, recent studies have shown evidence for regime shifts between floating and submerged invasive plants following biological control (Strange et al. 2018). Only recently has research started to look at ecosystem recovery as part of the evaluation of successful biological control programmes (Coetzee et al., 2020; Motitsoe et al. 2020).

BIOLOGICAL CONTROL IN EUROPE

Biological control is a highly regulated, science-based approach, which has become a valuable tool in the fight against invasive weeds in countries such as Australia, New Zealand, South Africa and the United States (Hill & Coetzee, 2017). In Europe, however, regulations and public perception have hampered or altogether hindered the adoption of this method (Shaw, 2008; Marchante et al., 2023), despite various attempts to publicize its effectiveness and prioritize potential targets (Gassmann et al., 2006; Djeddour & Shaw, 2010; Baars, 2011; Pratt et al., 2013).

This is not for lack of potential targets (Sheppard et al., 2006; Lesieur et al., 2023). In fact, approximately 12,000 alien species are currently recorded in Europe (European Commission, 2016) – 10-15% of which considered invasive (Kumschick et al., 2015) – and this number is only expected to increase in the future (Caffrey et al., 2014). Since 2012, these comprised 96 alien aquatic plant species, 18 of which are already listed by the European and Mediterranean Plant Protection Organization (EPPO) as IAAPs (Hussner, 2012). Despite these numbers, research into the management of IAAPs in Europe has been lagging behind compared to other regions of the world, especially with regards to the use of classical biological control (Sheppard et al., 2006).

GENERAL INTRODUCTION

In 2014, the European Union adopted a new Invasive Alien Species Regulation (EU No. 1143/2014), requiring all member states to take action against invasive alien species of Union Concern in the form of prevention, eradication and management strategies (Genovesi et al., 2015). The management measures listed by the regulation include “*physical, chemical or biological actions aimed at the eradication, population control or containment of a population of an invasive alien species*” (EU No. 1143/2014 - Art. 19.2). The inclusion of **biological actions** offers a step in the right direction, but has yet to address the regulatory nightmare of implementing biological control on a national level (Shaw et al., 2016).

Leveraging accidental introductions – which includes weed biocontrol agents established within the EU well into 2010 – a few programmes have implemented intentional redistribution as a tool to combat recognized environmental weeds present in Europe (Shaw et al., 2018; Marchante et al., 2023). Amongst these are the weevil *Stenopelmus rufinasus* Gyllenhal (Coleoptera: Curculionidae), used for the control of *Azolla filiculoides* Lam. (Azollaceae) in the UK; and the ragweed beetle *Ophraella communa* LeSage (Coleoptera: Chrysomelidae), providing significant and increasing economical and health benefits by controlling the allergenic *Ambrosia artemisifolia* L. (Asteraceae) in Italy and nearby countries (Mouttet et al., 2018; Schaffner et al. 2020). New programmes were also launched in recent years to intentionally introduce biocontrol agents against internationally recognised target weeds such as *Acacia longifolia* (Andrews) Willd (Fabaceae) in Portugal (López-Núñez et al., 2021), *Impatiens glandulifera* Royle (Balsaminaceae) and *Crassula helmsii* (Kirk) Cockayne (Crassulaceae) in the UK (Pollard et al., 2021; Varia et al., 2022), and *Fallopia japonica* (Hout.) Ronse Decr. (Polygonaceae) in the Netherlands (STOWA, 2022; Lesieur et al., 2023).

MODEL SPECIES

The present thesis focuses on two invasive aquatic plant species, using them as blueprints to follow the different steps of a biocontrol program (Figure V). The first species, *Iris pseudacorus* L. (Iridaceae), is a riparian macrophyte native to Europe, northern Africa and western Asia. Considered invasive in temperate wetlands across the world, the biocontrol program against this species is still in its infancy. The second species, *Azolla filiculoides* Lam. (Salvinaceae), is a floating fern native to the Americas and a common invader throughout Europe and sub-Saharan Africa. The species has been successfully biocontrolled in several African countries and is now the focus of interest in Europe. The following sections will present a quick overview of the two species.

IRIS PSEUDACORUS LINNAEUS

MORPHOLOGY

Iris pseudacorus L. is a perennial, emergent macrophyte with thick, tuberous rhizomes and showy yellow flowers (Sutherland, 1990). In its native range, the plant can grow up to 150 cm at maturity, with rhizomes 1 to 4 cm in diameter and roots up to 30 cm long. The leaves, around 10 per ramet, are arranged in fan-shaped fashion, overlapping at the base (Sutherland, 1990). Leaves are a glaucous green, 3 to 4 cm wide, with a characteristic raised midrib running across the entire sword-like shape. Showy yellow flowers, 8 to 10 cm in diameter, are borne on tall, erect peduncles. Flowers are hermaphrodite, with 3 sepals and 3 petals fused at the base of the perianth, forming a widening cone. Fruits are elongated, three-chambered capsules, 4 to 8 cm long and 5 to 9 mm wide (Tu, 2003). These can bear up to 120 disc-shaped seeds, densely packed in three vertical rows (Figure VI).



Figure VI – Illustration of *Iris pseudacorus* L. with details of the fruits (© Federico Banfi).

GENERAL INTRODUCTION

DISTRIBUTION

Iris pseudacorus native range extends from northern Africa throughout Europe, western Asia and the Middle East (Figure VII). Across its native distribution the species is considered Least Concern by the IUCN (Kavak, 2014). The plant has been historically exported for ornamental and horticultural purposes, but also as part of phytoremediation and erosion control programmes (Zhao et al., 2015; Venditti et al., 2022). Introduced to every continent except Antarctica, the species is now considered naturalized and often invasive in Canada, the United States, Argentina, Chile, Uruguay, South Africa, Australia, New Zealand, China, Japan and the Korean peninsula (Cody, 1961; Kadono, 2004; Howell, 2008; Masciadri et al., 2010; Ugarte et al., 2011; USDA, 2013; Chang et al., 2014; Jaca & Mkhize, 2015; Hayasaka et al., 2018; McGrannachan & Barton, 2019; Gervazoni et al., 2020; Morgan et al., 2022; POWO, 2023; Xiong et al., 2023; AVH, 2024).

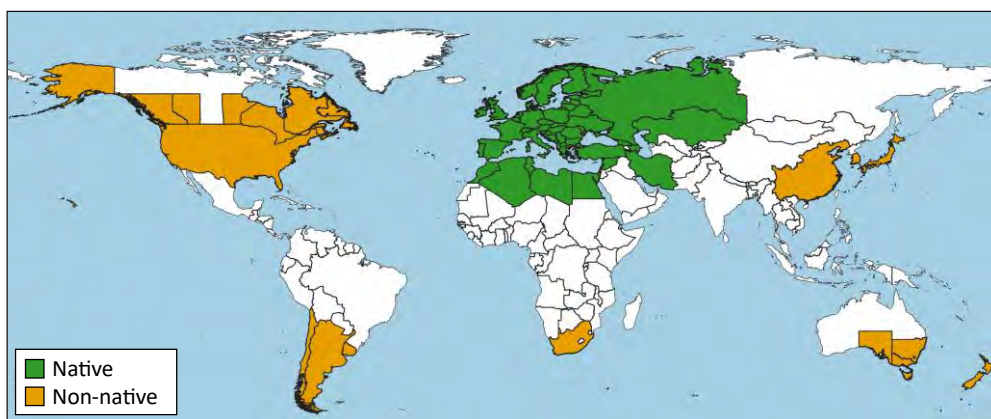


Figure VII – Global distribution and status of *Iris pseudacorus* L.

HABITAT

Iris pseudacorus is usually associated with humid habitats. It is found on the banks of lakes, ponds, rivers and streams, in wetlands, swamps and marshes, but also in woodlands, open woods and forest edges where the soil is moist or regularly flooded (Sutherland & Walton, 1990). Full grown plants can tolerate a

GENERAL INTRODUCTION

wide range of environmental conditions and stressors, such as submersion, prolonged drought, anoxic water conditions and high salinity levels (Sutherland, 1990; Schlüter & Crawford, 2001; Mopper et al., 2016; Gillard et al., 2021). Natural and anthropic disturbance events, including fires, floods, eutrophication, habitat alteration, vegetation removal and management activities have been reported to promote *I. pseudacorus* spread and establishment (Morgan et al., 2023). Being disturbance-adapted and commonly planted as an ornamental, it often occurs in human-modified habitats such as meadows, wet pastures, roadside ditches, irrigation channels and artificial wetlands (Morgan et al., 2023 and references therein).

IMPACTS

Iris pseudacorus is a fast-growing and fast-spreading invasive weed both in natural and man-made aquatic ecosystems. It can form dense thickets, outcompeting native wetland plants, reducing the habitat available to fish and waterfowl, and altering successional trajectories (Thomas, 1980; Tu, 2003; USDA, 2013; Morgan et al., 2023; GISD, 2024). The resulting transformation in the structure of the riparian vegetation has also been linked to a reduction of habitat for several important aquatic species (DNRP, 2020). In a recent study, *I. pseudacorus* invasion was shown to disrupt the composition and function of native plant communities across brackish estuarine gradients in North America (Gallego-Tévar et al., 2022). In Japan, *I. pseudacorus* density of infestation was linked to a significant decrease in the number of native plant species, and a concomitant increase in the number of invasives (Hayasaka et al., 2018). The intricate rhizome mat formed by *I. pseudacorus* colonies compacts the soil and elevates the topography, creating a drier habitat type with increased rates of siltation and sedimentation. This creates a positive feedback loop, preventing the germination and seedling growth of other plant species while improving

GENERAL INTRODUCTION

habitat suitability for *I. pseudacorus* (Sutherland, 1990; Tu, 2003; Morgan et al., 2023; Figure VIII).

Dense *I. pseudacorus* infestations are known to clog small streams, irrigation systems, and flood control structures (DNRP, 2020). Ecosystem processes and services provided by native aquatic and riparian vegetation can also be detrimentally altered by invasion (USDA, 2013). By decreasing stream width, promoting sedimentation and preventing access to water, *I. pseudacorus* infestations can restrict recreational and fishing activities, having an impact on tourism (McGrannachan & Barton, 2019). Furthermore, *I. pseudacorus* is considered unpalatable to livestock and wild animals due to the large amounts of glycosides found within its foliage and rhizomes (Fuller & McClintock, 1986; Tu, 2003; Bossuyt et al., 2005).



Figure VIII – Yellow flag iris invading a wetland outside of Buenos Aires, Argentina (© Ana Faltlhauser, 2021).

Iris pseudacorus is a very popular ornamental plant. Many cultivars have been developed, with features such as variegated leaves, double flowers and novel colours (Tu, 2003 and references therein). Despite its status as a weed in many

GENERAL INTRODUCTION

countries, rhizomes and seeds can still be purchased online and in dedicated plant nurseries (Raghu & Morin, 2017). Aside from its primary horticultural value, *I. pseudacorus* has been used for the phytoremediation of eutrophic systems and urban wastewaters (Yousefi & Mohseni-Bandpei, 2010; Zhao et al., 2015; Venditti et al., 2022; Zhang et al., 2022). This species is reported to reduce the concentrations of heavy metals (Branković et al., 2015), organic chemicals (Larue et al., 2010), insecticides (Wang et al., 2013), and bacterial loads (Sutherland, 1990; Jacobs et al., 2011). It is also a suitable plant to use in erosion control (Tu, 2003) and for phytoremediation of contaminated soils (Pérez-Sirvent et al., 2017).

MANAGEMENT

Physical as well as chemical control methods have been used to control *I. pseudacorus* invasion in its introduced range. Except in the case of small, isolated infestations, these methods seldom result in the complete eradication of the plant (Morgan et al. 2019). Hand-pulling, followed by excavation of the rhizomes, is considered one of the most effective control methods. However, this method is labour-intensive and extremely destructive for the environment. Furthermore, it must be repeated every 3 to 4 years to prevent re-establishment from the seedbank and rhizome fragments (Jacobs et al., 2011). Physical removal has to be coupled with spread limitation and biosecurity measures, as it may cause dislodged rhizome pieces to be transported downstream (especially in flowing waters), assisting their dispersal (USDA, 2013). Furthermore, these methods tend to create repeated disturbance to the ecosystem, at the detriment of native species and facilitating the establishment of other invasive species (DiTomaso & Kyser, 2016). Flower clipping has also been employed to reduce seed production, but with limited results (DiTomaso & Kyser, 2016). The use of benthic barriers (i.e. shadow mats) and simulated trampling by cattle have been recently proposed as an effective method of

GENERAL INTRODUCTION

control (Tarassof et al., 2016; Stoneburner et al., 2021). These methods are non-specific and need to be integrated with ecological restoration efforts. Chemical control of *I. pseudacorus* consists of direct herbicide (i.e. glyphosate, imazapyr or metsulfuron) application on the leaves of the plant (GISD, 2023). Aside from the harmful side effects of herbicides in freshwater ecosystems, their application can be rather impractical and expensive in large aquatic settings (DiTomaso & Kyser, 2016). In New Zealand, for instance, the cost of chemical control – including labour – of *I. pseudacorus* has been estimated in a range between \$350/hectare for small, isolated infestations and \$1350/hectare for large-scale application (McGrannachan & Barton, 2019).

AZOLLA FILICULOIDES LAMARCK

MORPHOLOGY

Azolla filiculoides is a small, free-floating aquatic fern, around 2.5 cm in diameter (Janes, 1998). The plant consists of a main rhizome branching into secondary ones, each divided in nodes bearing ventral adventitious roots hanging in the water, from which it absorbs nutrients. The rhizomes bear small, bright green to rusty red leaves, arranged in alternate fashion (Figure IX). The plant grows in symbiotic association with a nitrogen-fixing, heterocystic cyanobacterium which nests in cavities on the dorsal lobes of its leaves (Ashton & Walmsley, 1984). The water fern is able to propagate both vegetatively – by branching and fragmentation – and sexually, through the production of spores (Wagner, 1997). Spores are produced in spring and summer, and can overwinter and survive extreme desiccation (Lumpkin & Pluckett, 1982). Under ideal environmental conditions the plant will undergo both types of reproduction, doubling its biomass vegetatively every 4 to 5 days and producing hundreds of microspores each year (Ashton, 1982).

GENERAL INTRODUCTION

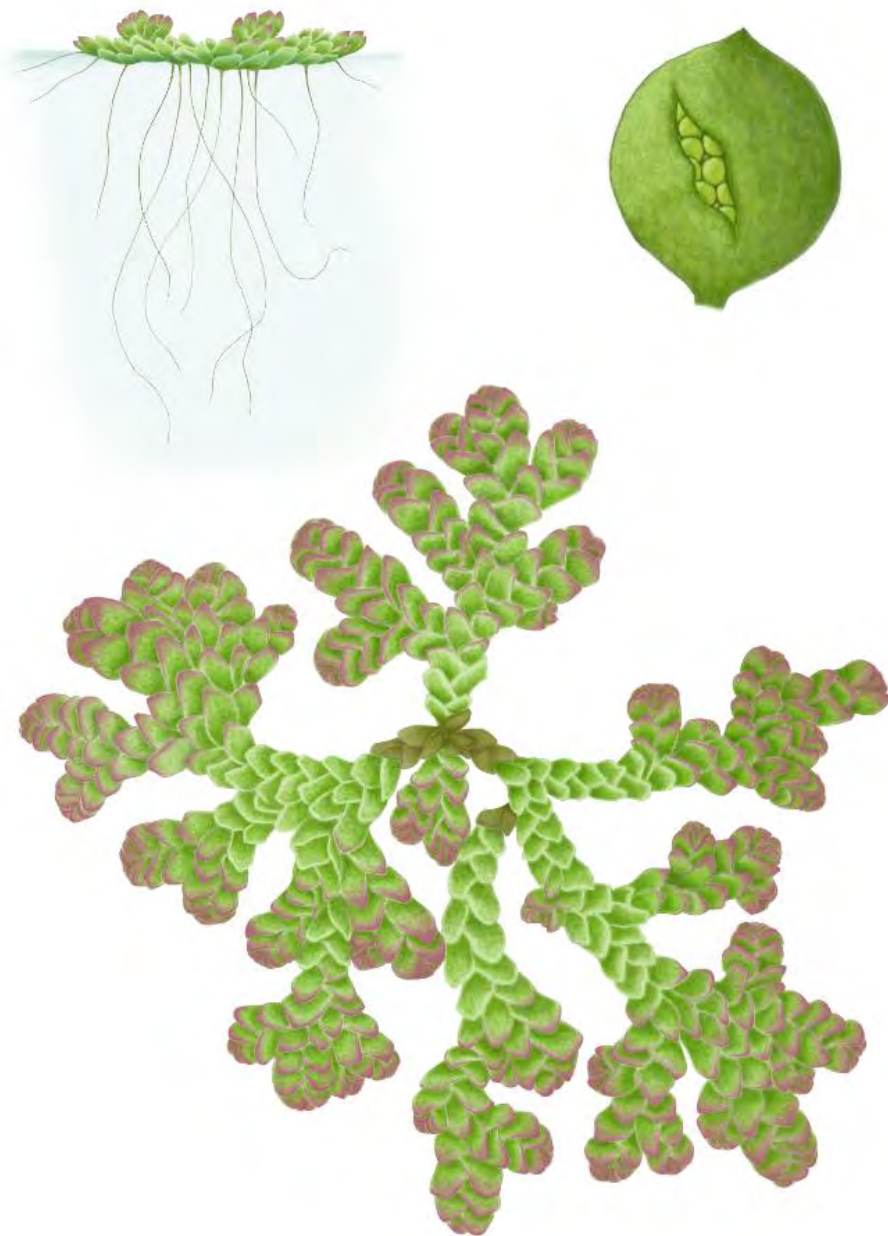


Figure IX – Illustration of *Azolla filiculoides* Lam. (side and top view) with details of the sporangium (© Federico Banfi).

GENERAL INTRODUCTION

DISTRIBUTION

Azolla filiculoides is native to the Americas, from western USA through Central and most of South America (Figure X). The species has not been evaluated by the IUCN Red List of Threatened Species. The plant has been historically introduced into Europe, North and sub-Saharan Africa, China, Japan, Australia and New Zealand, with many countries currently considering it invasive (Hill, 2014). The main pathway of introduction recorded is the ornamental trade; however, unintentional introduction through ballast water and intentional release as green manure are also mentioned (Lumpkin & Plucknett, 1982; Carrapiço, 2010).

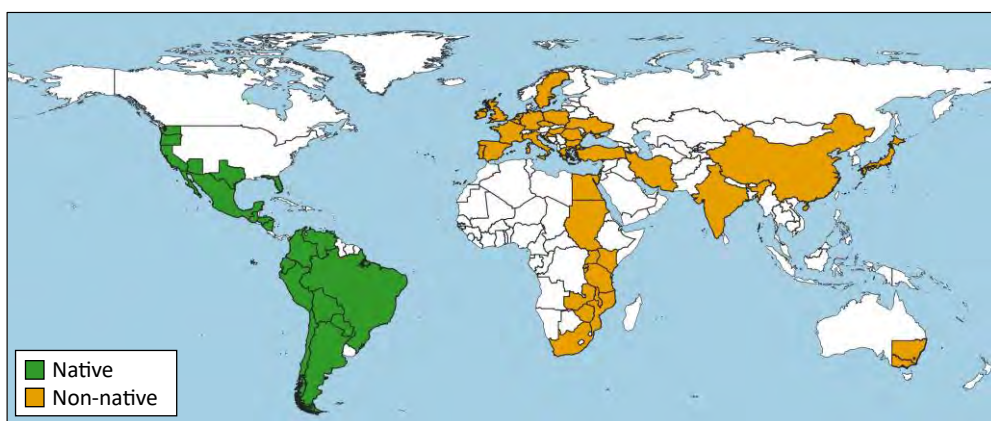


Figure X – Global distribution and status of *Azolla filiculoides* Lam. (Hill, 2014).

HABITAT

In its native range, *A. filiculoides* is commonly found in slow flowing streams and rivers, marshes, ponds, lakes and other standing bodies of water (Ashton, 1982). Water fern is phosphorus-limited and shows a high affinity towards eutrophicated, alkaline waters with organic bottoms (Janes, 1998). This species often invades disturbed and human-made habitats such as reservoirs, irrigation canals and roadside ditches. Local spread is often attributed to human activities, flood events and the movement of animals (Hill, 2014).

GENERAL INTRODUCTION

IMPACTS

In favourable conditions, *A. filiculoides* grows rapidly, easily covering the water surface with thick mats and outcompeting the indigenous floating and submerged vegetation (Janes et al., 1996; Paolacci et al., 2018; Figure XI). Decaying root and leaf matter below the mat, coupled with the lack of light penetration, create an anaerobic environment which hinders the survival of other aquatic organisms (Pinero-Rodriguez et al., 2020). Infestations can grow to hectares in size, with severe impacts on aquatic biodiversity, ecosystem services, and socio-economic activities linked with water utilization (Gratwicke & Marshall, 2001). Thick mats can clog water filters, pumps and irrigation systems, impede boating, fishing and other recreational activities. The weed also reduces water quality, increases water loss through evapotranspiration, and promotes the development of water-borne diseases (Strayer, 2010). Evaluating the economic impacts of water fern has been notoriously hard due to the difficulty to monetize environmental, cultural and aesthetic values affected by its invasion. An early evaluation from McConnachie and colleagues (2003) estimated *A. filiculoides* invasion in South Africa to be 600 USD per hectare per year; whereas Vilà et al. (2010) report actual expenditures of over 1 million EUR per year in a protected area in Spain. Finally, recent estimates of the management costs of *A. filiculoides* in Great Britain range from 9.4 to 18.9 million USD per year (Pratt et al., 2022). Besides its ornamental value, *A. filiculoides* is mainly used as green manure and inhibitor of weed growth in rice paddies (Kröck & Alkämper, 1991), as animal fodder, alternative compost in agricultural systems, and for water purification (Sanyahumbi et al., 1998; Forni et al., 2001).

GENERAL INTRODUCTION



Figure XI – Water fern invading a nature reserve in Flanders, Belgium (© Gianmarco Minuti, 2020).

MANAGEMENT

Isolated infestations of *A. filiculoides* can be physically removed with the help of rakes and nets (Hill, 1997). However, due to the rapid vegetative growth of the weed, continuous effort is required keep up with its daily biomass production. Furthermore, even when local eradication is achieved, recruitment from spores the following year is usually inevitable, and physical control applications are only regarded as a palliative measure. Methods of mechanical harvesting have been suggested (Ashton, 1992; Hussner et al., 2017), but apart from the potential side effects on aquatic ecosystems, the costs associated with large scale application of these measures are considered prohibitive (Hill, 2014). Herbicides used to control *A. filiculoides* include glyphosate, paraquat and diquat (Stejn et al., 1979; Axelsen & Julien, 1988; Ashton, 1992), all of which have been found toxic to fish and other aquatic organisms and are therefore banned from use in the EU and other countries (Hill, 2014).

GENERAL INTRODUCTION

So far, biological control has achieved the most success in the long-term management of *A. filiculoides* invasion. The first biocontrol programme against this weed was initiated in South Africa in 1995 with the release of the weevil *Stenopelmus rufinasus* Gyllenhal (Coleoptera: Curculionidae) (Hill, 1998). By the year 2002, the weevil had been released across 112 infestations, resulting in significant levels of control and even local eradication, to the point where *A. filiculoides* was considered no longer a threat to South African aquatic ecosystems (McConnachie et al., 2004). The net present value of the water fern biocontrol programme in South Africa was calculated at 206 million USD, with cost-benefit ratio of 1:15 (McConnachie et al., 2003). Ten years after first release, the weed was considered eradicated in 81% of previously infested sites, and the weevil was found at 73% of sites where the plant was still present (Hill et al., 2008). Due to these exceptional results, the biological control of *A. filiculoides* in South Africa is now widely regarded as one of the most successful programmes against an invasive aquatic weed (Coetzee et al., 2011).

Stenopelmus rufinasus has been present in Europe for over a hundred years, probably first brought into the UK together with the plant (Janson, 1921). Since then, it has been recorded in Ireland, France, Belgium, the Netherlands, Portugal, Spain and Poland (Carrapiço et al., 2011; Pratt et al., 2013; Wanat, 2018). Now considered naturalized, the weevil has been used in translocation and inundation programmes in the UK and Ireland to keep water fern infestations under control (Pratt et al., 2013; Reeder et al., 2018). A recent study evaluated the economic value of *S. rufinasus* biocontrol to Great Britain to range between 43.7 to 88.1 per pound spent on the augmentative programme (Pratt et al., 2022).

THESIS OUTLINE AND OBJECTIVES

The general objective of this thesis is to gain insight into the invasion biology and advance the biological control programs of two invasive aquatic plant species. Accordingly, the thesis is divided in two parts, structured in five consecutive chapters, which follow the most common stages of a modern biological control programme (Figure XII).

Part I, comprising **Chapters 1 through 4**, investigates the potential for biological control of *Iris pseudacorus* L., a European plant which has invaded various wetland habitats across the world.

Chapter 1 describes the process of identification and selection of candidate biocontrol agents with potential to be used against this invasive weed. This begins with an extensive literature review aimed at **compiling bibliographical records of natural enemies** of the plant. Then, field surveys are carried to identify **what organisms attack the plant across its native range**, which are then prioritized based on incidence, abundance, phenology, known host-range and damage type.

In **Chapter 2**, the bioclimatic suitability of the weed and those of the previously selected candidates are modelled and projected across various geographical and temporal ranges. The primary goal is **to identify areas at risk of invasion** and **regions of climatic mismatch** between the invasive weed and its natural enemies, as these are known to affect establishment and biological control. Furthermore, future scenarios are incorporated to the models predict **how climate change will affect** the establishment of the weed and its different candidates across various invaded ranges.

Chapter 3 focuses on safety protocols, presenting an overview of the results obtained from the host-specificity tests conducted, to date, on the main

GENERAL INTRODUCTION

candidate biocontrol agent. The main objective of the chapter is **to present a consolidated list of plants to be tested for host-specificity** of *I. pseudacorus* biocontrol agents. This is then used to **discuss the method of plant selection**, and its application to widespread invaders and complex phylogenies such as the case at hand. Finally, the preliminary results obtained on one of the candidate biocontrol agents, the flea beetle *Aphthona nonstriata* Goeze (Coleoptera: Chrysomelidae), are presented and used to **assess the risks associated with the potential future release** of this agent.

Part II of the thesis covers the last stage of a biological control programme, post-release evaluations. Given that the candidate biocontrol agents for *I. pseudacorus* have yet to be approved for release, the spotlight is shifted towards a second wetland species, the water fern *Azolla filiculoides* Lam. (Salvinaceae). Native to Central and South America this species has been exported worldwide and is now considered invasive in several regions of Africa, Asia and Europe. Water fern has been successfully managed via biological control in South Africa, and although never intentionally introduced, its biocontrol agent – the weevil *S. rufinasus* – is considered naturalized in several countries of the EU, making it a perfect target for post-release evaluations.

Chapter 5 presents the results of a case study carried out in natural wetlands in Belgium, where the effects of water fern invasion as well as those of its biological control agent were analysed by proxy of water parameters, macroinvertebrates and zooplankton communities. These results are then used to discuss a) the **impact of *A. filiculoides*** invasion on native aquatic ecosystems; b) the **effectiveness of *S. rufinasus*** in controlling water fern invasions; and c) the intricacies of evaluating the **recovery of aquatic ecosystems** after biocontrol.

GENERAL INTRODUCTION

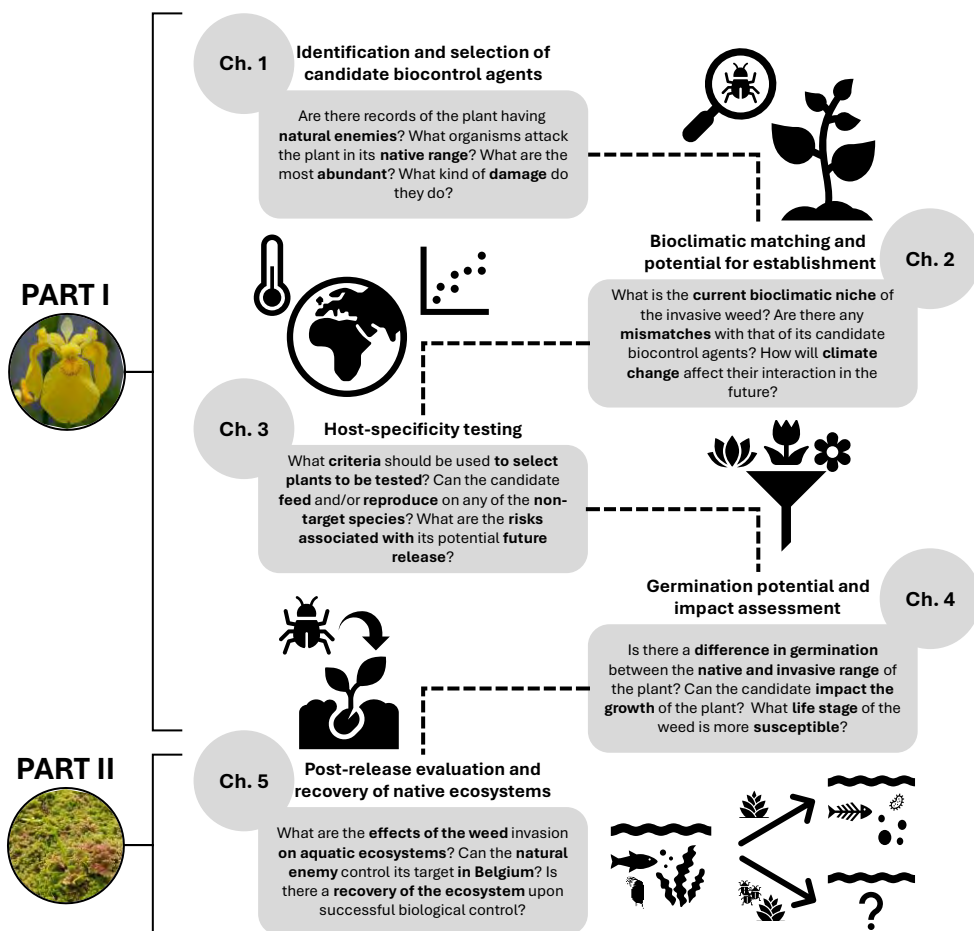


Figure XII - Outline of the thesis including the main research questions answered in each chapter. Part I, comprised of Chapters 1 through 4, investigates the potential for biological control of *Iris pseudacorus* L. (Iridaceae), a European plant which has invaded various wetland habitats across the world. Part II, represented by Chapter 5, tackles an often overseen aspect of biological control, post-release evaluation, and in particular it discusses the inclusion of ecosystem recovery in such evaluations. The spotlight is shifted towards a second wetland species, the water fern *Azolla filiculoides* Lam. (Salvinaceae), native to Central and South America and considered invasive in several regions of the world. Water fern has been successfully managed via biological control in South Africa, and its biocontrol agent – the weevil *Stenopelmus rufinus* Gyllenhal. (Coleoptera: Curculionidae) – is considered naturalized in several countries of the EU, making it a perfect target for post-release evaluations.

PART I

PRE-RELEASE STUDIES



YELLOW FLAG

CHAPTER 1

Selection and prioritization of natural enemies for the biological control of the invasive macrophyte *Iris pseudacorus* L. (Iridaceae)

Adapted from: Minuti, G., Coetzee, J.A., Ngxande-Koza, S., Hill, M.P., & Stiers, I. (2021). Prospects for the biological control of *Iris pseudacorus* L. (Iridaceae). *Biocontrol Science and Technology*, 31(3), 314-335.

ABSTRACT

Native to Europe, North Africa and western Asia, *Iris pseudacorus* L. (Iridaceae) has invaded natural and human-modified wetlands worldwide. This species is considered a noxious weed in several countries including Argentina, South Africa and New Zealand. Its broad ecological tolerance, high resilience and reproductive potential make current mechanical and chemical control measures cost-ineffective, and biological control is considered a suitable alternative. In order to prioritize candidate biocontrol agents, a list of organisms reported to attack the plant within its native range has been assembled, and information about their host-range and damaging potential gathered from literature. Furthermore, surveys for natural enemies of the plant were conducted in Belgium and northern Italy. The insect fauna associated with *I. pseudacorus* at the sites surveyed comprised mostly incidental visitors and polyphagous feeders, with the exception of the sawfly *Rhadinoceraea micans* Klug (Hymenoptera: Tenthredinidae), the seed weevil *Mononychus punctumalbum* Herbst (Coleoptera: Curculionidae), and the flea beetle *Aphthona nonstriata* Goeze (Coleoptera: Chrysomelidae). The potential of these species for biocontrol was evaluated, and *A. nonstriata* was given highest priority. A population of this species was imported to quarantine in South Africa, where it is currently undergoing host-specificity testing. Importation of the two

CHAPTER 1

remaining candidates is expected shortly. In conclusion, the prospects for the biological control of *I. pseudacorus* appear promising.

1. INTRODUCTION

Biological invasions are of great concern for biodiversity conservation and ecosystem integrity (Vilà et al., 2010), especially when it comes to wetlands, which are particularly vulnerable to invasive aquatic plants (Stiers et al., 2011; Hussner et al., 2017). Yellow flag, *Iris pseudacorus* L. (Iridaceae), is an herbaceous perennial, emergent macrophyte native to Europe, North Africa and western Asia, considered invasive in natural and human-modified wetlands worldwide. In the wild, this species is commonly found inhabiting swamps, marshes, and shallow waters along the shores of lakes, ponds or slow-flowing watercourses (Sutherland, 1990; Kavak, 2014). Exported internationally as an ornamental plant, escape from cultivation is presumably the main pathway of establishment of this species outside of its native range (Morgan et al., 2020).

To date, *I. pseudacorus* is considered naturalized and often invasive in Canada, the United States, Argentina, Chile, Uruguay, South Africa, China, Japan, Australia and New Zealand (Cody, 1961; Howell, 2008; Masciadri et al., 2010; Ugarte et al., 2011; USDA-APHIS, 2013; Jaca & Mkhize, 2015; Hayasaka et al., 2018). Although first records of establishment outside of its native range date as far back as 1771 on east coast of the United States (Wells & Brown, 2000), recognition of its invasive status is far more recent (USDA, 2013). In Argentina and New Zealand, this species was first observed in the wild in the 1930s, but has since spread over a wide geographic range invading various ecozones and habitat types (McGrannachan & Barton, 2019; Gervazoni et al., 2020). In South Africa, *I. pseudacorus* was first recorded as naturalised in 2004 (Jaca & Mkhize, 2015), but since then further introductions and local dispersion through water systems rapidly expanded its range, leading to its listing as a category 1A emerging invader (NEMBA, 2014).

CHAPTER 1

In most of its introduced range, *I. pseudacorus* has been linked to a variety of ecological and socio-economic impacts (Jacobs et al., 2011; Jaca & Mkhize, 2015; Gaskin et al., 2016; Hayasaka et al., 2018). This plant's rapid vegetative growth allows it to form dense, monospecific stands, which outcompete the native wetland vegetation resulting in a reduction of plant diversity and the loss of natural habitats for fish and waterfowl (Jacobs et al., 2011; Morgan et al., 2023). Furthermore, its rhizome system traps the sediment, progressively compacting the soil and altering the hydrology and functioning of aquatic ecosystems, which slowly shift towards drier, species-poorer habitats (Jacobs et al., 2011; Morgan et al., 2023). In the United States, *I. pseudacorus* flowers were observed to attract long-tongued flies and bumblebees, reducing pollination of native flowering plants (Dieringer, 1982).

Being disturbance-adapted, *I. pseudacorus* is often found in human-modified habitats such as meadows, wet pastures and artificial wetlands (Thomas, 1980; Stone, 2009; Jaca & Mkhize, 2015). In these suburban settings, infestations clog up irrigation channels and drainage ditches, increasing the risk of flooding and resulting in agricultural losses and high maintenance costs (USDA, 2013). Additionally, the plant may pose a threat to domestic animals, as its leaves and rhizomes contain high concentrations of glycosides making it poisonous if ingested (Jacobs et al., 2011).

Iris pseudacorus is a resilient weed: adapted to a variety of natural and disturbed environments, it can tolerate drought, submersion, salt stress, low pH and long-term anoxia (Schlüter & Crawford, 2001; Blokhina et al., 2003; Mopper et al., 2016). Its thick, tuberous rhizomes retain the ability to develop into new plants even after fragmentation (Jaca, 2013). Nonetheless, sexual reproduction plays an important role in its spread, both in the native and invasive range (Lamote et al., 2002; Gaskin et al., 2016). Its buoyant seeds, carried by flowing water and flood events, have been observed to survive desiccation and retain most of their

CHAPTER 1

viability for over two years (Authors, unpublished data). Altogether, these characteristics make *I. pseudacorus* a difficult target for manual or mechanical removal, which seldom, if ever, results in its eradication (Jaca & Mkhize, 2015; Hill & Coetzee, 2017). Chemical control using glyphosate is currently adopted to manage the weed in some countries (Jacobs et al., 2011). However, large-scale herbicide application is regarded as environmentally undesirable, especially in proximity of water systems (Myers et al., 2016).

Following these considerations, classical biological control is considered a viable alternative for the long-term management of *I. pseudacorus* invasions. In 2016, the first biocontrol programme for this species was initiated in South Africa (Hill & Coetzee, 2017), and the option is now under consideration by other countries such as New Zealand and Argentina (McGrannachan & Barton, 2019; Gervazoni et al., 2020). Field surveys conducted throughout the invaded range found no specialized natural enemies attacking *I. pseudacorus* in the wild, whereas generalist insects were observed to cause only minor damage to the plant, leaving most herbivore niches unexploited (Gervazoni, McGrannachan and Sandenbergh, pers. comm.). This supports the hypothesis that a lack of specialist herbivores may be among the factors determining the enhanced competitive ability of invasive *I. pseudacorus* populations in its invaded range, emphasizing the potential of biocontrol as a management option for this species (Blossey & Nötzold, 1995; Keane & Crawley, 2002). In this regard, the main objective of this study was to compile a list of organisms reported to attack *I. pseudacorus* within its native range, and conduct surveys of the insect fauna associated with the plant in an attempt to prioritize natural enemies as candidate biocontrol agents.

2. MATERIALS & METHODS

2.1. LITERATURE REVIEW

A comprehensive list of insect species reported to be associated with *I. pseudacorus* within its native range was compiled. Google, Google Scholar, Web of Science and CABI Invasive Species Compendium were searched using different combinations of the terms: ['*Iris pseudacorus*' OR 'yellow flag iris' OR 'yellow flag' OR 'pale-yellow iris' OR 'water iris' OR 'water flag'] AND ['natural enem*' OR 'invertebrat*' OR 'herbivor*' OR 'pest*' OR 'damag*' OR 'attack*']. The most common homotypic synonyms for the plant (i.e. *Iris palustris* Gaterau; *Limniris pseudacorus* (L.) Fuss and *Xiphion pseudacorus* (L.) Schrank) were also searched in combination with the abovementioned keywords. For each insect species, information regarding its geographic distribution, host-range and damage was gathered from the available literature and relevant online databases (Robinson et al., 2010; Pocock et al., 2015; Ellis, 2020).

2.2. FIELD SURVEYS

Iris pseudacorus has a widespread distribution in Europe and western Asia, being a common element of wetland ecosystems across different bioregions and climate types (Kavak, 2014). In the absence of specific information regarding the genetic and geographic origin of invasive *I. pseudacorus* populations, native range surveys followed an eco-climatic approach (Wapshere, 1985). In the invaded range, this species is found along the coasts of North America, southern Chile, north-eastern Argentina, Uruguay, South Africa, Japan, south-east Australia and New Zealand (GBIF, 2019), showing a marked preference for warm temperate climates (Cfa and Cfb for the greatest part, Csa and Cwb in minor proportions; Kottek et al., 2006). Accordingly, our surveys focused on humid areas of Belgium and northern Italy, as these share a similar climate to that invaded by the plant (Cfa and Cfb; Kottek et al., 2006). A

CHAPTER 1

total of 12 sites, spread across a variety of natural and human-modified habitats (IUCN, 2012), were visited multiple times between 2017 and 2018, covering different phenological phases of the plant (Supplementary Table S1.1).

At each site, three *I. pseudacorus* plots (1m² each, at least 10m apart from each other) were sampled as replicates. Wetland plant species co-occurring with *I. pseudacorus* were identified within a 9m² quadrat around each sampling plot, and their contribution to the vegetation matrix was estimated following the DAFOR scale (Kent & Coker, 1992). Each quadrat was monitored closely for 30 minutes, during which the insects found on *I. pseudacorus* were collected and observations were made on their presence on the co-occurring plant species.

Where possible, all parts of the plant (i.e. leaves rhizomes, roots, flowers and seed capsules) were inspected. Insects were collected using aspirators, fine-mesh nets and soft tweezers. Samples of various plant parts were gathered for subsequent dissection and placed into Berlese funnels to facilitate invertebrates' extraction. Immature insects were reared out to the adult stage in emergence boxes. All specimens were preserved in 90% ethanol and stored at 4°C in the dark. Identification was conducted via consultation with expert entomologists and taxonomists from the Royal Belgian Institute of Natural Sciences (RBINS). Finally, a population of a flea beetle species found feeding on *I. pseudacorus* in the field was reared in controlled conditions to observe its feeding behaviour and development on the plant.

2.3. SPECIES ACCUMULATION CURVES

A species accumulation curve (SAC) represents the number of species present in a particular area or habitat as a function of the effort required to observe them (Colwell et al., 2004). As native range surveys for natural enemies are often prone to geographical and temporal limitations, SACs can be used as an informative tool to evaluate the adequacy of the sampling effort and highlight the need for further investigations (Hortal et al., 2006). We constructed a SAC using

CHAPTER 1

a subset of herbivorous insects collected on *I. pseudacorus* during our surveys. Species occurring at only one sampling site and determined (from direct observations or literature review) not to feed on the plant were considered incidental visitors and excluded from the analyses. Individual-based rarefaction curves were generated using the software EstimateS[®] (Version 9.1.0; Colwell, 2013). Non-parametric, incidence-based estimators were adopted to predict true species richness and evaluate the adequacy of our sampling effort. The Chao estimator classifies rare species as occurring in exactly 1 (*uniques*) or 2 (*duplicates*) sampling units; the corresponding incidence-based coverage estimator (ICE) adds information based on species found in 10 or fewer units; whereas the Michaelis-Menten is an asymptotic estimator calculated as the mean score after 100 randomization (Chao et al., 2005; Hortal et al., 2006). These estimators were chosen as they are known to perform well in studies with small sample size (Hortal et al., 2006). A statistical extrapolation of the obtained species accumulation curve to three times its empirical size was also computed using a likelihood-based estimator with 95% bootstrap confidence intervals (Colwell et al., 2004).

2.4. CANDIDATES' PRIORITIZATION

Geographic distribution. It was assumed that organisms with a broad native distribution, ranging across different climate zones and habitat types, would be able to adapt to a broader suite of environmental conditions and therefore be more likely to establish upon introduction in the invaded range (Blossey, 1995; Paynter et al., 2017). Accordingly, species known from literature to be widespread throughout *I. pseudacorus* native range, and that were observed to be most common during our sampling, were given higher priority.

Damage type, timing and extent. In order to achieve substantial control over time, biocontrol agents should be able to decrease the density of their host and increase its susceptibility to biotic and abiotic stressors (Harris, 1973). *Iris*

CHAPTER 1

pseudacorus shows high stress-tolerance and competitive abilities, but studies investigating the mechanisms behind its competitiveness are scarce. This plant exhibits a fast foliar growth in early spring (Sutherland, 1990), which some authors have linked with overshadowing of smaller plants (Hayasaka et al., 2018). During summer, when leaves reach maximum height and photosynthetic activity peaks, resources are allocated to rhizomes and roots growth (Sutherland, 1990). Belowground biomass constitutes the greatest part of the total biomass, and has been observed to reach over 99% in plants grown in controlled conditions (Mopper et al., 2016). This characteristic, often linked with the resilience of this species (Sutherland, 1990), could enhance its ability to compete for space and resources. Degradation of the rhizome, typically occurring after 6-15 years, was observed to be strongly accelerated by mechanical damage, especially in wet soils (Sutherland, 1990). Following these considerations, priority was given to organism able to exert a significant pressure on newly emerged leaves and rhizomes of the plant, either by being individually damaging or through high and sustained infestation levels (Wapshire, 1985). Nonetheless, as *I. pseudacorus* is known to disperse both clonally and sexually, attention was paid to select also enemies attacking its reproductive structures. Candidates were classified based on damage type, timing and extent, which were assessed qualitatively in the field via direct observations and estimates of abundance, and verified from literature sources, when available.

Inferred host-range. The family Iridaceae differentiated during the late Cretaceous or early Tertiary and appears phylogenetically isolated within the order Asparagales (Goldblatt, 2000). The genus *Iris* (sub-family Iridoideae) diversified from the sub-Saharan African clades of the tribe Iridae (*i.e.* *Dietes*, *Bobartia*, *Ferraria* and *Moraea*) ca. 45mya, and its distribution is now restricted to temperate regions of the Northern Hemisphere (Goldblatt et al., 2008). Australian and Neotropical Iridaceae show an even higher degree of

CHAPTER 1

phylogenetic separation from *I. pseudacorus* (Goldblatt et al., 2008), putting them at a lower risk from strictly host-specific biocontrol agents (Briese, 2005). Information regarding the host-range of each organism reported or observed attacking *I. pseudacorus* within its native range was gathered from the literature and supplemented with field observations. Organisms recorded uniquely on species within the genus *Iris* were selected as potential biocontrol agents, and priority was given to those believed to feed and develop solely on *I. pseudacorus*.

3. RESULTS

3.1. LITERATURE REVIEW

Sutherland (1990) lists 22 invertebrates and 11 pathogens reported to attack *I. pseudacorus* across its native range. This list was updated by McGrannachan & Barton (2019) to include records of 36 arthropods and 67 fungi worldwide. Our review focused only on invertebrates, finding 42 insect species associated with *I. pseudacorus* within its native range (Supplementary Table S1.2). The majority of these insects (31 spp.) are reported to be polyphagous and were therefore considered unsuitable as potential biocontrol agents. The remaining 11 species were given further consideration. These were: the thrips *Iridothrips iridis* Watson (Thysanoptera: Thripidae); the aphid *Aphis newtonii* Theobald (Hemiptera: Aphididae); the flea beetle *Aphthona nonstriata* Goeze (Coleoptera: Chrysomelidae); the seed weevil *Mononychus punctumalbum* Herbst (Coleoptera: Curculionidae); the sawfly *Rhadinoceraea micans* Klug (Hymenoptera: Tenthredinidae); the scarce marsh neb *Monochroa divisella* Douglas (Lepidoptera: Gelechiidae); the noctuid moths *Amphipoea crinanensis* Burrows and *Oxytripia orbiculosa* Esper (Lepidoptera: Noctuidae); the leaf-mining flies *Cerodontha iraeos* Robineau-Desvoidy and *C. iridis* Hendel (Diptera: Agromyzidae); and the gall midge *Dicerura iridis* Kaltenbach (Diptera: Cecidomyiidae).

CHAPTER 1

3.2. FIELD SURVEYS

The surveys for natural enemies of *I. pseudacorus* took place during the spring and summer months in Europe, at sites where the plant was growing naturally. The sites surveyed were located mostly within marshes, swamps and lentic systems (natural and artificial ponds and lakes), with only a few occurring on the shores of streams or canals. In the field, *I. pseudacorus* was observed in isolated patches and in admixture with native wetland plants.

A total of 34 herbivorous insect species were recorded on *I. pseudacorus* during our surveys (Supplementary Table S1.3). These comprised five orders: Coleoptera (15 spp.), Hemiptera (8 spp.), Orthoptera (5 spp.), Lepidoptera (4 spp.) and Hymenoptera (2 spp.). The majority (ca. 65%) of these species were found dwelling on the leaves of the plant, whereas the remaining were recorded on flowers and fruits. Only one species, the seed weevil *M. punctumalbum*, emerged from the seed capsules collected in the field. Although no endophagous root feeders were recovered during our surveys, rhizome inspection highlighted the presence of feeding damage that was later attributed to the larvae of the flea beetle *A. nonstriata*. Almost 80% of the herbivorous insect species recovered during our surveys were determined to be polyphagous and thus excluded from the prioritization process (Supplementary Table S1.3). Only 5 species reported in the literature as attacking *I. pseudacorus* were recovered during our samplings. Two of them – the leaf beetle *Plateumaris sericea* L. (Coleoptera: Chrysomelidae) and the gold spot moth *Plusia festucae* L. (Lepidoptera: Noctuidae) – are known polyphagous and were thus discarded. The three remaining species – the iris sawfly *R. micans*, the seed weevil *M. punctumalbum*, and the flea beetle *A. nonstriata* – all allegedly host-specific according to the literature, were given further consideration.

CHAPTER 1

3.3. SPECIES ACCUMULATION CURVES

Of the 34 herbivorous species collected, 20 were encountered only at one sampling site and therefore considered incidental visitors. The remaining 14 species (used to compute species accumulation curves) made up between 64 and 93% of the total richness predicted by ICE (15 spp.), Chao 2 (14-17 spp.) and Michaelis-Menten (22 spp.) estimators (Figure 1.1). The $S(\text{est})$ curve nearly reached the asymptote at 14 spp., with statistical extrapolations indicating that a 3-fold increase in sampling effort would only yield 2 additional species. This suggests that ICE and Chao 2 predictions might represent better accuracy than Michaelis-Menten estimates, as observed in other studies dealing with small sample size (Hortal et al., 2006).

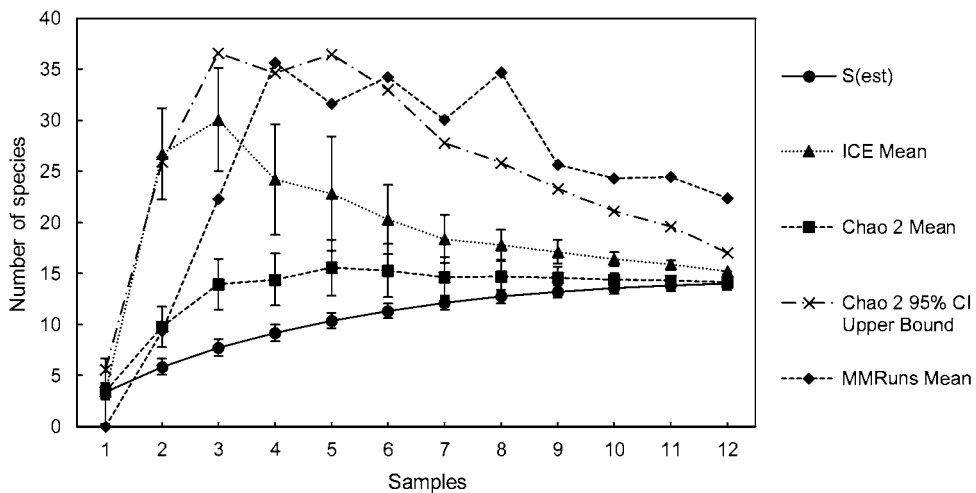


Figure 1.1 – Species accumulation curve of non-incidental herbivorous insect species associated with *Iris pseudacorus* at the sites surveyed in Belgium and northern Italy. Incidence-based Coverage Estimator (ICE Mean), bias-corrected Chao 2 (Mean and 95% CI Upper Bound) and Michaelis-Menten (MMRuns Mean) estimates are shown (error bars indicate standard deviation). When the rarefaction curve $S(\text{est})$ and the estimators converge at the highest observed richness, estimates can be considered as representative (Longino et al., 2002).

CHAPTER 1

3.4. CANDIDATES' PRIORITIZATION

The most promising candidate biocontrol agents were prioritized, amongst the species selected from the literature and the field surveys, based on their geographic distribution, damage potential, and known host-range (Table 1.1).

Table 1.1 – Species evaluated as candidate biocontrol agents for *I. pseudacorus* L., listed from the most to the least promising. Positive (+), negative (–), and dubious (?) attributes are based on literature information (Supplementary Table S1.2) and field observations (Supplementary Table S1.3).

#	Species evaluated	Geographic Range	Damage Potential	Known Host-range
1	<i>Aphthona nonstriata</i>	+	+	+
2	<i>Mononychus punctumalbum</i>	+	+	+
3	<i>Rhadinoceraea micans</i>	–	+	+
4	<i>Monochroa divisella</i>	–	+	+
5	<i>Amphipoea crinanensis</i>	–	?	+
6	<i>Oxytripia orbiculosa</i>	–	?	+
7	<i>Iridothrips iridis</i>	+	–	+
8	<i>Cerodontha iraeos</i>	+	–	+
9	<i>Cerodontha iridis</i>	–	+	?
10	<i>Aphis newtoni</i>	+	–	–
11	<i>Dicerura iridis</i>	–	–	–

Species for which host-specificity was dubious or which were reported feeding outside of the *Iris* genus were given lowest priority. These included the gall midge *D. iridis*, the aphid *A. newtoni* and the fly *C. iridis*. The thrips *I. iridis* and the leaf-mining fly *C. iraeos* are both widely distributed and recorded as monophagous on *I. pseudacorus*. However, the damage associated with their presence is often negligible making them sub-optimal candidates. Little information was found in the literature regarding the noctuid moths *O. orbiculosa* and *A. crinanensis*, which are both allegedly host-specific and associated with damage to the rhizome of the plant. The two species share a restricted and disjunct distribution

CHAPTER 1

across Europe, where they are considered rare and, in some cases, at risk. This could pose some challenges for their use in biological control. Similarly, the scarce marsh neb *M. divisella* is reported as uncommon and restricted to some areas of central Europe. However, the species is allegedly monophagous on *Iris* species, and considered a pest for the iris horticulture in Japan (Tabata & Kubota, 2017). Its larvae infest the apical shoots of the plant, inhibiting the development of the flower bud and thus sexual reproduction (Ellis, 2020). This could be considered an asset for biological control, assuming the level of host-specificity is accepted. Finally, highest priority was attributed to three species prioritized based on the literature review and encountered during the field samplings conducted as part of this study (Figure 1.2).

Larvae of the sawfly *R. micans* were recorded at two pond sites in Belgium. Often sporadic, the native distribution of the species is restricted to humid temperate regions of central and northern Europe. In spring, adult females lay their eggs between the leaf sheaths of *I. pseudacorus*, showing a preference for waterside plants (Halstead & Henricot, 2010). The larvae can be found feeding on the plant for a 3-4 week period in early summer, after which they burrow into the soil to overwinter at the prepupal stage (Boevé et al., 2013). Despite the short-period of activity and low abundance observed in the field, individual feeding was associated with severe defoliation of the plant, and no damage was observed on the neighbouring vegetation. To date, the host range of this species is believed to be restricted to the Iridaceae family, with larval feeding reported on *I. pseudacorus*, *I. laevigata*, *I. pumila*, *I. spuria* and *I. × germanica* (Ellis, 2020).

Another priority species, the seed weevil *M. punctumalbum* was recorded at all sites visited during the flowering season. This species has a wide distribution in the central and western Palearctic region, where it is commonly found in relation with various *Iris* species both in the wild and in horticultural settings (Skuhrovec et al., 2017). In the field, adult weevils are observed attacking the reproductive

CHAPTER 1

structures of the plant, perforating the fruits with their rostrum laying their eggs inside the seeds. After hatching, the larvae consume the seeds of the plant and then pupate within the mature fruit. New adults exit the fruit in late summer, then crawl into the soil to overwinter (Gültekin & Korotyaev, 2012). *Mononychus* species develop exclusively on *Iris* spp., and *M. punctumalbum* is reported to use *I. pseudacorus*, *I. germanica* L. and *I. spuria* L. as full hosts (i.e. for feeding and larval development), although adult feeding has been recorded on at least 7 other species within the genus (Gültekin & Korotyaev, 2012; Ellis, 2020).

Finally, *A. nonstriata* was the most common and abundant species encountered during our surveys. Present both in Belgium and northern Italy, adult flea beetles were observed across all habitat types surveyed. This species is frequently found in wetland habitats across Europe and western Asia in association with *I. pseudacorus* (Hubble, 2010). Its presence in the field caused tangible impact on the plant, especially evident during early spring when the population reached the highest densities (Steinhausen, 2005). The peculiar furrow-like damage left behind by *A. nonstriata* on *I. pseudacorus*' leaves is easily recognizable, and no other plant species in the field showed signs that could be attributed to the beetle. Despite its alleged monophagy (Baviera & Biondi, 2015), records of *A. nonstriata* feeding on *Iris* spp. other than *I. pseudacorus* (e.g. Wojciechowicz-Zytko et al., 2016) warrant further consideration.

3.5. NOTES ON THE BIOLOGY OF *APHTHONA NONSTRIATA*

A population of the flea beetle *A. nonstriata* was reared in controlled conditions to observe its feeding behaviour and development on *I. pseudacorus*. In controlled conditions, oviposition by adult females occurred a few centimetres below the soil surface, in close proximity to the plant stem. After hatching, larvae were observed to mine the plant stem downward, boring the external parts of the rhizomes and feeding within it. At completed development, larvae exited the plant roots and pupated in the soil. New adults emerged gradually during the

CHAPTER 1

summer months, exerting a low but sustained feeding pressure on the plant. These adults are believed to overwinter at the end of the summer (Biondi, pers. comm.). However, under controlled conditions, summer adults were able to oviposit and conclude another generation, indicating that warmer temperatures might allow the species to be multivoltine.

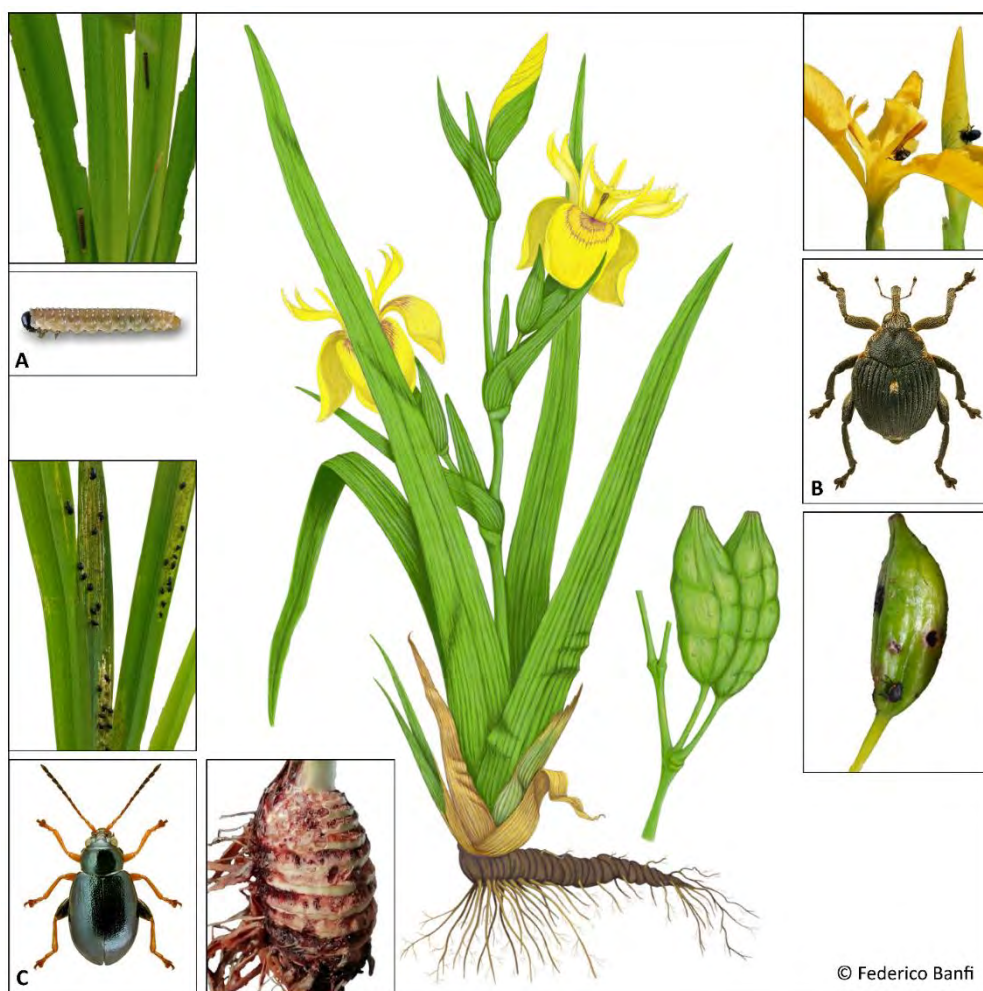


Figure 1.2 – Schematic representation of the damage caused to *Iris pseudacorus* by the three agents prioritized in the present study. **A:** *Rhadinoceraea micans* larvae (bottom panel) feeding on *I. pseudacorus* leaves (top panel); **B:** *Mononychus punctumalbum* (central panel) damaging the flowers of the plant (top panel) and new adults emerging from the seed capsules (bottom panel); **C:** *Aphthona nonstriata* adult (central panel) feeding on the leaves of the plant (top panel) and holes bored in the rhizomes by its larvae (right panel). Iconographies of *A. nonstriata* and *M. punctumalbum* by © Lech Borowiec, used with permission.

4. DISCUSSION

The native range of *I. pseudacorus* is vast, extending through most of Europe all the way to western Siberia, the Caucasus and the Middle East (Kavak, 2014). Our surveys, focused on wetland areas within the Atlantic and Continental bioregions of Europe, were intended to be a first, exploratory study of the insect fauna associated with this species. To our knowledge, field surveys to identify natural enemies of *I. pseudacorus* have not been conducted elsewhere within the native range of the plant. Given the geographical and temporal limitations of our study, further investigations should be carried across the species' native range to include other biogeographic regions (e.g. Mediterranean), climate types and habitats, as these are known to influence insect diversity (Goolsby et al., 2006). Research is already underway to elucidate the genetic origin of invasive *I. pseudacorus* populations and guide future studies. Our surveys found only a portion of the specialist natural enemies reported from the literature to attack *I. pseudacorus* within its native range. Among other factors, this could be attributed to i) the small geographic scale of the study; ii) asynchrony between sampling moments and period of activity of some species; or iii) the inability to access all parts of the plant at some locations. Nonetheless, the results of our SAC indicate that a significant proportion of the non-incidental herbivorous insects predicted to occur on *I. pseudacorus* at the localities visited were encountered during our surveys, confirming the validity of our sampling effort.

Most of the herbivorous insect species recorded on *I. pseudacorus* were incidental visitors, polyphagous feeders or species associated with other plants occurring at the site (e.g. the loosestrife flower weevil *Nanophyes marmoratus* Goeze and the rush sawfly *Dolerus ferrugatus* Serville). After excluding them from the prioritization process, three remaining species were evaluated as potential biocontrol agents. Preliminary surveys of the insect fauna associated with *I. pseudacorus* in South Africa found no sign of these species (Authors,

CHAPTER 1

unpublished data), but further analyses are needed to assess the presence of native analogues (Paynter et al., 2010).

The criteria applied for the prioritization of our agents have been employed in other studies (Paterson et al., 2014; Paynter et al., 2017; Egli & Olckers, 2020) and are known to be good preliminary indicators of chances of establishment, potential level of control and risk of non-target attack (Goolsby et al., 2006). The three prioritized candidates possess several characteristics suggesting their potential as biocontrol agents. Larvae of the sawfly *R. micans*, despite being active only for a short period of time, are individually highly damaging and able to completely defoliate their host. By feeding on the leaves of *I. pseudacorus*, these larvae are believed to sequester secondary plant metabolites which are stored in the haemolymph as defence against predators (Boevé et al., 2013). This characteristic suggests a high acquired specificity and lower predicted chances of attack from generalist predators in the invaded range (Sheppard, 2002). However, toxicity testing should be considered to determine if these metabolites could pose a risk to domestic animals or wildlife upon ingestion (Boevé et al., 2018). Additionally, *R. micans* larvae bear a strongly hydrophobic cuticle which allows them to move on the water's surface to reach new plants (Voigt et al., 2011). This detail, coupled with the oviposition preference shown by adult females, makes them an interesting option for release in extremely wet regions invaded by *I. pseudacorus* (e.g. wetlands of Argentina; Gervazoni et al., 2020), where other species might have a problem establishing (Van Driesche et al., 2009).

The weevil *M. punctumalbum* is the only candidate that attacks the reproductive structures of the plant. Its capacity to limit *I. pseudacorus* seed production (Skuhrovec et al., 2017) is regarded as a valuable asset for halting the spread of the weed in the invaded range. Although seed-destruction alone is not expected to cause a decline in the current density or distribution of the weed, long-term

CHAPTER 1

pressure by seed weevils was observed to reduce the hosts' reproductive fitness, hindering dispersal and limiting the insurgence of new invasions (Impson & Hoffmann, 2019). Future studies should investigate the seed production and field germination rates of *I. pseudacorus* across different ranges, in order to determine the potential of the weevil to control its spread (Clewley et al., 2012).

The flea beetle *A. nonstriata* was given highest priority in our study. The ability of root-feeding beetles to impact both the foliar and rhizome systems of their target is considered a fundamental resource to reduce invasiveness (Simelane, 2010). Six other *Aphthona* spp. have been used in the biological control of the leafy spurge complex (*Euphorbia* spp.) in North America, achieving medium to high levels of control (Bourchier et al., 2006; Winston et al., 2024). Root-boring larvae were observed to cause significant damage to the plant, sometimes leading to fungal and bacterial infections (Caesar, 2003). Further research should investigate potential interactions between *A. nonstriata* and plant pathogens, as these could contribute to the effectiveness of biocontrol (Willsey et al., 2017).

In some instances, cumulative stress exerted by multiple agents was observed to greatly increase the level of control achieved on the target weed (Hoffmann & Moran, 1998). The organisms presented in this study are deemed complementary in attacking different parts of the plant at different times, decreasing its competitiveness and increasing susceptibility to external stressors. All three candidates are thus advised for further investigations, prioritizing *A. nonstriata* based on its widespread distribution, broad period of activity, damage duality and suspected monophagy. In 2017, a population of this species was collected from the Snoekengracht Nature Reserve (Verrijck – Belgium) and imported to the quarantine facility of the Centre for Biological Control, Rhodes University (Makhanda, South Africa), where it is currently undergoing host-specificity testing.

5. CONCLUSION

Although emerging invaders such as *I. pseudacorus* are often not the focus of biological control programmes, there have been significant successes against incipient weeds in the past (Olckers, 2004). The effectiveness of these programmes highlights the potential of implementing biocontrol during early stages of invasion, minimizing the impact of the weed and the costs required for its control (Mgidi et al., 2007). International cooperation and joint research projects strongly contribute to the success of these programs by allowing research to be conducted concomitantly within the native and invasive range of the weed (Olckers, 2004). In this perspective, the current project fits within a broader collaboration between research institutes in Belgium (Vrije Universiteit Brussel), South Africa (Centre for Biological Control, Rhodes University), Argentina (Centro de Ecología Aplicada del Litoral and Fundación para el Estudio de Especies Invasivas) and New Zealand (Landcare Research). Because of the global extent of *I. pseudacorus* invasion, the results obtained through this collaboration will contribute substantially to the implementation of sustainable management plans aimed at alleviating the impact of this species on wetland ecosystems worldwide.

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SUPPLEMENTARY INFORMATION

Supplementary Table S1.1 – Sites surveyed for natural enemies of *Iris pseudacorus* in the native range. Common plant species co-occurring with *I. pseudacorus* at each site are listed from the most to the least abundant: *Ca* = *Carex* spp.; *Eq* = *Equisetum* spp.; *Le* = *Lycopus europaeus*; *Ls* = *Lythrum salicaria*; *Ma* = *Mentha aquatica*; *Pa* = *Phragmites australis*; *Tl* = *Typha latifolia*.

Country	Site coordinates	Habitat type (IUCN, 2012)	Sampling period	Co-occurring plant species
Belgium	50.835, 4.435	Artificial pond (15.2)	Jul 2017 Jun 2018	<i>Pa, Ls, Le</i>
Belgium	50.838, 4.435	Permanent stream (5.1)	Jul 2017 Jun 2018	<i>Eq, Ca, Tl</i>
Belgium	50.877, 4.466	Artificial pond (15.2)	Jul 2017 May 2018	<i>Ca, Tl, Ma, Ls</i>
Belgium	50.833, 4.836	Marsh (5.4)	Jul 2017 Apr 2018	<i>Ca, Ma, Eq, Tl, Pa</i>
Belgium	50.984, 5.100	Natural pond (5.7)	Jul 2017 May 2018	<i>Eq, Tl</i>
Belgium	51.088, 5.306	Permanent river (5.1)	Jul 2017 Apr 2018	<i>Eq, Tl</i>
Italy	45.885, 8.794	Swamp (5.7)	Aug 2017	<i>Pa, Ca</i>
Italy	45.800, 8.783	Lake shore (5.5)	Aug 2017	<i>Ca, Pa, Le</i>
Italy	45.912, 8.825	Lake shore (5.5)	Aug 2017	<i>Ls, Ma</i>
Italy	45.810, 8.607	Lake shore (5.5)	Aug 2017	<i>Ca, Pa, Ls, Le</i>
Italy	45.752, 8.678	Lake shore (5.5)	Aug 2017	<i>Pa, Ca, Le, Po, Eq</i>
Italy	45.898, 8.827	Swamp (5.7)	Aug 2017	<i>Pa, Ca</i>

CHAPTER 1

Supplementary Table S1.2 – List of insect species reported to attack *Iris pseudacorus* within its native range (adapted from McGrannachan & Barton, 2019). Information regarding geographic distribution, known host-range and damage type are provided (Spencer, 1990; Downes, 1955; Price et al., 2005; Kizil et al., 2008; Stekolshchikov, 2008; Bräu et al., 2010; Bukejs, 2010; Halstead & Henricot, 2010; Gültekin & Korotyaev, 2012; de Jong et al., 2014; Hoessini, 2014; Kučinić et al., 2014; Poelen et al., 2014; Baviera & Biondi, 2015; Whittaker, 2015; Pocock et al., 2015; Ricarte et al., 2017; Skuhrovec et al., 2017; Tabata & Kubota, 2017; Blackman & Eastop, 2020; de Prins et al., 2020; Ellis, 2020; Wagner, 2020). Species to be given further consideration, based on the available information, are indicated with an asterisk (*).

Species	Distribution	Host-range	Damage
THYSANOPTERA			
Thripidae			
<i>Frankliniella intonsa</i> Trybom	Widespread in Europe	Polyphagous	Mainly on flowers, larvae feed on the content of epidermal cells
* <i>Iridothrips iridis</i> Watson	Central and northern Europe, rare	Only recorded on <i>I. pseudacorus</i>	Feeds within submerged leaf sheaths of the plant
<i>Thrips flavus</i> von Schrank	Widespread in Europe	Polyphagous	Feed on flower buds
HEMIPTERA			
Aphididae			
<i>Aphis fabae</i> Scopoli	Widespread in Europe	Polyphagous	Large colonies can cause leaf deformation and death
* <i>Aphis newtoni</i> Theobald	Common in eastern Europe, but not abundant	Allegedly an <i>Iris</i> specialist, but recorded on <i>Gladiolus</i>	Forms ant-attended colonies feeding at the base of the leaves
<i>Dysaphis tulipae</i> Boyer de Fonscolombe	Cosmopolitan	Polyphagous, common on <i>Iris</i> species	Forms dense colonies on leaves, flowers and fruits
<i>Macrosiphum euphorbiae</i> Thomas	Cosmopolitan	Polyphagous, considered a pest of crops	Large infestations lead to stunted leaf growth, necrosis, etc.
<i>Myzus ascalonicus</i> Doncaster	Cosmopolitan	Polyphagous	Vector of plant viruses
Cicadellidae			
<i>Metalimnus formosus</i> Boheman	Widespread in Europe	Polyphagous on several riparian species	No information available

CHAPTER 1

Species	Distribution	Host-range	Damage
Cymidae			
<i>Cymus glandicolor</i> Hahn	Common in Europe and western Asia	Polyphagous, often associated with <i>Carex</i>	No information available
Myridae			
<i>Adelphocoris ticinensis</i> Meyer-Dur	Common in Europe and the Middle East	Polyphagous, associated with <i>Lythrum</i> and <i>Achillea</i>	No information available
Rhyparochromidae			
<i>Pachybrachius fracticollis</i> Schilling	Common in Europe and western Asia	Polyphagous, associated with <i>Carex</i>	No information available
COLEOPTERA			
Chrysomelidae			
* <i>Aphthona nonstriata</i> Goeze	Widespread in Europe and western Asia, very common and abundant	Allegedly host-specific, but recorded attacking several <i>Iris</i> species	Adults consume strips of leaf epidermis. Larvae descend to the rhizome where they continue as borers
<i>Plateumaris sericea</i> Linnaeus	Widespread in Europe and western Asia	Polyphagous, common on <i>Sparganium</i>	Adults found on flowers, larvae allegedly feed on the roots of the plant
Curculionidae			
* <i>Mononychus punctumalbum</i> Herbst	Widespread, common in Europe and western Asia	Allegedly host-specific, reported from at least 11 species of <i>Iris</i>	Adults attack flowers and fruits. Larvae feed and develop within the seeds of the plant
HYMENOPTERA			
Tenthredinidae			
* <i>Rhadinoceraea micans</i> Klug	Restricted to central and northern Europe, rare	Allegedly host-specific, reported from at least 5 species of <i>Iris</i>	Larvae are gregarious leaf feeders that may cause severe defoliation during early spring
LEPIDOPTERA			
Erebidae			
<i>Rhyarioides metelkana</i> Lederer	Restricted to central Europe	Polyphagous, hosts include <i>Caltha</i> and <i>Mentha</i> spp.	No information available
<i>Spilosoma urticae</i> Esper	Widespread in Europe	Polyphagous	Larvae are defoliators

CHAPTER 1

Species	Distribution	Host-range	Damage
Gelechiidae			
<i>*Monochroa divisella</i> Douglas	Restricted to central Europe, rare	Allegedly host-specific, considered a pest of the iris horticulture in Japan	Larvae infest the shoot apices, leading to the inhibition of flower bud development
Geometridae			
<i>Lycia zonaria</i> Schiffermuller	Central Europe	Polyphagous	Larvae are defoliators
Glyphipterigidae			
<i>Orthotelia sparganella</i> Thunberg	Widespread in Europe	Polyphagous, hosts include <i>Sparganium</i> , <i>Glyceria</i> and <i>Typha</i> spp.	Larvae are leaf and stem miners
Noctuidae			
<i>Acronicta rumicis</i> Linnaeus	Widespread in Europe	Polyphagous	Larvae are defoliators
<i>*Amphipoea crinanensis</i> Burrows	Restricted to northern Europe, rare	Allegedly host-specific, but little information available	Larvae are reported to feed inside the stems of the plant, but little information available
<i>Celaena leucostigma</i> Hubner	Widespread in Europe	Polyphagous on helophytes	Larvae are leaf miners and stem/roots borers
<i>Globia algae</i> Esper	Widespread in Europe	Polyphagous on helophytes	Larvae are leaf miners and stem borers
<i>Globia sparganii</i> Esper	Widespread in Europe	Polyphagous on helophytes	Larvae are leaf miner and stem borers
<i>Hydraecia micacea</i> Esper	Widespread in Europe	Polyphagous	Larvae are stem and root borers
<i>*Oxytripia orbiculosa</i> Esper	Disjunct in southern and eastern Europe, rare	Allegedly specific to <i>Iris pumila</i> , <i>I. pseudacorus</i> and <i>I. arenaria</i>	Larvae are leaf miner and later bore into the rhizomes
<i>Plusia festucae</i> Linnaeus	Widespread in Europe	Polyphagous	Larvae are defoliators
<i>Plusia putnami</i> Grote	Widespread in Europe	Polyphagous	Larvae are defoliators
<i>Xylena exsoleta</i> Linnaeus	Widespread in Europe	Polyphagous	No information available
<i>Xylena vetusta</i> Hubner	Widespread in Europe	Polyphagous	Larvae are defoliators

CHAPTER 1

Species	Distribution	Host-range	Damage
Nymphalidae			
<i>Coenonympha oedippus</i> Fabricius	Restricted to central Europe	Polyphagous, <i>Carex</i> and <i>Molina</i> spp. indicated as main hosts	Larvae are defoliators
Tortricidae			
<i>Aphelia paleana</i> Hübner	Widespread in Europe	Polyphagous	Larvae are defoliators
<i>Clepsis spectrana</i> Treitschke	Widespread in Europe	Highly polyphagous	Larvae are defoliators
<i>Paramesia gnomana</i> Clerck	Widespread in Europe	Polyphagous	Larvae are defoliators
<i>Sparganothis pilleriana</i> Schiffermuller	Widespread in Europe	Polyphagous	Larvae are defoliators
DIPTERA			
Agromyzidae			
* <i>Cerodontha iraeos</i> Robineau-Desvoidy	Widespread and common in Europe	Allegedly monophagous on <i>I. pseudacorus</i> , dubious records on other plants	Larvae create a short mine in the leaf blade, where pupation occurs. Larvae are solitary, damage is negligible
* <i>Cerodontha iridis</i> Hendel	Common in central Europe	Allegedly an <i>Iris</i> specialist, its use of <i>I. pseudacorus</i> as host is debated	Larvae create a short mine in the leaf blade, where pupation occurs. Gregarious, substantial damage
Cecidomyiidae			
* <i>Dicerura iridis</i> Kaltenbach	Central and Northern Europe	Oligophagous, reported on <i>I. pseudacorus</i> but also on <i>Acorus calamus</i>	Larvae feed through external digestion, causing a slight discolouration of the leaf tissue
Ceratopogonidae			
<i>Atrichopogon pollinivorus</i> Downes	Central Europe	Polyphagous, <i>Lonicera</i> spp. are indicated as primary hosts	Adults are pollen feeders. Little is known on the feeding habits of the larvae
Syrphidae			
<i>Eumerus strigatus</i> Fallen	Widespread in Europe	Polyphagous, considered a pest of flowering plants	Adults are pollinivorous, larvae feed on bulbs and rhizomes, causing rot

CHAPTER 1

Supplementary Table S1.3 – List of herbivorous insect species recorded on *Iris pseudacorus* during surveys in Belgium and northern Italy. Incidence expresses the number of sites at which the species was found. Abundance is the total number of individuals encountered during the survey. Position of the insect on the plant, life stage and known plant associations of each species are reported. Species prioritized as candidate biocontrol agents in this study are indicated with an asterisk (*).

Species	Incidence	Abundance	Position	Life stage	Known plant associations
ORTHOPTERA					
Tetrigidae					
<i>Tetrix subulata</i> L.	1	2	Leaves	Adult	Polyphagous (Bellmann & Luquet, 2009)
Tettigoniidae					
<i>Barbitistes alpinus</i> Fruhstorfer	1	1	Leaves	Adult	Polyphagous (Wagner, 2020)
<i>Leptophyes laticauda</i> Frivaldsky	1	2	Leaves	Nymph	Polyphagous (Wagner, 2020)
<i>Miramella alpina</i> Kollar	1	14	Leaves	Adult	Polyphagous (Asshoff & Hättenschwiler, 2005)
<i>Phaneroptera nana</i> Fieber	2	2	Leaves	Adult	Polyphagous (Davis, 1989)
HEMIPTERA					
Cicadellidae					
<i>Cicadella viridis</i> L.	3	6	Leaves	Adult	Polyphagous (Nickel & Remane, 2002)
Cixiidae					
<i>Cixius</i> sp.	1	1	Leaves	Adult	-
Coreidae					
<i>Coreus marginatus</i> L.	1	1	Fruits	Adult	Polyphagous (Ding et al., 2004)
Miridae					
<i>Adelphocoris</i> sp.	1	2	Leaves	Adult	-
Pentatomidae					
<i>Eysarcoris aeneus</i> Scopoli	1	1	Fruits	Adult	Polyphagous (Paik et al., 2007)

CHAPTER 1

Species	Incidence	Abundance	Position	Life stage	Known plant associations
<i>Halyomorpha halys</i> Stål	3	5	Fruits	Adult	Polyphagous (Leskey et al., 2012)
<i>Nezara viridula</i> L.	1	1	Fruits	Adult	Polyphagous (Todd, 1989)
<i>Palomena prasina</i> L.	2	3	Fruits	Adult	Polyphagous (Ozsahin et al., 2014)
COLEOPTERA					
Chrysomelidae					
<i>Altica palustris</i> Weise	1	1	Leaves	Adult	<i>Lythrum salicaria</i> (Fuss et al., 2005)
* <i>Aphthona nonstriata</i> Goeze	8	187	Leaves	Adult	<i>Iris pseudacorus</i> (Baviera & Biondi, 2015)
<i>Cassida viridis</i> L.	1	1	Leaves	Adult	Lamiaceae (Borowiec & Więtojańska, 2018)
<i>Chrysolina geminata</i> Paykull	1	1	Leaves	Adult	<i>Hypericum</i> spp. (Jolivet & Petitpierre, 1976)
<i>Chrysolina polita</i> L.	1	1	Leaves	Adult	Lamiaceae (Jolivet & Petitpierre, 1976)
<i>Donacia vulgaris</i> Zschach	1	2	Leaves	Adult	Ord. Poales (Kölsch & Pedersen, 2008)
<i>Exosoma</i> sp.	2	2	Flowers	Adult	-
<i>Gastrophysa viridula</i> De Geer	1	1	Leaves	Adult	<i>Rumex</i> spp. (Smith & Whittaker, 1980)
<i>Phyllobrotica quadrimaculata</i> L.	1	1	Leaves	Adult	<i>Scutellaria</i> spp. (Mitter et al., 1991)
<i>Plateumaris sericea</i> L.	2	3	Flowers	Adult	Polyphagous (Bukejs, 2010)
Curculionidae					
* <i>Mononychus punctumalbum</i> Herbst	6	91	Flowers Fruits	Adult	<i>Iris</i> spp. (Gültekin & Korotyaev, 2012)

CHAPTER 1

Species	Incidence	Abundance	Position	Life stage	Known plant associations
<i>Nanophyes marmoratus</i> Goeze	2	7	Flowers	Adult	<i>Lythrum salicaria</i> (Blossey & Schroeder, 1995)
Meloidae					
<i>Lytta vesicatoria</i> L.	2	2	Leaves	Adult	Polyphagous (Schlager, 2004)
Nitidulidae					
<i>Brassicogethes aeneus</i> Fabricius	4	8	Flowers	Adult	Brassicaceae (Ekbohm & Borg, 1996)
Tenebrionidae					
<i>Lagria hirta</i> L.	3	3	Leaves	Adult	Polyphagous (Szujewski, 2012)
HYMENOPTERA					
Tenthredinidae					
<i>Dolerus ferrugatus</i> Serville	2	4	Leaves	Larva	<i>Juncus effusus</i> (Borowski & Marczak, 2017)
<i>*Rhadinoceraea micans</i> Klug	2	23	Leaves	Larva	<i>Iris</i> spp. (Halstead & Henricot, 2010)
LEPIDOPTERA					
Crambidae					
<i>Anania hortulata</i> L.	1	1	Flowers	Adult	Polyphagous (Groenteman et al., 2017)
<i>Pyrausta aurata</i> Scopoli	1	1	Flowers	Adult	Lamiaceae (Dirimanov, 1970)
Noctuidae					
<i>Plusia festucae</i> L.	1	3	Leaves	Larva	Poaceae (Zahiri & Fibiger, 2008)
Tortricidae					
<i>Pandemis corylana</i> Fabricius	1	1	Leaves	Adult	Polyphagous (Ellis, 2020)

CHAPTER 1

CHAPTER 2

Predicting the effect of climate change on the invasion risk and biocontrol potential of the invasive macrophyte *Iris pseudacorus* L.

Adapted from: Minuti, G., Coetzee, J.A., & Stiers, I. (2023). Contrasting effects of climate change on the invasion risk and biocontrol potential of the invasive *Iris pseudacorus* L. between Northern and Southern Hemisphere. *Biological Control*, 184, 105290.

ABSTRACT

Iris pseudacorus is both a prized ornamental and an invasive aquatic plant that tends to grow dense monospecific stands, displacing local riparian vegetation and altering the hydrology of freshwater ecosystems. Originally from Europe, this species has historically invaded North America, China and Japan, and more recently spread through Argentina, South Africa and Australasia, where it is now a target for biological control. Field surveys within its native range have led to the selection of three candidate biocontrol agents. Prioritizing the best candidates for different regions constitutes a critical step, which could save significant time and resources before further cost-intensive experimental studies are conducted. Climate change is seldom taken into consideration in the prioritization process. In this regard, climatic suitability can be used to model the potential distributions of weeds and their candidate agents, both in space and time, thus allowing to identify areas at risk of invasion and predict where agents will be able to establish long-term. Accordingly, the objectives of this work were i) to predict *I. pseudacorus* invasions and range shifts in the context of climate change; ii) to identify wetland areas most at risk of invasion under present and future climatic conditions; and iii) to prioritize the best suite of candidate biocontrol agents for different invaded ranges, worldwide. To do so,

CHAPTER 2

we modelled the present and future (2040-2060) climatic suitability of *I. pseudacorus* and its candidate agents using the software MaxEnt. Our results highlight a clear distinction between predictions for the Northern and Southern Hemispheres. In North America and eastern Asia, the area climatically suitable for *I. pseudacorus* is expected to increase and shift northwards. As for its biocontrol agents, very low suitability is predicted across these regions, further decreasing under future climatic conditions. On the other hand, climatically suitable areas for the plant in South America, southern Africa and Australasia are predicted, on average, to reduce in response to climate change. A decrease in climatic suitability is also expected for its candidate biocontrol agents which, however, would still maintain a significant range overlap with their host. These results can be used to prioritize areas most at risk of invasion and identify which combination of candidates could potentially provide the best level of control across different invaded ranges.

1. INTRODUCTION

Biological invasions are recognized as a major driver of biodiversity loss and environmental degradation (Pyšek et al., 2020), especially in freshwater ecosystems (Santamaría, 2002). In particular, invasive alien aquatic plants (IAAPs) are considered a major threat to these habitats (Vilà et al., 2009). Yellow flag, *Iris pseudacorus* L. (Iridaceae), is a perennial helophyte native to Europe, North Africa and western Asia. Internationally sold as an ornamental plant, this species has historically invaded North America, China and Japan, and more recently spread through Argentina, South Africa and Australasia, where it is now a target for biological control (Hill & Coetzee, 2017; McGrannachan and Barton, 2019; Gervazoni et al., 2020). Its dense growth, paired with its ability to adapt to a wide range of environmental conditions, allows it to invade a variety of natural and human-disturbed habitats, displacing native communities and altering the hydrology of freshwater ecosystems (Sutherland, 1990; USDA, 2013). So far,

CHAPTER 2

three candidate biocontrol agents have been selected (Minuti et al., 2021; Chapter 1): the flea beetle *Aphthona nonstriata* Goeze (Coleoptera: Chrysomelidae); the seed weevil *Mononychus punctumalbum* Herbst (Coleoptera: Curculionidae); and the sawfly *Rhadinoceraea micans* Klug (Hymenoptera: Tenthredinidae).

Biological control has been used, with remarkable levels of success, against other aquatic weeds (Hill & Coetzee, 2017; Simberloff, 2021). As such, it holds the potential to be a sustainable solution to the problem, especially when other methods have been ineffective (Hussner et al., 2017). However, the costs and timeframes of developing and implementing biological control are not insignificant. For this reason, prioritizing the best suite of biocontrol agents and identifying areas of release are fundamental steps in order to maximize the return on investment (Mukherjee et al., 2021). A fundamental prerequisite for the effective biological control of an invasive species is the ability of its biocontrol agent(s) to establish and thrive within the invaded range. Matching environmental conditions between native range and release locations increases the chances of survival of the agents and its ability to reproduce, with a direct effect on biological control (Hoelmer & Kirk, 2005). Mismatches are largely, although not solely, driven by climatic factors (Harms et al., 2021). For instance, differences in precipitation and temperature values are reported to affect up to 31% of weed biocontrol programs (Harms et al., 2020). In this context, climate change is expected to significantly influence the distribution of invasive weeds, biocontrol agents, and their interactions (Bellard et al., 2013; Sun et al., 2020; Robinson et al., 2020; Essl et al., 2020). Species distribution models (SDMs) have been increasingly used to identify areas at risk of invasion and, more recently, to prioritize areas for collection and release of biocontrol agents (Mukherjee et al., 2011; Sutton 2019; Minuti et al., 2021; Chapter 1). These methods provide useful tools to project the current climatic niche of an

CHAPTER 2

organism not only in space but also in time, and can thus be used to analyze climate matching across future climate scenarios (Sun et al., 2017).

Accordingly, the objectives of this work were to use SDMs to i) model *I. pseudacorus* climatic niche and predict its range shifts in response to climate change; ii) identify the most vulnerable wetlands of international importance; and iii) prioritize the best combination of candidates for each invaded range (i.e. North America, Eastern Asia, South America, South Africa and Australasia) based on their current and future climatic suitability.

2. MATERIALS & METHODS

2.1. OCCURRENCE RECORDS AND VARIABLES SELECTION

Worldwide occurrence records for each species were sourced from the Global Biodiversity Information Facility portal (GBIF 2020, 2021a,b,c). Each dataset was cleaned using the package “*CoordinateCleaner*” (Zizka et al., 2019) in R software 3.6.1 (R Core Development Team, 2019). This allows to remove duplicate records and records assigned to grids centroids, urban areas and biodiversity institutions (i.e. museums, botanical gardens and universities). The datasets were then visually inspected in QGIS 3.14 (QGIS Development Team, 2020) to identify uncertain records (i.e. geographic outliers, sea-stranded occurrences, doubtful identifications). Uncertain records that could not be confirmed or corrected were excluded from the datasets. Occurrences were then filtered to minimize spatial autocorrelation using the packages ‘*ecospat*’ (Di Cola et al., 2017) and ‘*spThin*’ (Aiello-Lammens et al., 2015). First, in order to avoid pseudo-replication, occurrences were thinned to retain a single record per grid cell (5 arc minutes resolution). Then, a spatial autocorrelation analysis was computed and, based on its results, the data were filtered at distances which minimized the influence of spatial autocorrelation (Boria et al., 2014). This cleaning and filtering method is effective in minimizing the influence of

CHAPTER 2

geographic and environmental biases on the data (Sutton, 2019; Minuti et al., 2022). The final datasets consisted of 1140 records for *I. pseudacorus*, 334 records for *A. nonstriata*, 314 records for *M. punctumalbum*, and 180 records for *R. micans* (Supplementary Figure S2.1).

The predictors used for modelling the present and future climatic suitability of each species were selected amongst the bioclimatic variables developed for the BIOCLIM package (Booth et al., 2014), downloaded from WorldClim v2.1. (Fick & Hijmans, 2017). All 19 bioclimatic layers were downloaded at 5 arc minute spatial resolution for historical climate (1970-2020) and for the projected time period 2040-2060. In order to make future projections more reliable, we considered three Global Circulation Models (GCMs) from the most recent version of the World Climate Research Program Coupled Model Intercomparison Project (WCRP-CMIP6). These were the Model for Interdisciplinary Research on Climate (MIROC6; Shiogama et al., 2019), the Institut Pierre-Simon Laplace Climate Model (IPSL-CM6A-LR; Boucher et al., 2018), and the Hadley Centre Global Environment Model (HadGEM3-GC31-LL; Ridley et al., 2019). These computer-driven mathematical models use current knowledge of dynamical systems paired with predictions of future conditions to accurately simulate the general circulation of planetary atmosphere and oceans in space and time. For each GCM, we considered two Shared Socio-Economic Pathways (SSPs), the ‘middle of the road’ (SSP2 – 4.5) and the ‘fossil-fueled development’ (SSP5 – 8.5) scenarios (Riahi et al., 2017).

After computing pairwise Pearson’s correlation coefficients among predictor variables, a selection was made to minimize multicollinearity (Petitpierre et al., 2016). Amongst highly correlated variables ($|r| > 0.75$), those deemed to hold higher biological relevance for our species, such as temperature extremes and seasonal precipitation values, were retained. The reduced set of variables consisted of: minimum temperature of the coldest month (bio6); mean

CHAPTER 2

temperature of the warmest quarter (bio10); annual precipitation (bio12); precipitation seasonality (bio15); and precipitation of the warmest quarter (bio18). The latter has been subject to some criticism due to the presence of discontinuities across its interpolated surface (Escobar et al., 2014). However, such discontinuities are rare on a global scale, and usually limited to the Equatorial regions (Booth, 2022). Upon visual inspection of the layer in QGIS 3.14, no abrupt changes were observed within the areas of interest (i.e. regions already invaded or at risk of invasion by *I. pseudacorus*). Accordingly, the variable was retained for modelling our species. An exception was made for *M. punctumalbum*, for which bio12 and bio18 showed high correlation ($r > 0.8$), and bio18 was therefore excluded. The meaningfulness of these predictors was further assessed by visual inspection of their response curves during model tuning (Supplementary Figure S2.2).

2.2. MODEL CALIBRATION AND EVALUATION

Climatic suitability models were computed using the software MaxEnt 3.4.1 (Phillips et al., 2017). This program was chosen for its efficiency and numerous applications in presence-background modelling of invasive plant species and biological control agents (Mukherjee et al., 2011; Trethowan et al., 2011; Sutton 2019; Minuti et al., 2022).

After testing optimal model settings using the ‘*SDMtune*’ package (Vignali et al., 2021), MaxEnt settings were set as follows: convergence = 10^5 ; maximum number of iterations = 500; prevalence = 0.5; regularization multiplier = 1; features = lqph. For each model, 10 bootstrap replicates were computed, each randomly allocating 70% of the occurrence records to model training (calibration) and the remaining 30% to model testing (evaluation). The average of all replicates was used for further analyses. Output maps were produced using logistic suitability scores ranging from 0 (low suitability) to 1 (high suitability). Relative contribution of each predictor variable was assessed by

CHAPTER 2

their permutation importance (Phillips et al., 2017). After modelling, the Multivariate Environmental Similarity Surface (MESS) was inspected to assess coverage of environmental gradients upon model projection and identify areas of uncertainty (Elith et al., 2010). The respective ‘out-of-range’ environmental variables (i.e. limiting factors) were extrapolated from the dissimilarity maps (MoD) provided by the program output (Elith et al., 2011).

As a first metric of model performance, the area under the receiver operating characteristic curve (AUC) was used. AUC values range from 0.5, identifying models that are no better than random, to 1, indicating excellent model performance (Fielding & Bell, 1997). Model overfitting was evaluated by comparing the average omission rate (OR) of the model with theoretical expectations. OR is the proportion of test localities predicted to fall outside the projected suitable area once the model is converted into a binary prediction by means of a specific threshold (Boria et al., 2014). Overfit models tend to have higher omission rates than those predicted by the relative threshold, and MaxEnt provides various methods for model thresholding (Merow et al., 2013). In this study, the 10th percentile training presence omission rate (OR10), which sets the threshold at a value that rejects only the lowest 10% of possible predicted occurrences, was used (Boria et al., 2014). Accordingly, models with an OR10 significantly higher than the expected 0.1 were considered overfit (Sutton 2019; Martin et al., 2020). Additionally, as AUC statistic has been subject to some criticism (Lobo et al., 2008), the true presence ratio (TPR) and the Continuous Boyce Index (CBI; mean $\pm$ SD) were employed as additional measures of model accuracy (Hirzel et al., 2006). These threshold-independent metrics are considered more reliable when it comes to validating predictions and transferability of presence-background models (Manzoor et al., 2018). These metrics were calculated using the package ‘*ecospat*’ (Di Cola et al., 2017) in R software 3.6.1.

CHAPTER 2

2.3. SPECIES DISTRIBUTION MODELLING

The climatic suitability model for *I. pseudacorus* was trained using a combination of native and invaded range occurrences, as this allows the inclusion of the environmental variability acquired by the species in newly colonized areas and produces more reliable approximations than using native or invaded range records alone (Beaumont et al., 2009; Trethowan et al., 2011; Rodríguez-Merino et al., 2019; Martin et al., 2020). Following the method of Hill & Terblanche (2014), background climatic data were drawn from a customized mask generated by selecting Köppen-Geiger climate zones (Beck et al., 2018) containing at least one occurrence record for the species (Minuti et al., 2022). As none of the candidate biocontrol agents for *I. pseudacorus* is recorded outside of its native European range, only native occurrences were available to use for model training. The distribution of these organisms is less documented, thus, to select a background representative of where the species could potentially occur, a geographic mask of Europe was used (de Jong et al. 2014).

Each model was calibrated using current climatic layers and then projected over the entire world surface. Ten replicates were computed for each future climate projection (MIROC6-SSP2; MIROC6-SSP5; IPSL-SSP2; IPSL-SSP5; HadGEM-SSP2; HadGEM-SSP5), for a total of 60 future models. These were then averaged to produce a single output, expressing suitability on a continuous logistic scale. In order to quantify the geographic area predicted as suitable for each organism, continuous rasters were converted into binary maps by applying the relative OR10 minimum suitability threshold (Sutton, 2019; Martin et al., 2020). In this sense, raster cells with suitability values lower than the OR10 threshold were considered unsuitable, whereas cells with values higher than the OR10 threshold were considered suitable. Finally, the suitable area for each species across various invaded ranges was calculated using the ‘*raster*’ package (Hijmans, 2017).

CHAPTER 2

2.4. INVASION RISK AND BIOCONTROL POTENTIAL

To visualize the effect of climate change on the invasion risk of *I. pseudacorus* worldwide, continuous suitability maps for present and future climatic conditions were overlapped, and difference values extracted for each raster cell, using the *raster calculator* in QGIS. The resulting difference map was then overlapped with an environmental layer containing all wetlands of international importance at a global scale (<https://rsis Ramsar.org/>). Present climatic suitability, future climatic suitability, and difference values were extracted for each Ramsar site using the *point sampling tool* in QGIS. Ramsar sites located within regions climatically unsuitable for *I. pseudacorus* were excluded, whereas the remaining sites were categorized based on their present suitability (Low: 0.3 – 0.5; Medium: 0.5 – 0.7; High: > 0.7) and the prediction for increasing or decreasing climatic suitability under future climatic conditions.

To assess the potential of the candidate biocontrol agents to establish and control *I. pseudacorus* in various regions of interest, binary climatic suitability maps were overlapped in QGIS. Present climatic suitability maps for each candidate biocontrol agent were overlapped with that of *I. pseudacorus* to identify invaded areas where the species could establish and potentially control its target under current climatic conditions. The same procedure was adopted for future suitability maps. Finally, a difference operator was used to calculate the change in climatic suitability and potential climatic niche overlap between present and future projections. All outputs were clipped to the extent of areas currently invaded by *I. pseudacorus* in North America, South America, South Africa, eastern Asia and Australasia. The total area of present and future climatic suitability and niche overlap was calculated for each region of interest using the *raster package* (Hijmans, 2017).

3. RESULTS

3.1. MODEL EVALUATION AND VARIABLES CONTRIBUTION

The average AUC values of models trained upon current climate data were 0.710 ± 0.012 for *I. pseudacorus*; 0.898 ± 0.010 for *A. nonstriata*; 0.875 ± 0.014 for *M. punctumalbum*; and 0.956 ± 0.009 for *R. micans* (Supplementary Table S2.1). The model discrimination capacity, as measured by its AUC, expresses the probability that a presence selected at random will have higher predicted suitability than a randomly selected absence (or background). However, a good model is not expected to discriminate beyond the discrimination that the species itself makes in the environment. Therefore, models of generalist species tend to have lower discrimination capacity than those of specialist species (such is the case for *I. pseudacorus*), which does not imply that they are worse models (Sillero et al., 2021). There was no substantial difference between AUC values of present and future climate scenarios, nor across different future climate models, showing agreement among models projected upon different climatic conditions. For all models computed, omission rates (OR10) were consistently close to the predicted threshold of 0.1 (indicating no overfitting), true presence ratio (TPR) approached 90%, and CBI values were consistently higher than 0.93, confirming the robustness of model predictions.

The relative contribution of the climatic variables used as predictors varied slightly between the models for *I. pseudacorus* and its candidate biocontrol agents (Supplementary Table S2.1). Overall, minimum temperature of the coldest month (bio6) was the most influential variable for all four species. For *I. pseudacorus*, this was followed by annual precipitation (bio12), mean temperature of the warmest quarter (bio10) and precipitation of the warmest quarter (bio18), whereas precipitation seasonality (bio15) showed the lowest importance (Table 1). On the other hand, models built for the three candidate biocontrol agents showed little contribution of precipitation-related variables

CHAPTER 2

(bio12 and bio18), and were instead highly influenced by temperature-related ones (bio6 and bio10). An exception is observed for precipitation seasonality (bio15), which showed high contribution to models for *A. nonstriata* and *M. punctumalbum*, but little to no contribution to the models built for *R. micans*. Overall, there was no considerable difference in variable contribution to models based on present and future climate scenarios. The Multivariate Environmental Similarity Surface (MESS) showed good coverage of the environmental gradients during model projection, supporting the transferability of our models in time and space (Supplementary Figure S2.3).

3.2. INVASION RISK AND BIOCONTROL POTENTIAL

3.2.1. North America

The results of our models predict a northward expansion in the climatically suitable area for *I. pseudacorus* in North America (Figure 2.1). Ramsar sites located in southern United States ($n = 25$; 756,502 ha) show a decrease in suitability under future climatic conditions, whereas for those in northern US and Canada ($n = 27$; 347,318 ha), suitability is expected to increase (Figure 2.1A). The total area identified as suitable for *I. pseudacorus* will increase by 31.1% (1,515,271 ha) according to our models, with areas of gained suitability in Alaska, northern US and Canada, and regions of lost suitability in south-eastern United States (Figure 2.1B). At the same time, areas of climatic suitability for the three candidate biocontrol agents are predicted to decrease (Figure 2.1C-E), with *A. nonstriata* and *R. micans* showing little to no suitability across North America, and *M. punctumalbum* losing 43.9% (1,054,086 ha) of its currently suitable area in eastern US and Canada. Accordingly, the overlap between the climatic niche of *I. pseudacorus* and that of its candidate biocontrol agents combined is predicted to decrease from 45.25% (2,494,915 ha) to 20.98% (1,455,821 ha) under future climatic conditions (Figure 2.1F).

CHAPTER 2

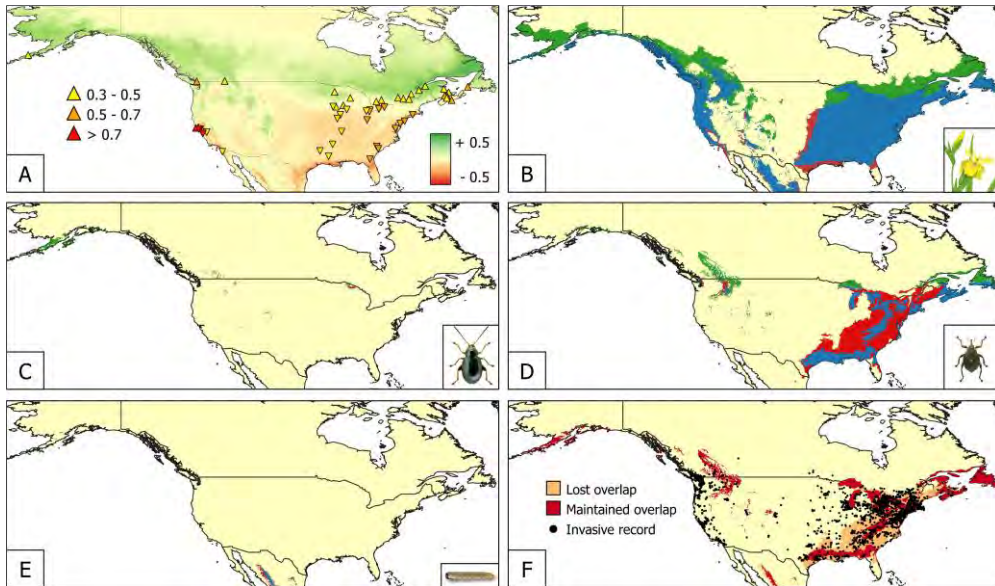


Figure 2.1 – Invasion risk, climatic suitability and biocontrol potential for *Iris pseudacorus* in North America. **(A)** Net change in suitability for *Iris pseudacorus* between present and future climatic conditions (green indicates an increase in suitability, red a decrease). Ramsar sites at risk of invasion are represented by triangles: colors represent their current suitability score; upward and downward direction indicate if suitability is predicted to increase or decrease in the future. Binary suitability maps for **(B)** *Iris pseudacorus*, **(C)** *Aphthona nonstriata*, **(D)** *Mononychus punctumalbum*, and **(E)** *Rhadinoceraea micans* show areas where suitability will be gained (green), lost (red), or maintained (blue) under future climatic conditions. **(F)** Climatic niche overlap between *I. pseudacorus* and its candidate biocontrol agents based on present and predicted future climatic conditions. Black dots indicate present occurrence records of the weed.

3.2.2. Eastern Asia

A similar northward shift in climatic suitability for *I. pseudacorus* is predicted across eastern Asia (Figure 2.2). The majority ($n = 67$; 1,244,394 ha) of Ramsar sites currently at risk of invasion show decreased suitability under future climatic conditions, while the remaining ($n = 26$; 234,171 ha) show increased suitability (Figure 2.2A). Nonetheless, most of the region is expected to remain highly suitable for the weed (Figure 2.2B). No suitability is predicted for the flea beetle *A. nonstriata*, both under current and future climate (Figure 2.2C). For the weevil *M. punctumalbum*, the limited suitable area currently predicted in northern Japan is expected to be completely lost due to climate change (Figure

CHAPTER 2

2.2D). Finally, for the sawfly *R. micans*, currently suitable areas in the Yunnan province of south-western China are predicted to decrease by 37.7% (174,441 ha) in the future (Figure 2.2E). Overall, the climatic niche overlap between *I. pseudacorus* and its candidate biocontrol agents is predicted to decrease from 17% (551,783 ha) to 10.5% (353,276 ha) in China and from 37.2% (136,684 ha) to 0.7% (2,590 ha) in Japan (Figure 2.2F).

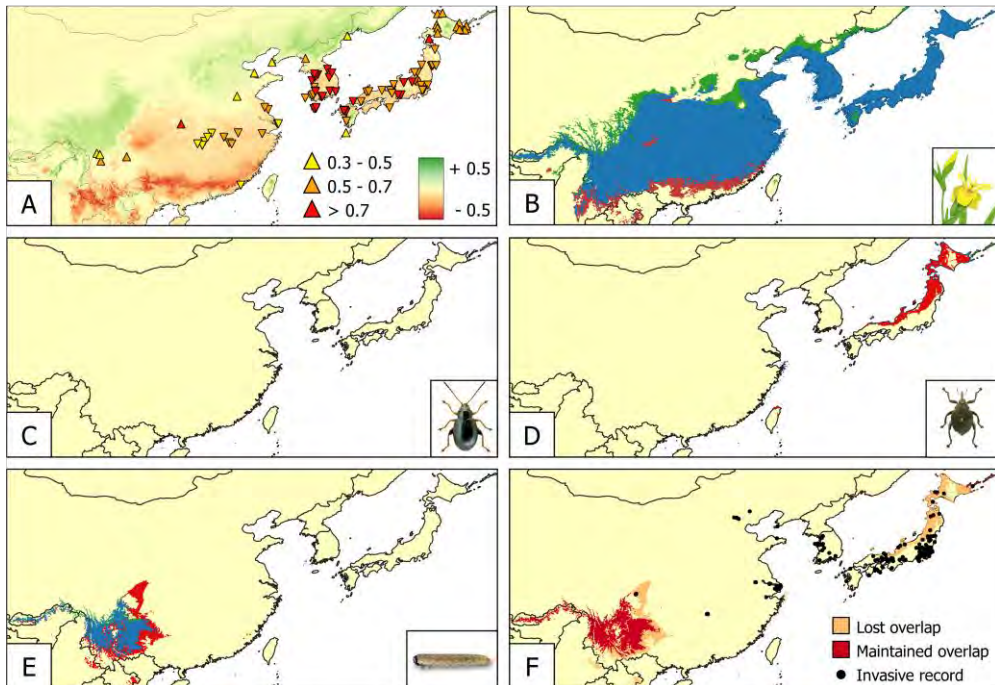


Figure 1.2 – Invasion risk, climatic suitability and biocontrol potential for *Iris pseudacorus* in Eastern Asia. **(A)** Net change in suitability for *Iris pseudacorus* between present and future climatic conditions (green indicates an increase in suitability, red a decrease). Ramsar sites at risk of invasion are represented by triangles: colors represent their current suitability score; upward and downward direction indicate if suitability is predicted to increase or decrease in the future. Binary suitability maps for **(B)** *Iris pseudacorus*, **(C)** *Aphthona nonstriata*, **(D)** *Mononychus punctumalbum*, and **(E)** *Rhadinoceraea micans* show areas where suitability will be gained (green), lost (red), or maintained (blue) under future climatic conditions. **(F)** Climatic niche overlap between *I. pseudacorus* and its candidate biocontrol agents based on present and predicted future climatic conditions. Black dots indicate present occurrence records of the weed.

CHAPTER 2

3.2.3. South America

The predicted climatic suitability for *I. pseudacorus* in South America shows an overall decrease in southern Brazil, Uruguay and north-east Argentina, and an expansion towards the southern border of Argentina and Chile (Figure 2.3).

Out of the 17 Ramsar sites currently at risk of invasion, 15 (2,138,866 ha) are expected to decrease in suitability in the future, and only 2 (6,257 ha) to increase (Figure 2.3A). The total area identified as suitable for the weed is predicted to decrease by 14% (245,352 ha), with only small regions of southern Chile becoming newly suitable to the weed (Figure 2.3B). The same pattern is observed for its candidate biocontrol agents. Climatically suitable areas are predicted to decrease by 42.6% (198,494 ha) for *A. nonstriata* (Figure 2.3C), by 11% (108,416 ha) for *M. punctumalbum* (Figure 2.3D), and by 82.2% (155,691 ha) for *R. micans*, with limited regions of newly acquired suitability in southern Argentina and Chile (Figure 2.3E). Despite this decrease, only a minor effect is expected on the overlap between the climatic niche of the weed and that of its candidate biocontrol agents, from 40.8% (716,135 ha) under current climate to 35.1% (533,464 ha) under future climatic conditions (Figure 2.3F).

CHAPTER 2

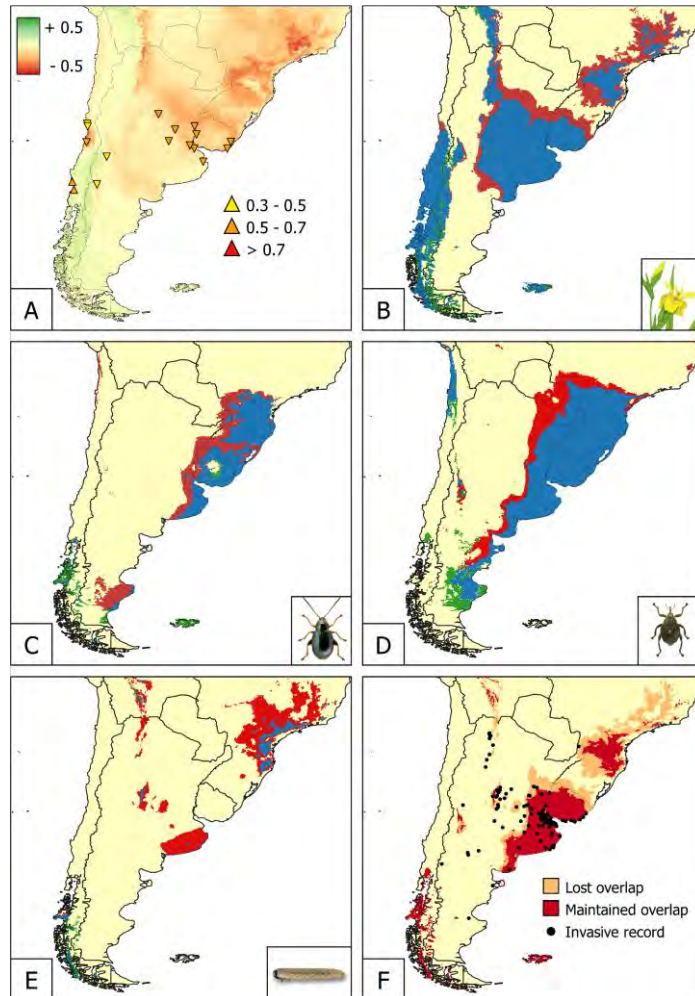


Figure 2.2 – Invasion risk, climatic suitability and biocontrol potential for *Iris pseudacorus* in South America. **(A)** Net change in suitability for *Iris pseudacorus* between present and future climatic conditions (green indicates an increase in suitability, red a decrease). Ramsar sites at risk of invasion are represented by triangles: colors represent their current suitability score; upward and downward direction indicate if suitability is predicted to increase or decrease in the future. Binary suitability maps for **(B)** *Iris pseudacorus*, **(C)** *Aphthona nonstriata*, **(D)** *Mononychus punctumalbum*, and **(E)** *Rhadinoceraea micans* show areas where suitability will be gained (green), lost (red), or maintained (blue) under future climatic conditions. **(F)** Climatic niche overlap between *I. pseudacorus* and its candidate biocontrol agents based on present and predicted future climatic conditions. Black dots indicate present occurrence records of the weed.

CHAPTER 2

3.2.4. South Africa

The models predict an overall decrease in climatic suitability for *I. pseudacorus* in southern Africa (Figure 2.4). Ramsar sites in the Western Cape, North-West and Limpopo regions (n = 11, 15,210 ha) are expected to decrease in suitability, whereas for those located in the Eastern Cape, Free State, Gauteng, Mpumalanga and Kwa-Zulu Natal regions (n=7, 265,908 ha), the models predict increased suitability (Figure 2.4A). A net decrease of 20.9% (152,767 ha) is predicted in the total area suitable for *I. pseudacorus*, with no areas of gained suitability (Figure 2.4B). A similar pattern is expected for its candidate biocontrol agents. Suitable areas for *A. nonstriata*, *M. punctumalbum*, and *R. micans* are predicted to decrease by 44.5% (28,796 ha), 16% (36,781 ha) and 43.3% (122,661 ha), respectively (Figure 2.4C-E). Nonetheless, the overlap in the climatic niche of the weed and its biocontrol agents shows little change, from 41.7% (314,014 ha) to 35.1% (210,178 ha) under future climatic conditions (Figure 2.4F).

CHAPTER 2

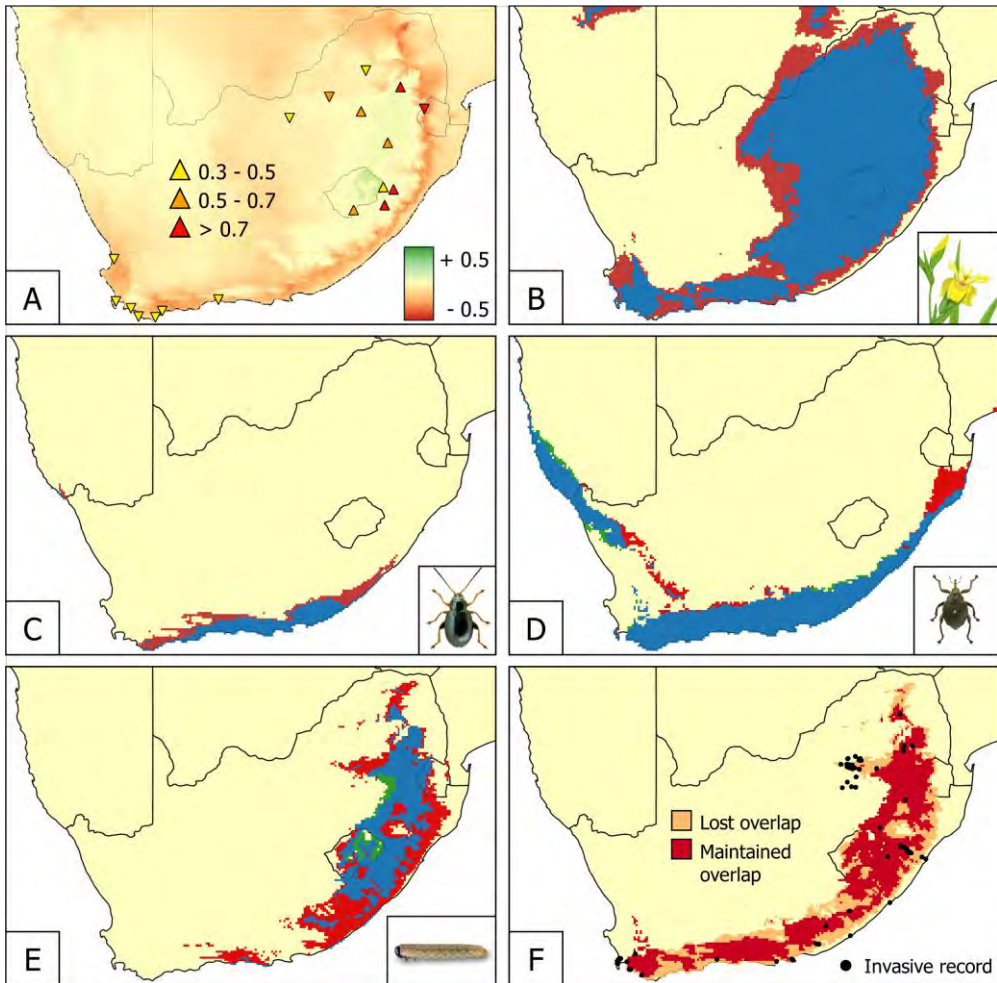


Figure 2.3 – Invasion risk, climatic suitability and biocontrol potential for *Iris pseudacorus* in South Africa. **(A)** Net change in suitability for *Iris pseudacorus* between present and future climatic conditions (green indicates an increase in suitability, red a decrease). Ramsar sites at risk of invasion are represented by triangles: colors represent their current suitability score; upward and downward direction indicate if suitability is predicted to increase or decrease in the future. Binary suitability maps for **(B)** *Iris pseudacorus*, **(C)** *Aphthona nonstriata*, **(D)** *Mononychus punctumalbum*, and **(E)** *Rhadinoceraea micans* show areas where suitability will be gained (green), lost (red), or maintained (blue) under future climatic conditions. **(F)** Climatic niche overlap between *I. pseudacorus* and its candidate biocontrol agents based on present and predicted future climatic conditions. Black dots indicate present occurrence records of the weed.

CHAPTER 2

3.2.5. Australasia

A contrasting scenario is predicted for Australasia, with the climatic suitability for *I. pseudacorus* showing a decrease in Australia and an increase, albeit small, in New Zealand (Figure 2.5). Accordingly, most Ramsar sites currently at risk of invasion in Australia show decreased suitability ($n = 27$, 610,528 ha), with some exceptions located in south-eastern Australia and Tasmania ($n = 10$, 16,922 ha), for which suitability is predicted to increase (Figure 2.5A). In contrast, in New Zealand a decrease is predicted for 3 Ramsar sites (27,858 ha) and an increase for 4 sites (39,328 ha). The total area identified as suitable for the weed is expected to decrease by 28.4% (469,202 ha) in Australia and increase by 1.75% (4,495 ha) in New Zealand (Figure 2.5B). The same pattern is predicted for *A. nonstriata* and *M. punctumalbum*, the former showing a 30.6% (107,868 ha) decrease in Australia and a 16.7% (34,320 ha) increase in New Zealand, and the latter a 25.7% (554,839 ha) decrease in Australia and a 27.6% (45,172 ha) increase in New Zealand (Figure 2.5C-D). Finally, the suitable area for *R. micans* is predicted to decrease in both ranges, by 55.4% (186,230 ha) in Australia and by 13.3% (20,376 ha) in New Zealand (Figure 2.5E). The climatic niche overlap between *I. pseudacorus* and its candidate biocontrol agents is predicted to decrease slightly from 85.9% (1,380,690 ha) to 83.5% (1,008,914 ha) in Australia, and increase from 80.7% (207,449 ha) to 89.9% (230,217 ha) in New Zealand (Figure 2.5F).

CHAPTER 2

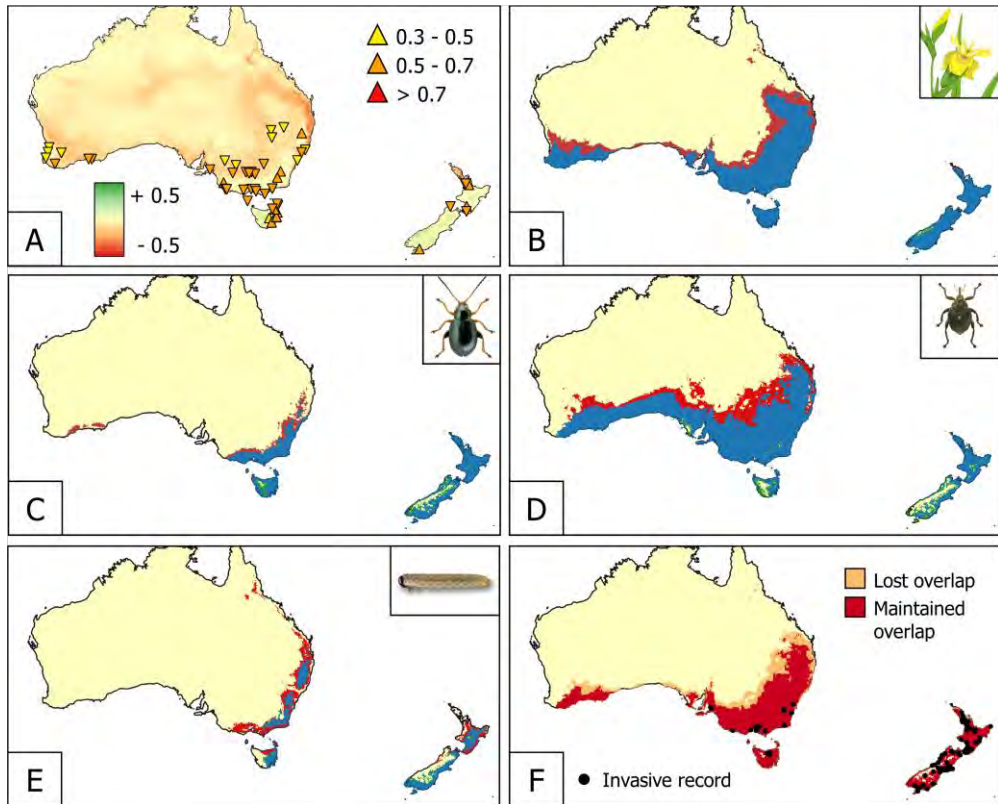


Figure 2.4 – Invasion risk, climatic suitability and biocontrol potential for *Iris pseudacorus* in Australasia. **(A)** Net change in suitability for *Iris pseudacorus* between present and future climatic conditions (green indicates an increase in suitability, red a decrease). Ramsar sites at risk of invasion are represented by triangles: colors represent their current suitability score; upward and downward direction indicate if suitability is predicted to increase or decrease in the future. Binary suitability maps for **(B)** *Iris pseudacorus*, **(C)** *Aphthona nonstriata*, **(D)** *Mononychus punctumalbum*, and **(E)** *Rhadinoceraea micans* show areas where suitability will be gained (green), lost (red), or maintained (blue) under future climatic conditions. **(F)** Climatic niche overlap between *I. pseudacorus* and its candidate biocontrol agents based on present and predicted future climatic conditions. Black dots indicate present occurrence records of the weed.

4. DISCUSSION

Modelling the environmental suitability of IAAPs can significantly improve the cost-effectiveness of management strategies by directing response efforts towards the most vulnerable areas and helping spread-limitation campaigns (Cordeiro et al., 2020). This holds especially true for weed biological control, where climatic mismatches have been known to affect the outcome of over one-third of reviewed programs (Harms et al., 2020). The present work is one of the few studies where the bioclimatic niche of the weed and its biocontrol agents have been modelled together (Trethowan et al., 2011; Sun et al., 2017), accounting for the potential effects of climate change (Sun et al., 2020). Our results highlight a clear distinction between invaded regions in the Northern and Southern Hemispheres. In North America and eastern Asia, the area climatically suitable for *I. pseudacorus* is expected to shift northwards, maintaining and even increasing its current extent. This could be attributed to the rise in temperatures expected in the near future, making southernmost regions too dry for the plant and at the same time helping it overcome cold stress limitations in northern regions. On the other hand, climatically suitable areas for the plant in South America, southern Africa and Australasia – with the exception of southern Chile and New Zealand – are predicted to reduce significantly in the future. This range contraction in the Southern Hemisphere has been predicted for other invasive species (e.g. Kriticos et al., 2010) and could be attributed to an increase in heat and dry stresses, excluding the plant from areas currently experiencing a humid temperate climate (Beck et al., 2018). It is important to note that, despite an average decrease worldwide, the predicted suitability for most Ramsar sites is predicted to remain well above the minimum suitability threshold used in this study. Therefore, these environments should be considered at risk of invasion by *I. pseudacorus*, at least from a climatic perspective. Paired with other local factors (e.g. habitat, soil type, distance from current invasions, etc.), this

CHAPTER 2

information can be used to prioritize wetland areas where management and prevention strategies should be deployed in the near future.

For the three candidate biocontrol agents, our models predict limited to no suitability in North America and eastern Asia, further decreasing under future climatic conditions. This pattern undermines the potential use of these biocontrol agents across invaded regions of the Northern Hemisphere. Such doubts are only reinforced by host-specificity issues linked to the plethora of *Iris* species native to these regions, many of which are cultivated for ornamental use (Cantarelli, 2022). The opposite is true for the Southern Hemisphere, where the decrease in climatic suitability for both *I. pseudacorus* and its candidate biocontrol agents could result in a maintained, or even increased (i.e. Australasia) climatic niche overlap in the future. This is an encouraging outcome for the prospective use of biological control in these regions. However, local differences in predictions are observed. The seed weevil *M. punctumalbum* shows the greatest predicted climatic suitability and overlaps with *I. pseudacorus* across all invaded regions, making it a priority candidate according to climatic suitability ranking (Sun et al., 2017). It is followed by the flea beetle *A. nonstriata*, whose climatic suitability and overlap are most relevant within humid temperate climates of South America, coastal South Africa and Australasia. These findings confirm previous studies highlighting the climatic mismatch between *A. nonstriata* and *I. pseudacorus* across the Southern Hemisphere (Minuti et al., 2022). Finally, suitability models of the sawfly *R. micans* predict a limited climatic overlap with its host plant, restricted to specific regions of China and north-eastern South Africa. Naturally, the models obtained are only as good as the data used for training them, and it is impossible to completely exclude the presence of biases in the open-source datasets used (Beck et al., 2014). *Mononychus punctumalbum* is known to have a wide distribution across the western Palearctic region, covering a variety of climate types from northern Spain, throughout central Europe, to drier regions of north-

CHAPTER 2

eastern Turkey (Gültekin & Korotyaev, 2012). This adaptation to a wide range of geographical and climatic conditions mirrors our findings and supports the prioritization of this candidate biocontrol agent. On the other hand, to our knowledge, specific literature on the distribution of *A. nonstriata* is lacking. The species is reported as present in most European countries (PESI, 2022a), but the majority of available records belong to humid temperate regions of central and northern Europe (Schmitt & Rönn, 2011; GBIF, 2021a). Assuming an under-reporting of the species across other European climate zones, our results would be an underestimation of the potential climatic suitability of *A. nonstriata*, which could therefore perform better than expected across *I. pseudacorus* invaded range. Finally, the sawfly *R. micans* is endemic to central and northern Europe, being reported as absent in most Mediterranean and eastern European countries (PESI, 2022b). This selectivity for highly humid climates could explain the limited suitability predicted by our models. The resulting climatic mismatch between *R. micans* and *I. pseudacorus* suggests that the former should be given low priority as a candidate biocontrol agent, compared with the other species examined (Harms et al., 2021). As observed in other studies (Trethowan et al., 2011; Sun et al., 2017), such a result provides a useful pre-evaluation, cutting the time and costs that would have been related to the pursuit of this agent.

Maximum entropy modelling as a technique requires the user to provide presence records of a species and the raster layers to use as predictors (Phillips et al., 2017). The models presented in this study employ only climatic predictors, as the objectives were to identify climatic mismatches between species and observe the effect of climate change on their suitability. As such, these models identify regions of similar climatic conditions to where the studied species are known to occur at the present time, and should not be interpreted as predicting actual limits to their potential distribution (Pearson et al., 2007). The accuracy of these models can be further improved by adding environmental and anthropic layers as explanatory variables (Cordeiro et al., 2020).

CHAPTER 2

Alternatively, mechanistic models can be built using information about the species' functional traits and physiological tolerances to environmental conditions (Kearney & Porter, 2009). Combining correlative and mechanistic models of species distribution can be used to refine predictions and provide independent evidence for the effect of climate change (Kearney et al., 2010).

The success of a biocontrol program is linked with the ability of biocontrol agents not only to survive but also to naturally disperse from the introduction site to new invasions (Pérez de la O et al., 2020). Our models show high connectivity among climatically suitable regions in the invaded range, especially across the Southern Hemisphere. This provides a great opportunity to prioritize release sites with optimal environmental conditions for agent survival, reproduction, and dispersal (Robertson et al., 2008). We acknowledge that local biotic and abiotic environmental factors will also play an important role in the ability of a candidate to effectively establish and control its host plant. Further research is needed to shed light on specific conditions that might favor or hinder these organisms, in order to improve the selection of release sites. The models presented here constitute a cost-effective pre-evaluation of the invasion potential of the weed and the biocontrol potential of its candidate agents. As such, they will serve as a useful tool for planning both large-scale and local management strategies, as well as reducing the time and costs of conducting further experimental studies on less-than-optimal candidates.

5. CONCLUSION

The present study produced climatic suitability models of the wetland invader *I. pseudacorus* and its three candidate biocontrol agents on a global scale. These can be used to identify areas at risk of invasion, to direct sampling and management efforts, as well as to avoid climate mismatches when planning biocontrol releases. Furthermore, by integrating current and future climatic scenarios, these models allow to predict the effect of climate change on the

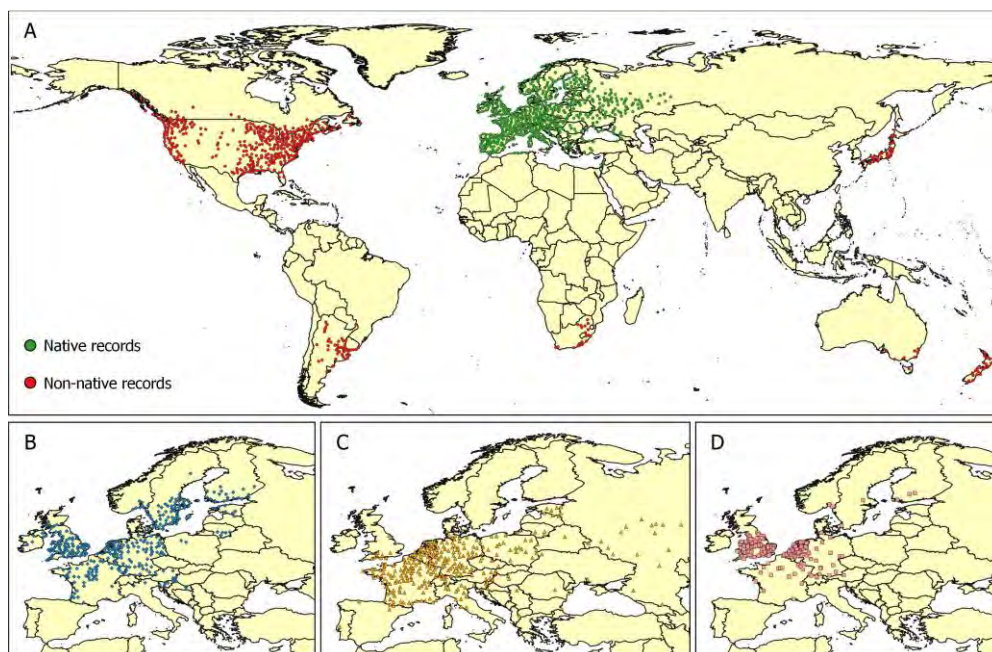
CHAPTER 2

potential distribution and climatic niche overlap of these four species. The results highlight a contrasting effect of climate change on the climatic suitability of *I. pseudacorus* and its candidate biocontrol agents between Northern and Southern Hemisphere. Regions of North America and eastern Asia are predicted to become increasingly suitable for *I. pseudacorus*, especially at higher latitudes, with little to no overlap with its candidate biocontrol agents. On the other hand, climatic suitability for the weed is predicted to decrease across the Southern Hemisphere, and as a result the potential overlap with its biocontrol agents will remain stable or even expand (i.e. Australasia). From a methodological perspective, this work constitutes one of the few examples where the climatic suitability of an invasive species and its candidate biocontrol agents have been modelled together, accounting for climate change. This methodology makes use of open-source datasets and does not require high computational power, and should therefore be considered as a first step when evaluating the climatic suitability of other invasive species targeted by biological control.

ACKNOWLEDGEMENTS

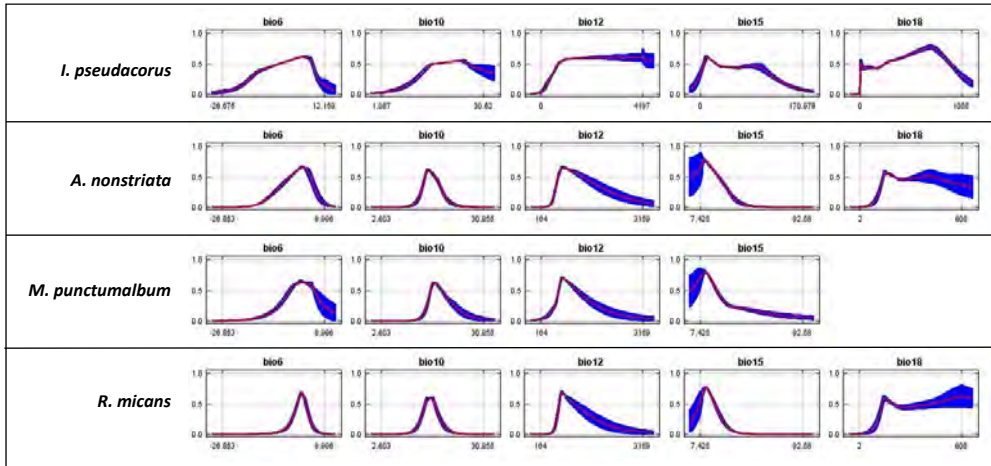
The PhD project of G.M. is funded by a strategic basic research fellowship of the Research Foundation – Flanders (FWO SB71). We thank the Vrije Universiteit Brussel (BAS 53 and BAS 42) and the Centre for Biological Control (Rhodes University) for logistic support. *Iris pseudacorus* biological control research in South Africa is funded through the Department of Environmental Affairs, Natural Resource Management Programs (previously the Working for Water Program). The South African Research Chairs Initiative of the Department of Science and Technology and the National Research Foundation of South Africa provided additional funding. Any opinion, finding, conclusion or recommendation expressed in this material is that of the authors, and the National Research Foundation does not accept any liability in this regard.

SUPPLEMENTARY INFORMATION

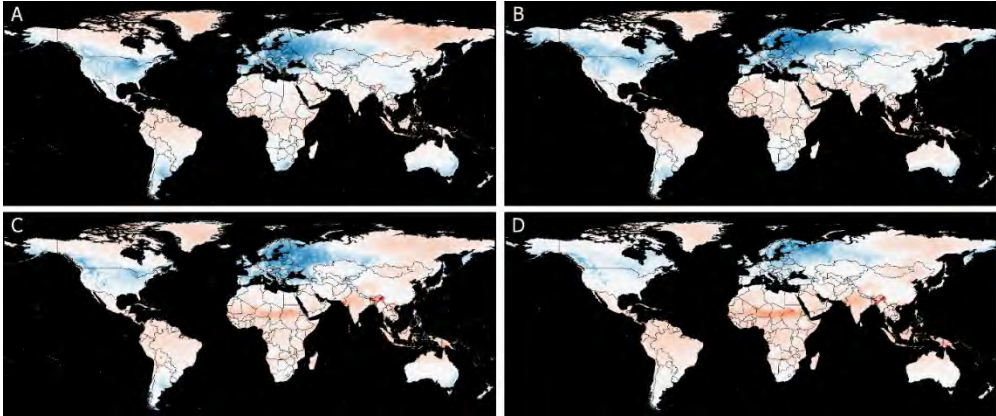


Supplementary Figure S2.1 – Occurrence records (after data cleaning and filtering) used for modelling their present and future climate suitability of **A)** *Iris pseudacorus*; **B)** *Aphthona nonstriata*; **C)** *Mononychus punctumalbum*; and **D)** *Rhadinoceraea micans* (see GBIF, 2020 and GBIF, 2021a,b,c for original datasets).

CHAPTER 2



Supplementary Figure S2.2 – Response curves of the climatic suitability models computed during the study. The curves show how the predicted climatic suitability (Y axis) changes in response to the variation of each environmental variable (X axis), independently of the other variables. The curves show the mean response (red) and the standard deviation (blue) of 10 replicate Maxent runs (Phillips et al., 2017).



Supplementary Figure S2.3 – Multivariate Environmental Similarity Surface (MESS) between the range of variables used for training the climatic suitability models and the range encountered in the projection layers. Areas having one or more environmental variables outside the range present in the training data are shown in red, so predictions in those areas should be treated with caution. **A)** Model for *I. pseudacorus*, projected on current climate; **B)** Model for *I. pseudacorus*, projected on future climate; **C)** Model for the biocontrol agents, projected on current climate; **D)** Model for the biocontrol agents, projected on future climate. Note that the regions of interest for our study show high environmental similarity with the training data (blue to white color), indicating reliable model transferability (Elith et al., 2010).

CHAPTER 2

Supplementary Table S2.1 – Details of the climatic suitability models computed in MaxEnt for *I. pseudacorus* and its candidate biocontrol agents. The total number of filtered occurrence records (n) is divided into training (70%) and testing (30%). Each future model is named after the relative Global Circulation Model and Shared Socio-economic Pathway used as prediction (see section 2.1 for details). Test AUC values close to 0.5 indicate a model no better than random, with values approaching 1 indicating perfect predictive performance (Fielding & Bell, 1997). As alternative evaluation metrics, the Continuous Boyce Index (CBI) and true presence ratio (TPR) are reported. CBI values range from -1 to 1, with positive values indicating model predictions consistent with the distribution of the presence data, values close to zero meaning that model predictions are no different from random, and negative values showing counter predictions (i.e. predicting no occurrence in areas where actual presence is recorded; Hirzel et al., 2006). The mean ($\pm$ SD) 10th percentile omission rate (OR10) was used to estimate overfitting, where values significantly different than the predicted 0.10 indicate an overfit model. The relative OR10 threshold was used to set the minimum suitability values to convert each model into binary predictions. The permutation importance (%) of the predictor variables is reported, and variables with the highest contribution to each model are indicated in bold. Variables are defined as follows: bio6 = minimum temperature of the coldest month; bio10 = mean temperature of the warmest quarter; bio12 = annual precipitation; bio15 = precipitation seasonality; bio18 = precipitation of the coldest quarter.

Sp	Model	Test AUC ($\pm$ SD)	CBI ($\pm$ SD)	TPR (%)	OR10 train/test	OR10 Threshold	bio6	bio10	bio12	bio15	bio18
<i>I. pseudacorus</i> (n = 1140)	Present climate	0.712 $\pm$ 0.012	0.996 $\pm$ 0.002	88.9	0.099/0.112	0.324	56.26	9.69	19.01	5.45	9.59
	HadGEM3_ssp245	0.723 $\pm$ 0.012	0.996 $\pm$ 0.003	89.8	0.098/0.099	0.323	52.07	8.91	28.84	3.72	6.47
	IPSL_ssp245	0.709 $\pm$ 0.012	0.994 $\pm$ 0.002	89.6	0.099/0.102	0.331	52.47	13.37	24.73	3.32	6.10
	MIROC6_ssp245	0.713 $\pm$ 0.012	0.994 $\pm$ 0.004	89.6	0.099/0.107	0.328	50.38	12.94	26.93	2.43	7.31
	HadGEM3_ssp585	0.719 $\pm$ 0.012	0.995 $\pm$ 0.002	89.2	0.098/0.110	0.314	51.17	14.29	20.98	2.86	10.70
	IPSL_ssp585	0.717 $\pm$ 0.012	0.995 $\pm$ 0.004	89.1	0.099/0.099	0.322	58.33	12.31	19.15	2.01	8.20
	MIROC6_ssp585	0.719 $\pm$ 0.012	0.997 $\pm$ 0.001	89.4	0.099/0.106	0.316	45.84	14.10	29.05	3.37	7.63
<i>A. nonstriata</i> (n = 334)	Present climate	0.898 $\pm$ 0.010	0.983 $\pm$ 0.007	88.4	0.097/0.116	0.249	58.47	24.02	3.10	9.05	5.37
	HadGEM3_ssp245	0.904 $\pm$ 0.009	0.982 $\pm$ 0.007	88.2	0.097/0.110	0.263	63.32	23.37	3.14	6.38	3.77
	IPSL_ssp245	0.905 $\pm$ 0.010	0.989 $\pm$ 0.005	89.0	0.097/0/106	0.262	58.73	22.15	3.21	13.07	2.85
	MIROC6_ssp245	0.896 $\pm$ 0.010	0.984 $\pm$ 0.006	88.5	0.097/0.114	0.262	59.55	21.81	3.34	10.08	5.22
	HadGEM3_ssp585	0.898 $\pm$ 0.010	0.983 $\pm$ 0.008	89.0	0.097/0.103	0.245	54.76	32.17	2.51	7.11	3.46
	IPSL_ssp585	0.901 $\pm$ 0.010	0.986 $\pm$ 0.003	89.3	0.097/0.112	0.272	57.86	21.61	2.48	14.01	4.03
	MIROC6_ssp585	0.900 $\pm$ 0.010	0.984 $\pm$ 0.007	89.7	0.098/0.102	0.261	61.27	22.44	2.25	9.09	4.96

CHAPTER 2

Supplementary Table S2.1 (Continued) – Details of the climatic suitability models computed in MaxEnt for *I. pseudacorus* and its candidate biocontrol agents. The total number of filtered occurrence records (n) is divided into training (70%) and testing (30%) Each future model is named after the relative Global Circulation Model and Shared Socio-economic Pathway used as prediction (see section 2.1 for details). Test AUC values close to 0.5 indicate a model no better than random, with values approaching 1 indicating perfect predictive performance (Fielding & Bell, 1997). As alternative evaluation metrics, the Continuous Boyce Index (CBI) and true presence ratio (TPR) are reported. CBI values range from -1 to 1, with positive values indicating model predictions consistent with the distribution of the presence data, values close to zero meaning that model predictions are no different from random, and negative values showing counter predictions (i.e. predicting no occurrence in areas where actual presence is recorded; Hirzel et al., 2006). The mean ($\pm$ SD) 10th percentile omission rate (OR10) was used to estimate overfitting, where values significantly different than the predicted 0.10 indicate an overfit model. The relative OR10 threshold was used to set the minimum suitability values to convert each model into binary predictions. The permutation importance (%) of the predictor variables is reported, and variables with the highest contribution to each model are indicated in bold. Variables are defined as follows: bio6 = minimum temperature of the coldest month; bio10 = mean temperature of the warmest quarter; bio12 = annual precipitation; bio15 = precipitation seasonality; bio18 = precipitation of the coldest quarter.

Sp	Model	Test AUC ($\pm$ SD)	CBI ($\pm$ SD)	TPR (%)	OR10 train/test	OR10 Threshold	bio6	bio10	bio12	bio15	bio18
<i>M. punctulatum</i> (n = 314)	Present climate	0.875 $\pm$ 0.014	0.980 $\pm$ 0.009	89.8	0.097/0.105	0.196	44.65	35.20	5.46	14.70	-
	HadGEM3_ssp245	0.877 $\pm$ 0.014	0.985 $\pm$ 0.009	88.2	0.099/0.125	0.212	43.48	44.29	4.82	7.41	-
	IPSL_ssp245	0.882 $\pm$ 0.013	0.983 $\pm$ 0.007	91.5	0.099/0.084	0.210	41.47	44.73	3.23	10.58	-
	MIROC6_ssp245	0.877 $\pm$ 0.014	0.982 $\pm$ 0.014	88.6	0.098/0.114	0.204	44.29	37.57	2.61	15.54	-
	HadGEM3_ssp585	0.866 $\pm$ 0.015	0.981 $\pm$ 0.011	86.9	0.099/0.116	0.202	42.59	43.31	2.82	11.28	-
	IPSL_ssp585	0.883 $\pm$ 0.013	0.986 $\pm$ 0.006	90.7	0.099/0.086	0.203	42.03	41.87	2.33	13.76	-
	MIROC6_ssp585	0.878 $\pm$ 0.014	0.985 $\pm$ 0.005	88.6	0.098/0.110	0.198	42.56	40.32	6.69	10.43	-
<i>R. micans</i> (n = 180)	Present climate	0.956 $\pm$ 0.009	0.946 $\pm$ 0.022	88.7	0.094/0.111	0.214	78.86	9.82	3.45	1.21	6.67
	HadGEM3_ssp245	0.961 $\pm$ 0.007	0.931 $\pm$ 0.034	88.5	0.093/0.115	0.229	80.30	12.35	2.12	1.43	3.81
	IPSL_ssp245	0.960 $\pm$ 0.008	0.935 $\pm$ 0.051	88.7	0.091/0.100	0.222	77.96	14.08	3.27	0.16	4.54
	MIROC6_ssp245	0.957 $\pm$ 0.008	0.943 $\pm$ 0.027	88.7	0.091/0.111	0.217	77.94	11.73	4.79	0.57	4.97
	HadGEM3_ssp585	0.959 $\pm$ 0.008	0.962 $\pm$ 0.012	90.2	0.090/0.104	0.236	77.71	9.47	6.31	0.61	5.89
	IPSL_ssp585	0.958 $\pm$ 0.008	0.994 $\pm$ 0.034	85.7	0.091/0.133	0.256	79.14	10.78	4.40	0.70	4.99
	MIROC6_ssp585	0.961 $\pm$ 0.007	0.952 $\pm$ 0.028	87.2	0.092/0.119	0.248	83.38	7.81	2.78	0.28	5.75

CHAPTER 2

CHAPTER 3

Evaluating the host-range of *Aphthona nonstriata* Goeze (Coleoptera: Chrysomelidae), a candidate biocontrol agent for *Iris pseudacorus* L.

Minuti, G., Cantarelli, M., Ngxande-Koza, S., Gervazoni, P., Stiers, I. & Coetzee, J.A. [Manuscript in preparation].

ABSTRACT

Iris pseudacorus L. (Iridaceae) is considered invasive in Canada, the United States, Argentina, Uruguay, South Africa, China, Japan, Australia and New Zealand. Due to its resilience to mechanical and chemical control methods, a classical biological control program was initiated against this species. The flea beetle *Aphthona nonstriata* Goeze (Coleoptera: Chrysomelidae) was prioritized as a candidate biocontrol agent. The next step of the program consists in testing its host-specificity against a tailored list of test plants. This is not an easy task, as the family Iridaceae contains ca. 2500 species, of which over 300 within the *Iris* genus alone. Plants to be included in host-specificity testing are often selected on a regional or national level, following heterogeneous and often unclear protocols. In the current study, we propose a standardized but customizable scoring system to prioritize plants to be included in host-specificity testing. We then use the system to compile an exhaustive list of taxa within the family Iridaceae to be considered for the host-specificity testing of candidate biocontrol agents for *I. pseudacorus*, on a global scale. Finally, we present preliminary results of the ongoing host-specificity testing of *A. nonstriata* and discuss the future perspectives on the use of this candidate agent. Of over 400 taxa evaluated, ca. 25% were selected as priority, providing a

CHAPTER 3

robust backbone for the host-specificity testing of *I. pseudacorus* across different invaded regions of the world. The results of the host-specificity trials highlighted the capacity of *A. nonstriata* to feed on a variety of genera of Iridaceae, challenging previous claims of monophagy. However, no second generation was recorded on any species outside the *Iris* genus. Due to the particular phylogeography of the family Iridaceae, and in particular the isolation of the genus *Iris* to the Northern Hemisphere, it is clear that higher risks would be associated with releasing the beetle across invaded regions of North America and Eastern Asia. On the other hand, despite the absence of native *Iris* species from the Southern Hemisphere, the plethora of native Iridaceae poses a challenge to conduct thorough host-specificity testing. Nonetheless, our preliminary results on *A. nonstriata* strengthens the hypothesis of the beetle being an *Iris* specialist, and thus not posing a significant threat to native plants.

1. INTRODUCTION

Classical biological control of weeds (i.e. invasive alien plants) has a rich history marked by numerous successes (McFadyen, 2000; Schwarzländer et al., 2018; Winston et al., 2024). The method owes its safety record (Hinz et al., 2020) to strict testing protocols (Wapshere, 1974; van Klinken, 1999, Schaffner, 2001), that are kept up-to-date by new technologies and ongoing refinements (e.g. Briese & Walker, 2002; Heard & Van Klinken 2004; Lesieur et al., 2020; Hinz et al., 2024). Host-specificity testing is considered the pillar of biocontrol, essential to circumscribe the fundamental host range of a biocontrol agent and predicting its potential field host range once introduced in a new environment (see van Klinken, 1999 for details). During host-specificity tests, the biocontrol agent is exposed to several plant species to identify its feeding preferences, potential damage and development on non-host plants. Which species and how many to test for host-specificity has been a recurrent debate among biocontrol practitioners and policy makers, with the latter often advocating for the

CHAPTER 3

inclusion of safeguard species – i.e. species of economic or ecological importance that are unrelated to the target plant (Briese & Walker, 2008). The plant selection and testing procedure is usually the most time-consuming part of the evaluation of a biocontrol agent. In order to be approved for release, an agent must not pose any significant risk to other plant species present in the region. Unfortunately, although nature has no political borders, legislation often dictates that these testing take place on a country-by-country basis. For the purpose, abridged plant test lists can be used to reduce time and effort, while still providing reliable results (Lesieur et al., 2020). However, these test lists are specific to a particular geographic area, and do not provide enough information to evaluate the release of the biocontrol agent elsewhere. This is particularly problematic for widespread weeds, for which testing of a biological control agent then needs to be repeated in several countries according to the local flora.

An example of such a global invader is *Iris pseudacorus* L. (Iridaceae), commonly known as yellow flag. Native to Europe, North Africa and Western Asia, this herbaceous perennial helophyte has been exported worldwide through the ornamental trade and is now considered invasive in Canada, the United States, Argentina, Uruguay, South Africa, China, Japan, Australia and New Zealand (Cody, 1961; Kadono, 2004; Howell, 2008; Masciadri, et al., 2010; Ugarte et al., 2011; USDA, 2013; Jaca & Mkhize, 2015; Gervazoni et al., 2020; Xiong et al., 2023). Due to its resilience to mechanical and chemical control methods, biological control is being evaluated as an alternative for this species across the Southern Hemisphere (Hill & Coetzee, 2017, McGrannachan & Barton, 2019; Gervazoni et al., 2020). Following field surveys across the native range of the plant in Europe, three candidate biocontrol agents were shortlisted for testing. Among these candidates, the flea beetle, *Aphthona nonstriata* Goeze (Coleoptera: Chrysomelidae) was given highest priority (Minuti et al., 2021; Chapter 1). This is a congeneric of *Aphthona* spp. used in the biocontrol programme against leafy spurge (*Euphorbia esula* L., Euphorbiaceae) in North

CHAPTER 3

America, which produced very successful results, reducing plant density by up to 95% (Larson et al., 2008). Although little is known on the biology and life-history of *A. nonstriata*, there are some reports of it feeding on other species within the *Iris* genus (e.g. Wojciechowicz-Zytko et al., 2016). The *Iris* genus alone comprises over 300 species widely distributed throughout the Northern Hemisphere. Furthermore, many of these species hold significant economic value for the ornamental trade, both across their native and non-native range (POWO, 2023). Going one step further, the family Iridaceae contains ca. 2500 species spread across 68 genera worldwide, with various centers of diversity, including southern Africa (Goldblatt, 2000). It is therefore not a surprise that selecting plants to test for host-specificity of *A. nonstriata* could be quite a colossal feat. Given that biological control is *per se* a discipline reliant on international effort and collaboration, this example highlights the need for a consolidated protocol for widespread invaders.

In the current study, we compiled an exhaustive list of taxa within the family Iridaceae to be considered for the host-specificity testing of *A. nonstriata* and other candidate biocontrol agents for *I. pseudacorus*, worldwide. Using this list, we propose a scoring system to be used to prioritize species for host-specificity testing, in order to facilitate programs dealing with widely invasive plants such as our target. We then present preliminary results of the ongoing host-specificity testing of *A. nonstriata* and discuss the future perspectives on the use of this candidate biocontrol agent.

2. MATERIALS & METHODS

2.1. SELECTION OF TEST PLANTS FOR HOST SPECIFICITY TESTING

A scoring system was developed to help with the selection of plant species to be tested for host-specificity. To do so, a modernized version of the centrifugal phylogenetic method (Wapshere, 1974; Briese & Walker, 2002) was followed.

CHAPTER 3

This takes into consideration the phylogenetic relatedness, geographic overlap, and ecological similarity of each plant to the target plant species (Briese, 2005). Additionally, because numerous species within the family Iridaceae hold an important ornamental value and/or are threatened within some regions of the world, these criteria were considered during the prioritization process. In the present study, phylogenetic relatedness was used as a backbone to develop and validate the results of the scoring system. Phylogenetic relatedness was based on taxonomy and adjusted to the available molecular studies (Goldblatt, 2000; Wilson, 2006; Goldblatt & Manning, 2008; Wilson, 2009; Wilson, 2011; Wheeler & Wilson, 2014; Feng et al., 2022; Boltenkov & Artyukova, 2023). In doing so, two separate lists were constructed: a first based on the phylogenetic relationships between *I. pseudacorus* and its congeneric species, and a second summarizing the generic relationships within the Iridaceae family (i.e. considering each genus as a separate taxon). These were then grouped into clades based on the “degree of phylogenetic separation” from the target plant (*sensu* Briese, 2005).

Geographic overlap, ecological similarity to the target plant species, conservation status and economic importance were then used to develop a score for each taxon. Bibliographical information was gathered through different online resources (eFloras, 2008; IUCN, 2022; Atlas of Living Australia, 2023; Pacific Bulb Society, 2023; USDA, 2023; GBIF, 2023; NatureServe, 2023; SANBI, 2023; POWO, 2023 and references therein) and climatic distributions compared using the most up-to-date Köppen-Geiger climate classification (Beck et al., 2018; Supplementary Figure S3.1). Since geographic overlap and ecological similarity are considered primary criteria for risk assessment based on the centrifugal phylogenetic method (Briese, 2005), these were given higher weight in the scoring system, whereas conservation status and economic importance were considered secondary criteria (Table 3.1). Scores were evaluated independently by two of the authors (GM, MC). Given the high phylogenetic relatedness within the *Iris* genus, a conservative threshold of 0.5 was applied to

CHAPTER 3

prioritize species for host-specificity testing. At a family level, a threshold of 0.7 was deemed sufficient to include most at-risk taxa.

Table 3.1 – Criteria used to prioritize plant taxa to be included in the host-specificity priority list of *I. pseudacorus* biocontrol agents. The final score was calculated as a weighted average of the scores three criteria (weight importance is indicated as %).

Score	Geographic Overlap (60%)	Ecological Similarity (30%)	Conservation Status and Economic Importance (10%)
1	The taxon is naturalized in one or more countries where <i>I. pseudacorus</i> is considered invasive	The taxon's climatic niche and habitat type partially overlap to that of <i>I. pseudacorus</i>	The taxon is considered vulnerable and/or holds minor economic importance in one or more countries where <i>I. pseudacorus</i> is considered invasive
2	The taxon is native to one or more countries where <i>I. pseudacorus</i> is considered invasive	The taxon's climatic niche and habitat type strongly overlap to that of <i>I. pseudacorus</i>	The taxon is considered threatened and/or holds medium economic importance in one or more countries where <i>I. pseudacorus</i> is considered invasive
3	Within its native range, the taxon's distribution is in geographic overlap with areas where <i>I. pseudacorus</i> is considered invasive	The taxon's climatic niche and habitat type completely overlap to that of <i>I. pseudacorus</i>	The taxon is considered endangered and/or holds high economic importance in one or more countries where <i>I. pseudacorus</i> is considered invasive

2.2. SHORT-TERM FEEDING TRIALS

A preliminary experiment was carried out to assess if *A. nonstriata* would be able to feed on native South African Iridaceae. In February 2020, 12 plant species from 8 different genera were acquired from local nurseries and brought into the quarantine facility of the Centre for Biological Control (Rhodes University, Makhanda, South Africa). The plants were re-potted and left to acclimatize for 2 weeks before the beginning of the experiment. In the meantime, a new generation of *A. nonstriata* was reared on *I. pseudacorus* plants previously grown within the

CHAPTER 3

quarantine facility. Upon emergence from the pupal stage, 5 naïve adult insects were restricted to a 20 cm leaf section of the different test plants by means of a plastic enclosure. The setup, consisting of a no-choice design, was replicated between 3 and 9 times, according to plant availability. A control (*Iris pseudacorus*) was present for each replication. The insects were left on the plants for 1 week (11th to 18th of March), during which daily checks were made to record feeding and mortality.

At the end of the 7 days, each tested leaf was cut and digitalized with a high-resolution scanner. The scans were then analyzed with the digital imaging software ImageJ v1.54f (Schindelin et al., 2012). This program allows the user to highlight and measure regions of interest (ROI) both manually and through the recognition of particular color patterns or thresholds. This function was used to identify damaged and decayed leaf portions and discern them from healthy ones. The feeding behaviour of *A. nonstriata* produces recognizable furrow-like damage between two adjacent leaf nerves, which allows discrimination from other forms of leaf decay (Minuti et al., 2021; Chapter 1). For each leaf, total surface eaten by the insects was accurately measured and used to calculate the feeding per insect per day. This measure was compared between each test plant and the control (i.e. *I. pseudacorus*) using a Welch two sample t-test or a Wilcoxon rank sum test with continuity correction, conditional to the data meeting the assumptions for parametric distribution. Mortality rates were compared using a Fisher exact test. Finally, Spearman's rank correlation was used to test relationships between feeding, mortality, and the phylogenetic distance from *I. pseudacorus*. All statistical analyses were computed in R version 4.3.1 (R Core Team, 2023).

2.3. LONG-TERM DEVELOPMENT TRIALS

Host range determination of *A. nonstriata* was based on experiments carried out in controlled conditions between October 2018 and December 2021, both in the

CHAPTER 3

native range – in the greenhouse of the Vrije Universiteit Brussel (Brussels, Belgium) – and in the invaded range – within the quarantine facility of the Centre for Biological Control (Makhanda, South Africa). The plants to be tested were selected among species previously prioritized through our scoring system (see section 2.1. for details), according to local and seasonal availability. Particular care was taken to select, if possible, test plants belonging to different phylogenetic clades (Goldblatt, 2000; Wilson, 2006; Goldblatt & Manning, 2008; Wilson, 2009; Wilson, 2011; Wheeler & Wilson, 2014). A total of 35 test plants (Belgium: 7; South Africa: 28) were used in the experiment. These represented 26 different species, covering 13 phylogenetic clades, 11 genera of Iridaceae and one outgroup – *Carex pendula* Huds. (Cyperaceae) – chosen as it commonly grows in admixture with *I. pseudacorus* across its native European range.

In a first experiment, the host-specificity of the candidate was determined through no-choice, adult oviposition and development trials. In these, newly emerge, naïve adult insects were placed into mesh cages with one individual plant. Three to five replicates were used for each test plant and for the relative control (i.e. *I. pseudacorus*). The insects were left to feed and reproduce on the plant for one week, after which they were removed from the cages. Plants were monitored during the following three months and, once emerged, the new adults were removed and counted.

For those species on which *A. nonstriata* was observed to develop in no-choice conditions, choice trials were conducted as a second experiment. These followed the same design as the no-choice trials, with the difference that each cage contained two individual plants – one test plant and one *I. pseudacorus* as control – and therefore the insect was given the choice on which species to feed and lay eggs. After a week, adults were removed from the cages, and the two plants were placed into separate enclosures and monitored regularly to record the emergence of new individuals over the following three months. Due to the

CHAPTER 3

long-term testing procedure, and in order not to influence the development of the insects, leaves could not be cut to precisely quantify feeding damage using imaging software. Consequently, damage to the test plants was monitored throughout the two experiments and visually quantified as a percentage of the total leaf area available for each test plant.

As plants were tested at different moments, each time using new controls (*I. pseudacorus*), all results were scaled for comparison. This was done by dividing the results obtained on test plants (i.e. damage percentage and number of newly emerged insects during the no-choice and choice trials) by those obtained on the relative control. A ratio lower than 1 indicates a performance of the insect on the test plant inferior to that on the control, whereas a ratio higher than 1 indicates a better performance.

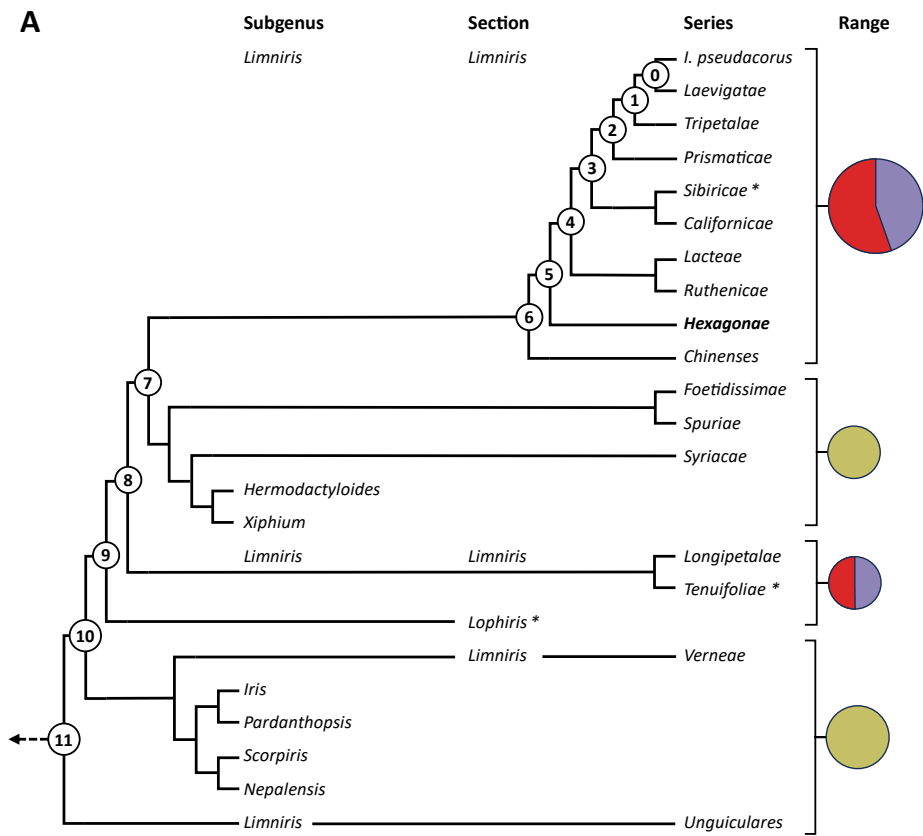
Spearman's rank correlation was used to test relationships between insect emergence, damage, and the phylogenetic distance from *I. pseudacorus*. All statistical analyses were computed in R version 4.3.1 (R Core Team, 2023).

3. RESULTS

3.1. PROPOSED LIST OF TEST PLANTS FOR HOST-SPECIFICITY TESTING

In total, 337 *Iris* species and 67 other genera of Iridaceae were evaluated (Supplementary Table S3.1; S3.2). Based on their final score, 64 species *Iris* species were selected as priority for host-specificity testing (Figure 3.1A; Table 3.2). These include 28 species native to North America, 21 native to eastern Asia, as well as 16 Eurasian species of global horticultural importance. Similarly, 41 genera of Iridaceae from which to select species for host-specificity testing were prioritized (Figure 3.1B; Table 3.2). South Africa accounts for the majority ($n = 23$), followed by South America ($n = 13$), North America ($n = 5$), Australasia ($n = 5$), and Eurasia ($n = 1$).

CHAPTER 3



CHAPTER 3

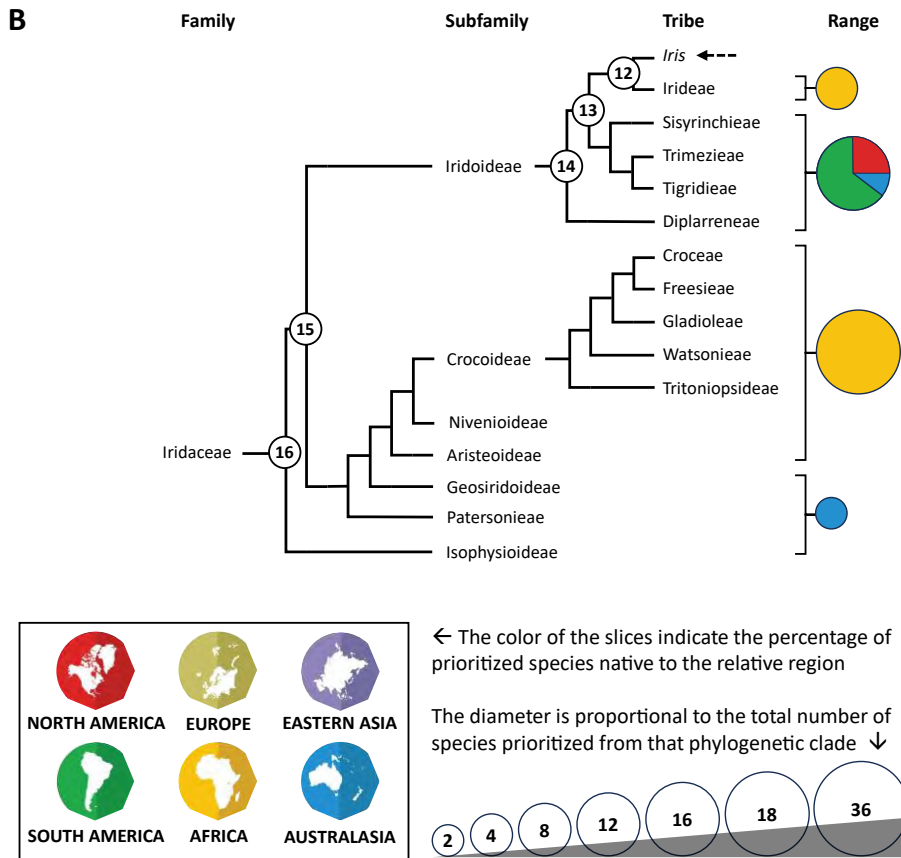


Figure 3.1 – Simplified phylogenetic relationships within **A**) the genus *Iris* (Wilson, 2006; Wilson, 2009; Wilson, 2011; Wheeler & Wilson, 2014) and **B**) the family Iridaceae (Goldblatt, 2000; Goldblatt & Manning, 2008). Numbers in circles indicate increasing degrees of phylogenetic separation from *I. pseudacorus* (*sensu* Briese, 2005). Clades for which monophyly is inconsistent across different phylogenetic studies are marked with an asterisk (*). Colored circles on the right represent the number of taxa prioritized in this study, as well as their geographic range of origin.

CHAPTER 3

Table 3.2 - List of taxa prioritized for the host-specificity testing of biocontrol agents targeting *Iris pseudacorus* L. The rank (#) represents the degree of phylogenetic separation from the target weed (see Figure 3.1). Taxa tested for the host-specificity of *A. nonstriata* within the context of the current paper are indicated in bold.

#	Prioritized taxa	Native Range
0	<i>I. versicolor</i> , <i>I. virginica</i> <i>I. ensata</i>, <i>I. laevigata</i>, <i>I. maackii</i>	North America Eastern Asia
1	<i>I. setosa</i> , <i>I. tridentata</i> , <i>I. hookeri</i> <i>I. setosa</i>	North America Eastern Asia
2	<i>I. prismatica</i> <i>I. sibirica</i> , <i>I. sanguinea</i> , <i>I. typhifolia</i>	North America Eastern Asia
3	<i>I. fernaldii</i> , <i>I. tenax</i> , <i>I. munzii</i> , <i>I. purdyi</i> , <i>I. tenuissima</i> , <i>I. bracteata</i> , <i>I. douglasiana</i> , <i>I. innominata</i> , <i>I. macrosiphon</i> , <i>I. chrysophylla</i> , <i>I. hartwegii</i>	North America
4	<i>I. lactea</i> , <i>I. uniflora</i>	Eastern Asia
5	<i>I. brevicaulis</i> , <i>I. fulva</i> , <i>I. giganteaerulea</i> , <i>I. hexagona</i> , <i>I. savannarum</i>	North America
6	<i>I. rossii</i> , <i>I. odaesanensis</i> , <i>I. koreana</i> , <i>I. speculatrix</i> , <i>I. minutoaurea</i> , <i>I. proantha</i>	Eastern Asia
7	<i>I. foetidissima</i>, <i>I. orientalis</i>, <i>I. spuria</i> , <i>I. graminea</i> , <i>I. masia</i> , <i>I. reticulata</i> , <i>I. xiphium</i>	Eurasia
8	<i>I. longipetala</i> , <i>I. missouriensis</i>	North America
9	<i>I. lacustris</i> , <i>I. cristata</i> , <i>I. tenuis</i> <i>I. japonica</i> , <i>I. gracilipes</i> , <i>I. confusa</i> , <i>I. tectorum</i>	North America Eastern Asia
10	<i>I. verna</i> , <i>I. × germanica</i> , <i>I. aphylla</i> , <i>I. florentina</i> , <i>I. graminea</i> , <i>I. pallida</i> , <i>I. pumila</i> , <i>I. tigridia</i> , <i>I. variegata</i> , <i>I. domestica</i> , <i>I. dichotoma</i>	Eurasia
11	<i>I. unguicularis</i>	Eurasia
12	<i>Dietes</i>, <i>Moraea</i>, <i>Bobartia</i>, <i>Ferraria</i>	South Africa
13	<i>Sisyrinchium</i> , <i>Olsynium</i> , <i>Herbertia</i> , <i>Nemastylis</i> , <i>Salpingostylis</i> <i>Libertia</i> , <i>Sisyrinchium</i> , <i>Orthrosanthus</i> , <i>Olsynium</i> , <i>Trimezia</i> , <i>Calydorea</i> , <i>Cypella</i> , <i>Herbertia</i> , <i>Gelasine</i> , <i>Eleutherine</i> , <i>Ennealophus</i> , <i>Phalocallis</i> , <i>Mastigostyla</i> <i>Libertia</i> , <i>Orthrosanthus</i>	North America South America Australasia
14	<i>Diplarrena</i>	Australasia
15	<i>Romulea</i> , <i>Babiana</i> , <i>Chasmanthe</i> , <i>Ixia</i> , <i>Tritonia</i> , <i>Dierama</i> , <i>Sparaxis</i> , <i>Syringodea</i> , <i>Hesperantha</i> , <i>Geissorhiza</i> , <i>Freesia</i> , <i>Devia</i> , <i>Crocasmia</i> , <i>Gladiolus</i> , <i>Watsonia</i> , <i>Lepeirousia</i> , <i>Tritoniopsis</i> , <i>Aristea</i> <i>Crocus</i> <i>Patersonia</i>	South Africa Eurasia Australasia
16	<i>Isophysis</i>	Australasia

CHAPTER 3

3.2. SHORT-TERM FEEDING TRIALS

The feeding of *A. nonstriata* during the short-term trials showed a significant negative correlation (Spearman's $\rho = -0.68$, $P < 0.001$) with the phylogenetic distance of the test plants from *I. pseudacorus* (Figure 3.2). Feeding on the two varieties of *I. × germanica* tested showed no statistical difference compared to *I. pseudacorus*. Closely related *Dietes* and *Moraea* spp. (subfamily Iridoideae, tribe Irideae) were fed upon significantly less than *I. pseudacorus*. A first exception was found in *Trimezia gracilis*, which despite being in a different tribe (Trimezieae), was subject to similar levels of feeding damage. Similarly, feeding on *Watsonia borbonica* Goldblatt (subfamily Crocoideae) showed no significant difference compared to *I. pseudacorus*. This is of particular interest, given that *A. nonstriata* feeding was almost negligible on all other Crocoideae tested, including *Watsonia angusta*, a congeneric of *W. borbonica*. Mortality rates recorded during the experiment ranged between 10% and 24%, with *I. pseudacorus* being on the lower end of the spectrum (12%). Insect mortality showed no significant correlation with phylogenetic distance from *I. pseudacorus* (Spearman's $\rho = -0.04$, $P = 0.69$) and no significant differences were observed between the species tested and the control. Finally, no significant correlation was observed between insect feeding and mortality rate (Spearman's $\rho = 0.123$, $P = 0.214$).

CHAPTER 3

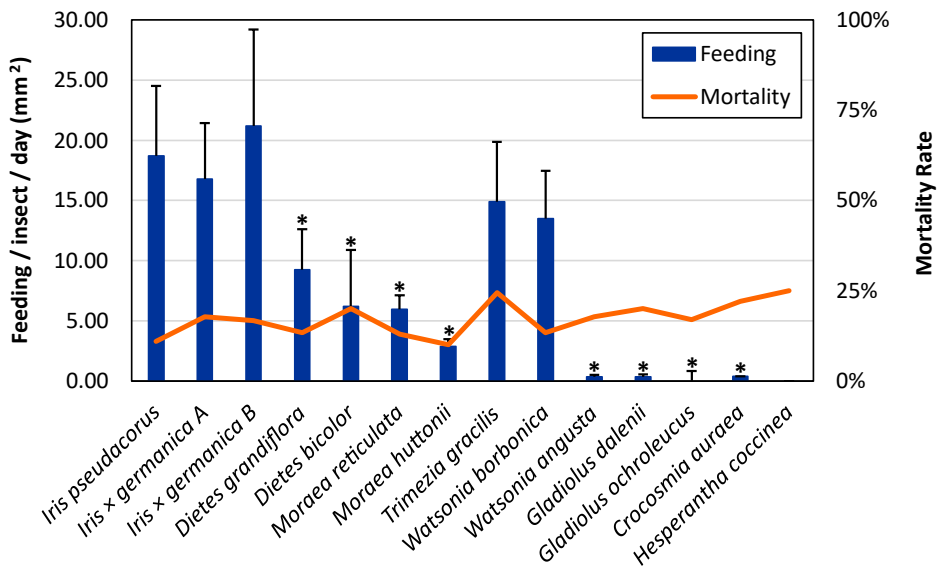


Figure 3.2 – Feeding per insect per day (mean $\pm$ SD) and mortality rate (%) of *Aphthona nonstriata* on various Iridaceae tested during short-term trials. Significant differences in feeding between each test plant and the control (*Iris pseudacorus*) are indicated with an asterisk. Species are listed in increasing degrees of phylogenetic separation from the target plant (*sensu* Briese, 2005).

3.3. LONG-TERM DEVELOPMENT TRIALS

3.3.1. No-choice trials

Of the 35 plants (26 species) tested in no-choice trials, 31 (22 species) were subjected to some level of feeding by *A. nonstriata* adults (Figure 3.3). However, feeding damage was negatively correlated with increasing phylogenetic distance from *I. pseudacorus* (Spearman's $\rho = -0.72$, $P < 0.001$), and most test plants showed $< 50\%$ damage compared to the relative control. Only 9 test plants (7 species) supported the development of a second generation of the insect. These are all European and eastern Asian species, representatives of the subgenera *Limniris* (*I. laevigata*, *I. sibirica*, *I. foetidissima*, *I. spuria*, *I. orientalis* and *I. confusa*) and *Pardanthopsis* (*I. domestica*). Noticeably, insect emergence was not recorded on *I. ensata* – a close relative of *I. pseudacorus* –, on North American irises of the subgenus *Limniris* series *Hexagonae*, and on three

CHAPTER 3

varieties of the common garden cultivar *I. × germanica* (Figure 3.3A). No second generation was recorded on any species outside of the *Iris* genus. Accordingly, the number of emerged insects was significantly correlated with the phylogenetic distance of the test plants from *I. pseudacorus* (Spearman's $\rho = -0.51$, $P < 0.01$), and with the relative feeding damage recorded during the experiment (Spearman's $\rho = 0.68$, $P < 0.001$).

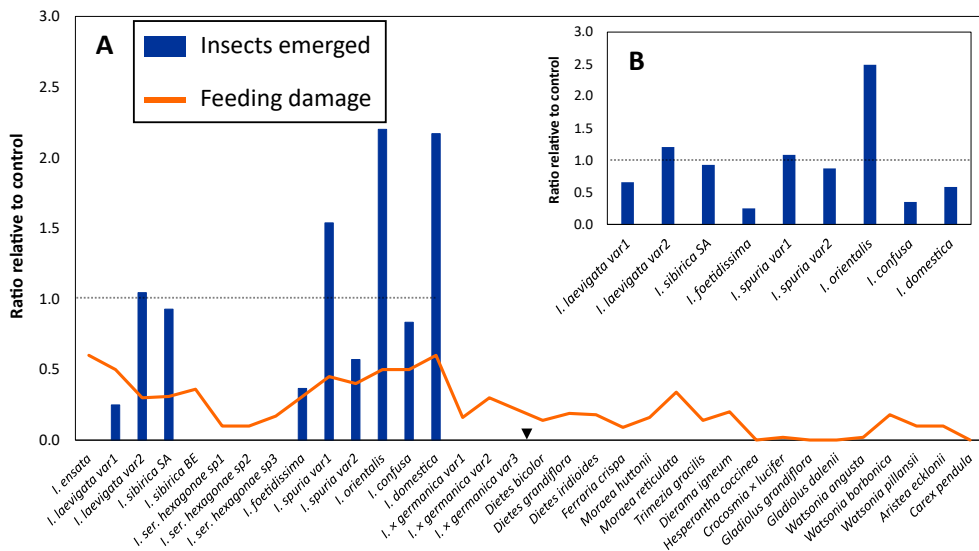


Figure 3.3 – Results of the **A)** no-choice or **B)** choice host-specificity trials of *Aphthona nonstriata* on various plant species, listed in increasing degrees of phylogenetic separation from *I. pseudacorus* (the black triangle indicates the distinction between *Iris* species and other genera). Blue bars represent the number of insects emerged from each plant, the orange line indicates the feeding damage observed on the leaves at the end of the experiment. To compare trials conducted at different moments in time, all results are scaled based to the relative control (*I. pseudacorus*): a ratio lower than 1 (dotted line) indicates a performance of the insect on the test plant inferior to that on the control, and vice versa. For quantitative emergence see Supplementary Table S3.3.

3.3.2. Choice trials

All plants tested in choice trials supported the development of a second generation of the insect (Figure 3.3B). For most test plants, relative emergence was similar between choice and no-choice trials, with a few species (*I. foetidissima*, *I. confusa* and *I. domestica*) showing a pronounced decreased.

CHAPTER 3

Emergence on some plant species (e.g. *I. laevigata*, *I. sibirica*, and *I. spuria*) was dependent on the variety tested (Figure 3.3B). The only species that showed significantly higher levels of emergence in both no-choice and choice trials was *I. orientalis*, an Eurasian representative of the subgenus *Limniris*, series *Spuriae*. In choice trials, no significant correlation was observed between number of insects emerged and phylogenetic distance from *I. pseudacorus* (Spearman's $\rho = -0.31$, $P = 0.387$), nor between emergence and feeding damage (Spearman's $\rho = -0.08$, $P = 0.813$).

4. DISCUSSION

4.1. PRIORITISATION OF PLANT SPECIES FOR HOST-SPECIFICITY TESTING

The distribution of the *Iris* genus is restricted to the Northern Hemisphere (POWO, 2023), thus only species native to North America or eastern Asia were found to overlap with *I. pseudacorus* across its non-native range. Interestingly, a significant proportion of these species are classified within the *Iris* subgenus *Limniris*, of which *I. pseudacorus* is a representative. Although *Iris* subgenera are mostly adapted to semiarid or rocky montane habitats, *Limniris* species are often found in mesic or even humid environments (Wilson, 2009). These include some North American species within the section *Limniris*, series *Californicae*, *Laevigatae*, *Longipetalae*, *Hexagonae*, *Prismatica*, *Tripetalae*, and *Vernae*, as well as native eastern Asian representatives of the series *Chinenses*, *Lacteae* and *Sibiricae*, and several species of mixed distribution within the section *Lophiris*. Given the polyphyletic relationships still existent within the subgenus (Wilson, 2009; Wilson, 2011), it is unclear if the adaptation to humid habitats constitutes an ancestral trait or if it evolved independently across different lineages. Nonetheless, the high ecological similarity of these species with the target weed should make them a priority for host-specificity testing. Species from other *Iris* subgenera are native to Europe and western Asia and, with a few exceptions, do not show high ecological similarity with *I. pseudacorus*.

CHAPTER 3

Some of these species, however, are exported, sold and naturalized worldwide as ornamentals, and therefore hold important economic value. Common examples include the widespread hybrid *Iris* × *germanica* L. (*I. pallida* Lam. × *I. variegata* L.), and the common garden irises *I. orientalis* Mill. and *I. spuria* L., to name just a few. To our judgement, these species should be included in the priority list for host-specificity testing across different ranges. Such inclusion would be most relevant for countries within the Southern Hemisphere which native flora do not include *Iris* species, but where a large and active horticultural society is present (e.g. The New Zealand Iris Society).

Given the significant phylogenetic radiation of the family Iridaceae out of Australasia and across the Southern Hemisphere (Goldblatt et al., 2008), most genera include at least a few species whose native range geographically overlaps with regions invaded by *I. pseudacorus* in these countries. This is reflected in our evaluation, for which most genera (ca. 60%) received a high score (> 0.7). Accordingly, as a conservative measure, whole genera have been selected as high priority for host-specificity testing. Of course, the choice of test species to be tested within each priority genus will have to be conducted on a case-to-case basis, based on local ecological and economic requirements, and following national frameworks (Briese & Walker, 2008; Lesieur et al., 2019).

The family Iridaceae includes a considerable number of taxa of horticultural importance, as well as many endemic and endangered species (Goldblatt & Manning, 2008). For instance, the South African genera *Bobartia* and *Ferraria*, whose distribution is restricted to semi-arid and Mediterranean climates of the Western Cape region, enumerate several species reported as vulnerable or threatened (SANBI, 2023). These should, evidently, be prioritized in the host-specificity procedure in the country. On the other hand, despite a lack of geographic overlap with the target species, the Eurasian genus *Crocus* should be prioritized based on its horticultural importance.

CHAPTER 3

Overall, our priority list includes 105 taxa (65 species, 41 genera) to be prioritized for host-specificity testing of candidate biocontrol agents for *I. pseudacorus* across different invaded regions of the world. These represent 25% of all taxa evaluated, effectively reducing the time and effort required to draw specific test lists across different countries. The scoring method developed for this evaluation is versatile: scoring criteria can be added and modified, and the weights and the thresholds adjusted to fit the requirements of different species. This is expected to be particularly useful in biocontrol programs where the target species is widely distributed, and numerous close relatives are present across the invaded range. Such is the case for the African lovegrass *Eragrostis curvula* (Schrad.) Nees (Poaceae) and the flax-leaf fleabane *Erigeron bonariensis* L. (Asteraceae) in Australia (Chen et al., 2024; Yell et al., 2024).

4.2. PRELIMINARY HOST-SPECIFICITY TESTING OF *APHTHONA NONSTRIATA*

The results of the host-specificity trials highlighted the capacity of *A. nonstriata* adults to feed on a variety of species from different genera within the family Iridaceae, challenging the claims of monophagy previously attributed to the beetle (Baviera & Biondi, 2015). However, the feeding recorded decreased significantly with increasing phylogenetic distance from *I. pseudacorus*, with a few exceptions (i.e. *Trimezia gracilis* Herbert and *Watsonia borbonica* (Pourr.) Goldblatt). These are believed to be time-dependent artifacts, as the same levels of damage were not observed in long-term experiments. Nonetheless, further investigation into the phytochemical properties of these species is necessary in order to unravel the reasons behind this incongruence (e.g. Rapo et al., 2019). Contrary to other studies on rapid preliminary characterization of host-specificity (e.g. Heard & Van Klinken, 2004), we found no direct relationship between acceptance of a test plant for feeding and the ability of our candidate biocontrol agent to develop on the said species. That is, despite *A. nonstriata* feeding on various species of Iridaceae, no second generation was recorded on

CHAPTER 3

any species outside the *Iris* genus. Within the genus, significant differences were observed between species and even between cultivars of the same species. For instance, *A. nonstriata* was able to develop on the eastern Asian *I. laevigata* Fisch., but not on *I. ensata* Thunb., despite the two species being sympatric and closely related to the target weed. Similarly, the beetle produced a second generation on a cultivar of the Asian *I. sibirica* L. tested in South Africa, but not on cultivars tested in Belgium, highlighting the need for genetic characterization of test plants, especially when dealing with horticultural varieties and highly hybridized species (Reid et al., 2023). Another species of particular economic importance, the hybrid *I. × germanica* L., did not support a second generation of the biocontrol agent. However, given the plethora of different cultivars of these species around the world, it would be informative to test plants from local nurseries across various ranges before completely excluding the possibility of acceptance of this plant as host by the beetle. Full development was reached on all other Eurasian and eastern Asian species tested, despite their differing degrees of phylogenetic separation from *I. pseudacorus*. Surprisingly, the beetle showed even higher performances on the widely cultivated Eurasian species *Iris orientalis* Mill., both in no-choice and choice trials. At the same time, no development was observed on tested species of *Iris* series *Hexagonae*, the only North America taxon included in our experiments. Further testing, including closely related species native to different continents, is needed in order to understand if this might constitute part of a broader phylogeographic pattern.

5. CONCLUSION

Altogether, the priority list developed within this study can function as a working platform for the host-specificity testing of candidate biocontrol agents for *I. pseudacorus*. That is, although local frameworks will still require each separate country to develop its own test list, the presence of a list encompassing different regions invaded by the weed will simplify the process significantly. The

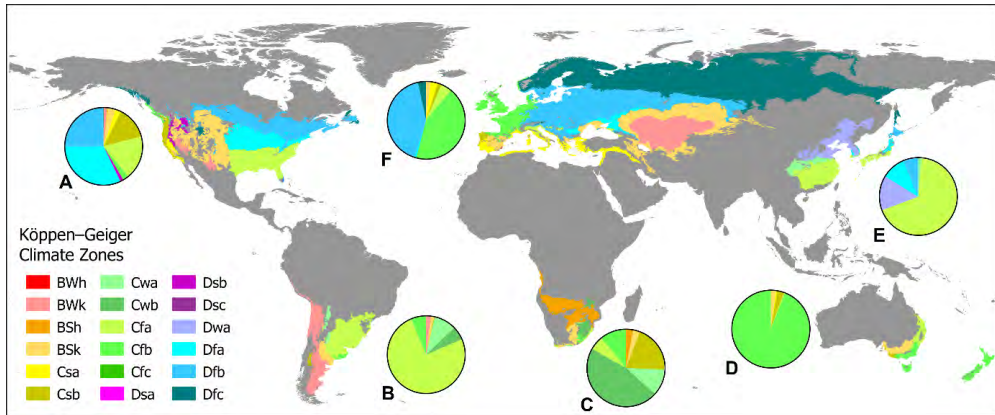
CHAPTER 3

preliminary results on *A. nonstriata* seem to be strengthening the hypothesis of the beetle being an *Iris* specialist, although not limited to *I. pseudacorus*, as previously assumed. Adult feeding was also observed on other Iridaceae outside of the *Iris* genus, highlighting the need to conduct a thorough risk assessment before release. Due to the particular phylogeography of the family Iridaceae, it is clear that higher risks would be associated with releasing the beetle across invaded regions of North America and Eastern Asia. On the other hand, the absence of native *Iris* species in the Southern Hemisphere shows more promise for the use of this candidate in Argentina, South Africa and Australasia.

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SUPPLEMENTARY INFORMATION



Supplementary Figure S3.1 - Köppen-Geiger climate zones occupied by *Iris pseudacorus* in its native and various invaded ranges. Pie charts represent the relative density of occurrence points within each range (A: North America; B: South America; C: South Africa; D: Australasia; E: Eastern Asia; F: Europe). Climate types are defined as follows: BW = desertic; BS = semi-arid; Cs = Mediterranean; Cw = humid subtropical; Cf = humid temperate and oceanic; Ds/Dw = humid continental and subarctic (adapted from Beck et al., 2018).

CHAPTER 3

Supplementary Table S3.1 - Scores given to the *Iris* species evaluated for the host-specificity testing of biocontrol agents to be used against *Iris pseudacorus* L. (Iridaceae). Category scores were assigned – according to the criteria detailed in Table 3.1 – based on the information gathered in Annex I. GEO: geographic overlap; ECO: ecological similarity; CS/EI: Conservation status and/or economic importance.

Species	GEO	ECO	CS/EI	Score
<i>I. versicolor</i>	3	3	3	1
<i>I. virginica</i>	3	3	3	1
<i>I. brevicaulis</i>	3	3	2	0.97
<i>I. ensata</i>	3	3	2	0.97
<i>I. laevigata</i>	3	3	2	0.97
<i>I. prismatica</i>	3	3	2	0.97
<i>I. setosa</i>	3	3	2	0.97
<i>I. fulva</i>	3	3	1	0.93
<i>I. japonica</i>	3	3	1	0.93
<i>I. lacustris</i>	3	3	1	0.93
<i>I. sanguinea</i>	3	3	1	0.93
<i>I. tridentata</i>	3	3	1	0.93
<i>I. cristata</i>	3	2	2	0.87
<i>I. domestica</i>	3	2	2	0.87
<i>I. giganteaerulea</i>	3	2	2	0.87
<i>I. gracilipes</i>	3	2	2	0.87
<i>I. hexagona</i>	3	2	2	0.87
<i>I. longipetala</i>	3	2	2	0.87
<i>I. missouriensis</i>	3	2	2	0.87
<i>I. fernaldii</i>	3	2	1	0.83
<i>I. hookeri</i>	3	2	1	0.83
<i>I. rossii</i>	3	2	1	0.83
<i>I. savannarum</i>	3	2	1	0.83
<i>I. tenax</i>	3	2	1	0.83
<i>I. dichotoma</i>	3	1	3	0.8
<i>I. munzii</i>	3	2	0	0.8
<i>I. sibirica</i>	2	3	3	0.8
<i>I. odaesanensis</i>	3	1	2	0.77
<i>I. purdyi</i>	3	1	2	0.77
<i>I. tenuissima</i>	3	1	2	0.77
<i>I. × robusta</i>	3	1	2	0.77
<i>I. bracteata</i>	3	1	1	0.73
<i>I. douglasiana</i>	3	1	1	0.73

CHAPTER 3

Species	GEO	ECO	CS/EI	Score
<i>I. innominata</i>	3	1	1	0.73
<i>I. koreana</i>	3	1	1	0.73
<i>I. lactea</i>	3	1	1	0.73
<i>I. maackii</i>	2	3	1	0.73
<i>I. macrosiphon</i>	3	1	1	0.73
<i>I. speculatrix</i>	3	1	1	0.73
<i>I. tenuis</i>	3	1	1	0.73
<i>I. chrysophylla</i>	3	1	0	0.7
<i>I. hartwegii</i>	3	1	0	0.7
<i>I. minutoaurea</i>	3	1	0	0.7
<i>I. proantha</i>	3	1	0	0.7
<i>I. verna</i>	3	1	0	0.7
<i>I. confusa</i>	2	2	2	0.67
<i>I. tectorum</i>	2	2	2	0.67
<i>I. typhifolia</i>	2	2	2	0.67
<i>I. delavayi</i>	2	2	1	0.63
<i>I. wilsonii</i>	2	2	1	0.63
<i>I. × nelsonii</i>	2	2	1	0.63
<i>I. oxypetala</i>	3	0	0	0.6
<i>I. foetidissima</i>	1	3	2	0.57
<i>I. orientalis</i>	1	3	2	0.57
<i>I. spuria</i>	1	3	2	0.57
<i>I. uniflora</i>	2	1	2	0.57
<i>I. × germanica</i>	1	3	2	0.57
<i>I. bulleyana</i>	2	1	1	0.53
<i>I. chrysographes</i>	2	1	1	0.53
<i>I. dabashanensis</i>	2	1	1	0.53
<i>I. forrestii</i>	2	1	1	0.53
<i>I. henryi</i>	2	1	1	0.53
<i>I. probstii</i>	2	1	1	0.53
<i>I. tigridia</i>	2	1	1	0.53
<i>I. wattii</i>	2	1	1	0.53
<i>I. × ampliflora</i>	2	1	1	0.53
<i>I. × flexicaulis</i>	2	1	1	0.53
<i>I. × fulvala</i>	2	1	1	0.53
<i>I. × thompsonii</i>	2	1	1	0.53
<i>I. × vinicolor</i>	2	1	1	0.53
<i>I. × violipurpurea</i>	2	1	1	0.53

CHAPTER 3

Species	GEO	ECO	CS/EI	Score
<i>I. anguifuga</i>	2	1	0	0.5
<i>I. calcicola</i>	2	1	0	0.5
<i>I. cathayensis</i>	2	1	0	0.5
<i>I. farreri</i>	2	1	0	0.5
<i>I. junzifengensis</i>	2	1	0	0.5
<i>I. ruthenica</i>	2	1	0	0.5
<i>I. × setosothungbergii</i>	2	1	0	0.5
<i>I. aphylla</i>	1	2	2	0.47
<i>I. florentina</i>	1	2	2	0.47
<i>I. graminea</i>	1	2	2	0.47
<i>I. pallida</i>	1	2	2	0.47
<i>I. pumila</i>	1	2	2	0.47
<i>I. reticulata</i>	1	2	2	0.47
<i>I. unguicularis</i>	1	2	2	0.47
<i>I. variegata</i>	1	2	2	0.47
<i>I. xiphium</i>	1	2	2	0.47
<i>I. clarkei</i>	1	2	1	0.43
<i>I. cuniculiformis</i>	2	0	1	0.43
<i>I. goniocarpa</i>	2	0	1	0.43
<i>I. leptophylla</i>	2	0	1	0.43
<i>I. tenuifolia</i>	2	0	1	0.43
<i>I. bungei</i>	2	0	0	0.4
<i>I. tibetica</i>	2	0	0	0.4
<i>I. mandshurica</i>	1	1	2	0.37
<i>I. barbatula</i>	1	1	1	0.33
<i>I. barnumiae</i>	1	1	1	0.33
<i>I. curvifolia</i>	1	1	1	0.33
<i>I. dolichosiphon</i>	1	1	1	0.33
<i>I. halophila</i>	0	3	1	0.33
<i>I. milesii</i>	1	1	1	0.33
<i>I. mzchetica</i>	0	3	1	0.33
<i>I. ventricosa</i>	1	1	1	0.33
<i>I. × sancti-cyri</i>	1	1	1	0.33
<i>I. brandzae</i>	0	3	0	0.3
<i>I. collettii</i>	1	1	0	0.3
<i>I. decora</i>	1	1	0	0.3
<i>I. neoensata</i>	1	1	0	0.3
<i>I. neosetosa</i>	1	1	0	0.3

CHAPTER 3

Species	GEO	ECO	CS/EI	Score
<i>I. staintonii</i>	1	1	0	0.3
<i>I. subdichotoma</i>	1	1	0	0.3
<i>I. humilis</i>	0	2	2	0.27
<i>I. kobayashii</i>	1	0	1	0.23
<i>I. loczyi</i>	1	0	1	0.23
<i>I. aschersonii</i>	0	2	0	0.2
<i>I. grant-duffii</i>	0	2	0	0.2
<i>I. latistyla</i>	1	0	0	0.2
<i>I. qinghainica</i>	1	0	0	0.2
<i>I. songarica</i>	1	0	0	0.2
<i>I. arenaria</i>	0	1	2	0.17
<i>I. atrofusca</i>	0	1	2	0.17
<i>I. atropurpurea</i>	0	1	2	0.17
<i>I. bismarckiana</i>	0	1	2	0.17
<i>I. hermona</i>	0	1	2	0.17
<i>I. histrioides</i>	0	1	2	0.17
<i>I. hookeriana</i>	0	1	2	0.17
<i>I. lortetii</i>	0	1	2	0.17
<i>I. ludwigii</i>	0	1	2	0.17
<i>I. suaveolens</i>	0	1	2	0.17
<i>I. adriatica</i>	0	1	1	0.13
<i>I. alexeenkoi</i>	0	1	1	0.13
<i>I. attica</i>	0	1	1	0.13
<i>I. aucheri</i>	0	1	1	0.13
<i>I. benacensis</i>	0	1	1	0.13
<i>I. bloudowii</i>	0	1	1	0.13
<i>I. boissieri</i>	0	1	1	0.13
<i>I. calabra</i>	0	1	1	0.13
<i>I. cedreti</i>	0	1	1	0.13
<i>I. colchica</i>	0	1	1	0.13
<i>I. danfordiae</i>	0	1	1	0.13
<i>I. darwasica</i>	0	1	1	0.13
<i>I. filifolia</i>	0	1	1	0.13
<i>I. formosana</i>	0	1	1	0.13
<i>I. grossheimii</i>	0	1	1	0.13
<i>I. haynei</i>	0	1	1	0.13
<i>I. hellenica</i>	0	1	1	0.13
<i>I. imbricata</i>	0	1	1	0.13

CHAPTER 3

Species	GEO	ECO	CS/EI	Score
<i>I. jacquinii</i>	0	1	1	0.13
<i>I. juncea</i>	0	1	1	0.13
<i>I. kashmiriana</i>	0	1	1	0.13
<i>I. kemaonensis</i>	0	1	1	0.13
<i>I. kirkwoodiae</i>	0	1	1	0.13
<i>I. latifolia</i>	0	1	1	0.13
<i>I. lutescens</i>	0	1	1	0.13
<i>I. marsica</i>	0	1	1	0.13
<i>I. masiae</i>	0	1	1	0.13
<i>I. notha</i>	0	1	1	0.13
<i>I. orjenii</i>	0	1	1	0.13
<i>I. pamphylica</i>	0	1	1	0.13
<i>I. perrieri</i>	0	1	1	0.13
<i>I. planifolia</i>	0	1	1	0.13
<i>I. pontica</i>	0	1	1	0.13
<i>I. pseudonotha</i>	0	1	1	0.13
<i>I. reichenbachii</i>	0	1	1	0.13
<i>I. relictata</i>	0	1	1	0.13
<i>I. rutherfordii</i>	0	1	1	0.13
<i>I. sabina</i>	0	1	1	0.13
<i>I. sari</i>	0	1	1	0.13
<i>I. serotina</i>	0	1	1	0.13
<i>I. sintenisii</i>	0	1	1	0.13
<i>I. stenophylla</i>	0	1	1	0.13
<i>I. stolonifera</i>	0	1	1	0.13
<i>I. taochia</i>	0	1	1	0.13
<i>I. timofejewii</i>	0	1	1	0.13
<i>I. tingitana</i>	0	1	1	0.13
<i>I. tuberosa</i>	0	1	1	0.13
<i>I. vorobievii</i>	0	1	1	0.13
<i>I. westii</i>	0	1	1	0.13
<i>I. × binata</i>	0	1	1	0.13
<i>I. × kobasensis</i>	0	1	1	0.13
<i>I. × neumayeri</i>	0	1	1	0.13
<i>I. aitchisonii</i>	0	1	0	0.1
<i>I. cycloglossa</i>	0	1	0	0.1
<i>I. edomensis</i>	0	1	0	0.1
<i>I. farashae</i>	0	1	0	0.1

CHAPTER 3

Species	GEO	ECO	CS/EI	Score
<i>I. furcata</i>	0	1	0	0.1
<i>I. gatesii</i>	0	1	0	0.1
<i>I. glaucescens</i>	0	1	0	0.1
<i>I. heracleana</i>	0	1	0	0.1
<i>I. histrio</i>	0	1	0	0.1
<i>I. korolkowii</i>	0	1	0	0.1
<i>I. lusitanica</i>	0	1	0	0.1
<i>I. narcissiflora</i>	0	1	0	0.1
<i>I. nectarifera</i>	0	1	0	0.1
<i>I. nusairiensis</i>	0	1	0	0.1
<i>I. palaestina</i>	0	1	0	0.1
<i>I. pseudopumila</i>	0	1	0	0.1
<i>I. reichenbachiana</i>	0	1	0	0.1
<i>I. setina</i>	0	1	0	0.1
<i>I. sprengeri</i>	0	1	0	0.1
<i>I. statellae</i>	0	1	0	0.1
<i>I. susiana</i>	0	1	0	0.1
<i>I. svetlanae</i>	0	1	0	0.1
<i>I. tschandalasica</i>	0	1	0	0.1
<i>I. vartanii</i>	0	1	0	0.1
<i>I. wallisiae</i>	0	1	0	0.1
<i>I. × seminaturalis</i>	0	1	0	0.1
<i>I. xanthospuria</i>	0	1	0	0.1
<i>I. zagrica</i>	0	1	0	0.1
<i>I. acutiloba</i>	0	0	2	0.07
<i>I. assadiana</i>	0	0	2	0.07
<i>I. auranitica</i>	0	0	2	0.07
<i>I. damascena</i>	0	0	2	0.07
<i>I. iberica</i>	0	0	2	0.07
<i>I. ivanovae</i>	0	0	2	0.07
<i>I. lineata</i>	0	0	2	0.07
<i>I. potaninii</i>	0	0	2	0.07
<i>I. afghanica</i>	0	0	1	0.03
<i>I. antilibanotica</i>	0	0	1	0.03
<i>I. avromanica</i>	0	0	1	0.03
<i>I. bakeriana</i>	0	0	1	0.03
<i>I. basaltica</i>	0	0	1	0.03
<i>I. bicapitata</i>	0	0	1	0.03

CHAPTER 3

Species	GEO	ECO	CS/EI	Score
<i>I. bostrensis</i>	0	0	1	0.03
<i>I. bucharica</i>	0	0	1	0.03
<i>I. camillae</i>	0	0	1	0.03
<i>I. caucasica</i>	0	0	1	0.03
<i>I. crocea</i>	0	0	1	0.03
<i>I. graeberiana</i>	0	0	1	0.03
<i>I. junonia</i>	0	0	1	0.03
<i>I. kolpakowskiana</i>	0	0	1	0.03
<i>I. lazica</i>	0	0	1	0.03
<i>I. maculata</i>	0	0	1	0.03
<i>I. magnifica</i>	0	0	1	0.03
<i>I. maracandica</i>	0	0	1	0.03
<i>I. mariae</i>	0	0	1	0.03
<i>I. microglossa</i>	0	0	1	0.03
<i>I. narbutii</i>	0	0	1	0.03
<i>I. nigricans</i>	0	0	1	0.03
<i>I. orchiodes</i>	0	0	1	0.03
<i>I. paradoxa</i>	0	0	1	0.03
<i>I. revoluta</i>	0	0	1	0.03
<i>I. rosenbachiana</i>	0	0	1	0.03
<i>I. scariosa</i>	0	0	1	0.03
<i>I. schachtii</i>	0	0	1	0.03
<i>I. vicaria</i>	0	0	1	0.03
<i>I. willmottiana</i>	0	0	1	0.03
<i>I. winogradowii</i>	0	0	1	0.03
<i>I. × brzhezitzky</i>	0	0	1	0.03
<i>I. × caeciliae</i>	0	0	1	0.03
<i>I. × ketzkhoveli</i>	0	0	1	0.03
<i>I. × koenigii</i>	0	0	1	0.03
<i>I. alberti</i>	0	0	0	0
<i>I. albomarginata</i>	0	0	0	0
<i>I. almaatensis</i>	0	0	0	0
<i>I. austrotschatkalica</i>	0	0	0	0
<i>I. baldshuanica</i>	0	0	0	0
<i>I. cabulica</i>	0	0	0	0
<i>I. capnoides</i>	0	0	0	0
<i>I. carterorum</i>	0	0	0	0
<i>I. chrysopetala</i>	0	0	0	0

CHAPTER 3

Species	GEO	ECO	CS/EI	Score
<i>I. doabensis</i>	0	0	0	0
<i>I. drepanophylla</i>	0	0	0	0
<i>I. ferdowsii</i>	0	0	0	0
<i>I. fosteriana</i>	0	0	0	0
<i>I. furseorum</i>	0	0	0	0
<i>I. galatica</i>	0	0	0	0
<i>I. griffithii</i>	0	0	0	0
<i>I. haussknechtii</i>	0	0	0	0
<i>I. heweri</i>	0	0	0	0
<i>I. hippolyti</i>	0	0	0	0
<i>I. hoogiana</i>	0	0	0	0
<i>I. hymenospatha</i>	0	0	0	0
<i>I. hyrcana</i>	0	0	0	0
<i>I. inconspicua</i>	0	0	0	0
<i>I. kamelinii</i>	0	0	0	0
<i>I. khassanovii</i>	0	0	0	0
<i>I. kopetdagensis</i>	0	0	0	0
<i>I. kurbanovii</i>	0	0	0	0
<i>I. kuschakewiczii</i>	0	0	0	0
<i>I. kuschkenensis</i>	0	0	0	0
<i>I. leptorrhiza</i>	0	0	0	0
<i>I. linifolia</i>	0	0	0	0
<i>I. linifoliiformis</i>	0	0	0	0
<i>I. lokiae</i>	0	0	0	0
<i>I. longiscapa</i>	0	0	0	0
<i>I. lycotis</i>	0	0	0	0
<i>I. marivanica</i>	0	0	0	0
<i>I. meda</i>	0	0	0	0
<i>I. nantouensis</i>	0	0	0	0
<i>I. narynensis</i>	0	0	0	0
<i>I. nicolai</i>	0	0	0	0
<i>I. odontostyla</i>	0	0	0	0
<i>I. paropamisensis</i>	0	0	0	0
<i>I. persica</i>	0	0	0	0
<i>I. peshmeniana</i>	0	0	0	0
<i>I. petrana</i>	0	0	0	0
<i>I. petri</i>	0	0	0	0
<i>I. platyptera</i>	0	0	0	0

CHAPTER 3

Species	GEO	ECO	CS/EI	Score
<i>I. polakii</i>	0	0	0	0
<i>I. popovii</i>	0	0	0	0
<i>I. porphyrochrysa</i>	0	0	0	0
<i>I. postii</i>	0	0	0	0
<i>I. psammocola</i>	0	0	0	0
<i>I. pseudocapnoides</i>	0	0	0	0
<i>I. pseudocaucasica</i>	0	0	0	0
<i>I. pseudomeda</i>	0	0	0	0
<i>I. pskemensis</i>	0	0	0	0
<i>I. ramsayi</i>	0	0	0	0
<i>I. regis-uzziae</i>	0	0	0	0
<i>I. rodionenkoi</i>	0	0	0	0
<i>I. rudolphii</i>	0	0	0	0
<i>I. schelkownikowii</i>	0	0	0	0
<i>I. schmakovii</i>	0	0	0	0
<i>I. sisianica</i>	0	0	0	0
<i>I. sophenensis</i>	0	0	0	0
<i>I. stocksii</i>	0	0	0	0
<i>I. straussii</i>	0	0	0	0
<i>I. subdecolorata</i>	0	0	0	0
<i>I. swensoniana</i>	0	0	0	0
<i>I. tadshikorum</i>	0	0	0	0
<i>I. tarhunensis</i>	0	0	0	0
<i>I. tubergeniana</i>	0	0	0	0
<i>I. urmiensis</i>	0	0	0	0
<i>I. victoris</i>	0	0	0	0
<i>I. vvedenskyi</i>	0	0	0	0
<i>I. warleyensis</i>	0	0	0	0
<i>I. wendelboi</i>	0	0	0	0
<i>I. winkleri</i>	0	0	0	0
<i>I. × sinistra</i>	0	0	0	0
<i>I. xanthochlora</i>	0	0	0	0
<i>I. yebrudii</i>	0	0	0	0
<i>I. yedisuensis</i>	0	0	0	0
<i>I. zaprjagajevii</i>	0	0	0	0
<i>I. zetterlundii</i>	0	0	0	0
<i>I. zhaoana</i>	0	0	0	0

CHAPTER 3

Supplementary Table S3.2 - Scores given to the genera evaluated for the host-specificity testing of biocontrol agents to be used against *Iris pseudacorus* L. (Iridaceae). Category scores were assigned – according to the criteria detailed in Table 3.1 – based on the information gathered in Annex I. GEO: geographic overlap; ECO: ecological similarity; CS/EI: Conservation status and/or economic importance.

Genus	GEO	ECO	CS/EI	Score
<i>Iris</i>	3	3	3	1
<i>Libertia</i>	3	3	3	1
<i>Romulea</i>	3	3	3	1
<i>Sisyrinchium</i>	3	3	3	1
<i>Watsonia</i>	3	3	3	1
<i>Crocasmia</i>	3	3	2	0.97
<i>Dietes</i>	3	3	2	0.97
<i>Trimezia</i>	3	3	2	0.97
<i>Calydorea</i>	3	3	1	0.93
<i>Cypella</i>	3	3	1	0.93
<i>Diplarrena</i>	3	3	1	0.93
<i>Herbertia</i>	3	3	1	0.93
<i>Aristea</i>	3	2	3	0.9
<i>Babiana</i>	3	2	3	0.9
<i>Chasmanthe</i>	3	2	3	0.9
<i>Gladiolus</i>	3	2	3	0.9
<i>Hesperantha</i>	3	2	3	0.9
<i>Isophysis</i>	3	3	0	0.9
<i>Ixia</i>	3	2	3	0.9
<i>Moraea</i>	3	2	3	0.9
<i>Tritonia</i>	3	2	3	0.9
<i>Dierama</i>	3	2	2	0.87
<i>Bobartia</i>	3	2	1	0.83
<i>Geissorhiza</i>	3	2	1	0.83
<i>Gelasine</i>	3	2	1	0.83
<i>Lapeirousia</i>	3	2	1	0.83
<i>Nemastylis</i>	3	2	1	0.83
<i>Orthrosanthus</i>	3	2	1	0.83
<i>Patersonia</i>	3	2	1	0.83
<i>Syringodea</i>	3	2	1	0.83
<i>Freesia</i>	2	3	3	0.8
<i>Salpingostylis</i>	3	2	0	0.8

CHAPTER 3

Genus	GEO	ECO	CS/EI	Score
<i>Eleutherine</i>	3	1	1	0.73
<i>Olsynium</i>	3	1	1	0.73
<i>Tritoniopsis</i>	2	3	1	0.73
<i>Ennealophus</i>	3	1	0	0.7
<i>Ferraria</i>	2	2	3	0.7
<i>Phalocallis</i>	2	3	0	0.7
<i>Sparaxis</i>	2	2	3	0.7
<i>Codonorhiza</i>	2	2	1	0.63
<i>Klattia</i>	2	2	1	0.63
<i>Micranthus</i>	2	2	1	0.63
<i>Nivenia</i>	2	2	1	0.63
<i>Pillansia</i>	2	2	1	0.63
<i>Thereianthus</i>	2	2	1	0.63
<i>Alophia</i>	3	0	0	0.6
<i>Crocus</i>	1	3	3	0.6
<i>Devia</i>	2	2	0	0.6
<i>Duthieastrum</i>	3	0	0	0.6
<i>Melasphaerula</i>	2	2	0	0.6
<i>Schizorhiza</i>	2	2	0	0.6
<i>Witsenia</i>	2	2	0	0.6
<i>Mastigostyla</i>	2	1	1	0.53
<i>Radinosiphon</i>	2	1	1	0.53
<i>Afrocrocus</i>	2	1	0	0.5
<i>Afrosolen</i>	2	1	0	0.5
<i>Xenoscapa</i>	2	1	0	0.5
<i>Solenomelus</i>	1	2	0	0.4
<i>Tigridia</i>	1	1	2	0.37
<i>Tapeinia</i>	1	1	0	0.3
<i>Hesperoxiphion</i>	0	1	1	0.13
<i>Cobana</i>	0	1	0	0.1
<i>Geosiris</i>	0	1	0	0.1
<i>Larentia</i>	0	1	0	0.1
<i>Savannosiphon</i>	0	1	0	0.1
<i>Cyanixia</i>	0	0	1	0.03
<i>Cipura</i>	0	0	0	0
<i>Zygotritonia</i>	0	0	0	0

CHAPTER 3

Supplementary Table S3.3 – Results of the host-specificity trials conducted with *Aphthona nonstriata* on various plant species and varieties. The facility where testing occurred (BE: Belgium; ZAR: South Africa), the number of replicates (*N*), number of insects tested (*F0*) and number of emerged insects (*F1*) are indicated for each test species and for the relative control plant (i.e. *Iris pseudacorus*). The ratio of emergence between test and control is used to compare data from trials conducted at different moments. Only plants for which insects emergence was recorded in no-choice trials were tested in choice trials.

Trials	Testing Facility	Species	Test plant			Control plant			Ratio
			<i>N</i>	<i>F0</i>	<i>F1</i>	<i>N</i>	<i>F0</i>	<i>F1</i>	
NO-CHOICE	BE	<i>Iris ensata</i>	4	80	0	4	80	435	0
	ZAR	<i>I. laevigata</i> var1	5	50	71	3	30	170	0.25
	ZAR	<i>I. laevigata</i> var2	5	50	209	3	30	119	1.05
	ZAR	<i>I. sibirica</i> SA	3	30	107	2	20	77	0.93
	BE	<i>I. sibirica</i> BE	4	80	0	4	80	435	0
	ZAR	<i>I. ser. Hexagonae</i> sp1	5	50	0	3	30	50	0
	ZAR	<i>I. ser. Hexagonae</i> sp2	3	30	0	2	20	19	0
	ZAR	<i>I. ser. Hexagonae</i> sp2	3	30	0	2	20	19	0
	BE	<i>I. foetidissima</i>	4	80	159	4	80	435	0.37
	ZAR	<i>I. spuria</i> var1	3	30	61	2	20	27	1.54
	ZAR	<i>I. spuria</i> var2	5	50	43	3	30	44	0.57
	ZAR	<i>I. orientalis</i>	5	50	264	3	30	71	2.2
	ZAR	<i>I. confusa</i>	5	50	32	3	30	23	0.83
	ZAR	<i>I. domestica</i>	5	50	98	3	30	11	5.34
	ZAR	<i>I. × germanica</i> var1	3	30	0	2	20	23	0
	ZAR	<i>I. × germanica</i> var2	3	30	0	2	20	25	0
	BE	<i>I. × germanica</i> var3	4	80	0	4	80	435	0
	ZAR	<i>Dietes bicolor</i>	5	50	0	3	30	95	0
	ZAR	<i>D. grandiflora</i>	5	50	0	5	50	131	0
	ZAR	<i>D. iridioides</i>	5	50	0	3	30	17	0
	ZAR	<i>Ferraria crispa</i>	3	30	0	2	20	66	0
	ZAR	<i>Moraea huttonii</i>	4	40	0	2	20	20	0
	ZAR	<i>M. reticulata</i>	4	40	0	2	20	21	0
	ZAR	<i>Trimezia gracilis</i>	5	50	0	3	30	70	0
	ZAR	<i>Dierema igneum</i>	3	30	0	2	20	30	0
	ZAR	<i>Hesperantha coccinea</i>	3	30	0	2	20	35	0
	BE	<i>Crocasmia × lucifer</i>	3	60	0	4	80	435	0
	ZAR	<i>Gladiolus grandiflora</i>	3	30	0	2	20	21	0
	BE	<i>G. dalenii</i>	4	80	0	3	60	119	0

CHAPTER 3

Trials	Testing Facility	Species	Test plant			Control plant			Ratio
			N	F0	F1	N	F0	F1	
	ZAR	<i>Watsonia angusta</i>	5	50	0	3	30	29	0
	ZAR	<i>W. borbonica</i>	5	50	0	3	30	32	0
	ZAR	<i>W. pillansii</i>	3	30	0	2	20	21	0
	ZAR	<i>Aristea ecklonii</i>	5	50	0	3	30	25	0
	BE	<i>Carex pendula</i>	4	80	0	3	60	188	0
CHOICE	ZAR	<i>I. laevigata</i> var1	5	50	52	5	50	76	0.66
	ZAR	<i>I. laevigata</i> var2	5	50	74	5	50	58	1.21
	ZAR	<i>I. sibirica</i> SA	3	30	89	3	30	97	0.93
	ZAR	<i>I. spuria</i> var1	3	30	67	3	30	61	1.08
	ZAR	<i>I. spuria</i> var2	5	50	108	5	50	126	0.87
	ZAR	<i>I. orientalis</i>	5	50	308	5	50	122	2.52
	ZAR	<i>I. confusa</i>	5	50	23	5	50	66	0.35
	ZAR	<i>I. domestica</i>	4	40	57	4	40	98	0.58

CHAPTER 4

Raising winter temperatures will favor the germination of *Iris pseudacorus* L. (Iridaceae) in Argentina - A flea beetle might hold the key to control seedling recruitment

Minuti, G., Gervazoni, P., Nicoló, R., Delange, O., Cantarelli, M., Franceschini, C., Sosa, A., Stiers, I., & Coetzee, J.A. [Manuscript in preparation].

ABSTRACT

The European macrophyte *Iris pseudacorus* has invaded temperate wetlands around the world and is considered a suitable target for biological control across the Southern Hemisphere. The primary candidate biocontrol agent is *Aphthona nonstriata*, a flea beetle that damages the foliar tissue of the plant in its adult stage, and feeds on the rhizomatic structures as a larva. Studies across the invaded range of the plant suggest sexual reproduction as its main means of dispersal. This, coupled with the challenges posed by climate change on weed biocontrol, calls into question the ability of *A. nonstriata* to control the recruitment and spread of its target. Accordingly, the objectives of the current study were a) to compare the germination and early growth of *I. pseudacorus* seeds from Belgium (native range) and Argentina (invaded range), using the absence of cold stratification as a proxy of climate change; and b) to evaluate the ability of *A. nonstriata* to stunt the growth and photosynthetic activity of different plant stages of *I. pseudacorus*, including seedlings. Our results confirmed that invasive seeds have a higher germination potential compared to seeds from the native range. Furthermore, seeds from Argentina performed significantly better in the absence of cold stratification, suggesting an adaptation to warmer winter temperatures. The impact assessment proved that

CHAPTER 4

A. nonstriata is able to affect *I. pseudacorus*' growth and survival. Plants grown by rhizome fragmentation showed decreased photosynthetic activity and biomass accumulation when exposed to herbivory by the beetle. Impact on plants grown from seeds was significantly higher, with herbivory causing severe defoliation and consistently leading to plant mortality within 4 to 6 weeks. Altogether, the present results offer new knowledge on the role of germination and climate change in the invasion biology of *I. pseudacorus*, and their implication for biological control. Further research is needed to develop management strategies counteracting the sexual dispersal of this weed. Although our results do not preclude the need to research a biocontrol agent attacking *I. pseudacorus* sexual reproductive structures, they suggest that *A. nonstriata* could be able to control its seedling recruitment.

1. INTRODUCTION

Despite their limited spatial extension, freshwater ecosystems provide countless services and constitute an invaluable habitat for biodiversity (Kingsford et al., 2016; Lynch et al., 2023a,b). Sadly, these habitats are especially vulnerable to biological invasions, with a quarter of the world's worst invasive plants being wetland species (Zedler & Kercher, 2004). These species can act as ecosystem engineers by outcompeting native macrophytes, modifying habitat structures and functions, and altering the chemo-physical characteristics of aquatic ecosystems, with direct ecological and economic consequences, as well as deleterious impacts on human livelihood and well-being (Strayer, 2010; Lovell et al., 2016; Keller et al., 2018; Hassan & Nawchoo, 2020). Several studies on the underlying mechanisms and traits associated with invasive alien aquatic plants have highlighted propagule pressure and dispersal as key factors for their invasion success (Catford et al., 2009; Thomaz et al., 2015; Molina-Montenegro et al., 2018). These are linked to the production of numerous sexual and asexual

CHAPTER 4

propagules (i.e. seeds, spores and vegetative fragments), their buoyancy and tolerance to desiccation (Hussner et al., 2021).

Climate change and its ramifications (e.g. raising temperature, sea level rise and extreme weather events) are expected to have an effect on the patterns and outcomes of biological invasions by altering the biotic and abiotic conditions of the recipient environment, as well as opening new pathways of introduction for invasive species and modifying current modes and rates of dispersal (Travis et al., 2013; Reid et al., 2019; Robinson et al., 2020). This effect is dependent on the species and the geographic area considered, but for some invasive aquatic plants, an increase in the potential distribution is predicted under warmer climates (Bellard et al., 2013; Gillard et al., 2017a,b,c; Bellard et al., 2018; Nikkel et al., 2023). Climate change can also affect cold stratification, germination and early plant growth, influencing the potential reach and outcome of biological invasions (Gillard et al., 2017a). These in turn will likely affect management actions aimed at eradicating, containing, and mitigating invasions (Robinson et al., 2020). This is particularly relevant with respect to classical biological control (Kriticos et al., 2011). In fact, due to the interconnected nature of ecological systems, it has been increasingly difficult to predict the effect of climate change on invasive alien plants, weed biocontrol agents, and their interactions under climate change (Sun et al., 2017; Sun et al., 2020; Sun et al., 2022a,b).

The success of biological control is dependent on the ability of the agent to regulate the population of the weed. As such, it is pivotal to assess the effectiveness of perspective agents before release (Hinz et al., 2024). Arguably the most important factor driving plant demography is photosynthetic rate (Schaffner et al., 2020), and recent studies have shown the potential of biocontrol agents to reducing the photosynthetic activity – and associated growth – of several weed species (Marlin et al., 2013; Cowie et al., 2016; 2018; Miller et al., 2019; Paterson et al., 2024).

CHAPTER 4

Yellow flag, *Iris pseudacorus* L. (Iridaceae), is a perennial emergent macrophyte native to Europe, northern Africa and western Asia (Sutherland, 1990). Within its native range, yellow flag can be found at the edges of streams and ponds, in open and forested flood plains, along shorelines, and in freshwater and brackish marshes (Sutherland, 1990). Historically exported as an ornamental plant, but also used in some countries for wastewater treatment and phytoremediation (Beaury et al., 2021; Venditti et al., 2022; Khatib et al., 2022), this species has often escaped cultivation and is now considered invasive in wetlands all over the world (Gervazoni et al., 2023; Minuti et al., 2023; Chapter 2).

In Argentina, *I. pseudacorus* was first recorded in 1931 and has since been reported from 7 of the 15 continental ecoregions of the country (Gervazoni et al., 2020), confirming its ability to adapt to a wide range of environmental conditions and ecological communities (Mopper et al., 2016; Gerwing et al., 2021). However, most records (> 60%) of the plant are concentrated in the Pampa area surrounding Buenos Aires, characterized by a warm temperate climate similar to that found across the native European range of *I. pseudacorus* (Gervazoni et al., 2020; Minuti et al., 2022). This region hosts numerous wetlands and riparian grasslands, harboring a great diversity of aquatic plants and associated fauna that could be threatened by *I. pseudacorus* invasion (Gervazoni et al., 2020). Indeed, studies in other regions invaded by yellow flag have highlighted the negative effects on biodiversity (Hayasaka et al., 2018; Gallego-Tévar et al., 2022) and ecosystem structure (Jacobs et al., 2011; USDA, 2013) caused by dense infestations.

Iris pseudacorus is an extremely tolerant and resilient weed (Schlüter & Crawford, 2001; Blokhina et al., 2003; Mopper et al., 2016; Gallego-Tévar et al., 2024), making its eradication via manual, mechanical and chemical control methods often ineffective (Jaca & Mkhize, 2015; Sandenbergh et al., 2024). Over the last five years, a biological control program was launched targeting this weed

CHAPTER 4

across various invaded regions of the Southern Hemisphere (Hill & Coetzee, 2017; McGrannachan & Barton, 2019; Gervazoni et al., 2020). Field surveys conducted across the weed's native range prioritized a flea beetle, *Aphthona nonstriata* Goeze (Coleoptera: Chrysomelidae), as the primary candidate biocontrol agent (Minuti et al., 2021; Chapter 1). The adults of this species feed upon the leaves of yellow flag, while its larval stages attack the rhizomatic system, exerting direct and indirect control over the vegetative structures of the plant (Minuti et al., 2021; Chapter 1).

However, like many other emergent invasive alien aquatic plants, *I. pseudacorus* can reproduce both vegetatively and sexually. Vegetative reproduction occurs via cylindrical rhizomes that grow radially forming large clonal populations. These rhizomes can also fragment and be transported by flowing water to initiate satellite colonies (Sutherland, 1990). Similarly, yellow flag seeds have a hard external coat and an internal air space allowing them to float and remain buoyant for several months, which allows them to spread over long distances (Coops & Van Der Velde, 1995). As is the case for other aquatic perennials (Barrett, 2015), vegetative reproduction was initially considered the predominant mode of spread for this species. However, molecular studies carried out both in the native and introduced range found high genetic differentiation among yellow flag populations, suggesting the spread of this species to be strongly driven by sexual reproduction (Lamote et al., 2002; Gaskin et al., 2016).

Since biological invasions by aquatic plants are strongly linked to the ability of an alien to establish and spread, knowledge about mechanisms of reproduction, dispersion and establishment in suitable areas is essential to design and deploy effective prevention and control strategies (Catford et al., 2009; Hussner et al., 2017). High variability in sexual and vegetative reproduction is known to potentially affect the outcome of biological control where agents only target certain plants structures (West et al., 2023). Thus, the observed seed dispersal

CHAPTER 4

of *I. pseudacorus* calls into question the ability of *A. nonstriata* to control its spread across the invaded range.

Accordingly, the objectives of the current study were twofold. First, we wanted to compare the germination and early growth of *I. pseudacorus* seeds between its native European and invaded South American range, while estimating the influence of increased winter temperatures (i.e. the presence or absence of cold stratification). Secondly, we wanted to evaluate the ability of *A. nonstriata* to stunt the growth and photosynthetic efficiency of *I. pseudacorus*, at various insect densities and across different life stages of the plant.

2. MATERIALS & METHODS

2.1. GERMINATION EXPERIMENT

Field samplings were carried out in September 2021 in Belgium and in February 2022 in Argentina. A total of 12 yellow flag populations (Belgium: 6; Argentina: 6) were sampled to collect fresh seeds to use for the germination experiment (Supplementary Table S4.1). At least 30 fruits were collected per population. Where possible, stems were selected from discrete patches at least 10 m apart, in an attempt to collect from distinct individuals. Mature fruits were stored in paper bags and transferred to the laboratory facility of the respective country of collection (Belgium: Vrije Universiteit Brussel, Brussels; Argentina: Fundación para el Estudio de Especies Invasivas, Buenos Aires). Once in the laboratory, the fruits were opened, and the seeds extracted and stored in dark paper envelopes with silica gel at room temperature. Two months prior to the beginning of the experiment, 120 mature seeds were selected for each population. Half of them ($n = 60$) were cold treated by placing them in a dark container with water at 4°C to mimic natural vernalization (Coops & Van der Velde, 1995). The other half of the seeds – hereafter referred to as the control – were left in a dark environment at room temperature for the duration of the pre-treatment. After two months, all

CHAPTER 4

seeds were retrieved, lightly washed in deionized water, and placed equidistantly upon moist paper towel in petri dishes ($\varnothing$ 8.5 cm). For each population, 3 petri dishes containing 20 cold-treated seeds and 3 petri dishes containing 20 control seeds were established. The petri dishes were randomly placed in germination chambers set to a 12h/12h photoperiod with gradual temperature gradient from 15°C /25°C. These conditions correspond to high summer temperatures (i.e. July – August) in the region of Brussels, Belgium, and mid-spring temperatures (i.e. October – November) in the area of Buenos Aires, Argentina (Supplementary Figure S4.1). The germination chambers were checked every 2-3 days for 6 consecutive months. At each check, the seeds were sprayed with distilled water to avoid desiccation, and any sign of germination – defined here as the protrusion of the radicle from the testa – was recorded. Attention was put into preventing the proliferation of mold during the experiment by removing any fungal development with a dry paper towel. Once the seeds were germinated and the first cotyledon had fully developed, they were transplanted into a multi-pot tray containing a 1:1 mixture of sand and potting soil. The tray, which dimensions ($2.5 \times 2.5 \times 3.0$ cm) were limited by the available space, was encased in a glasshouse box and kept within the germination chamber. This step was necessary to allow vertical growth of the plants while retaining high moisture conditions, which are fundamental for seedlings survival. After two weeks, once the plants had grown to 10 cm in height and become less dependent on moisture, they were removed from the glasshouse box and transplanted into a larger multi-pot tray ($4.0 \times 4.0 \times 5.0$ cm), still within the growth chambers, to allow for further growth. Each seedling was kept in the growth chambers for a total of six weeks from the moment of first germination. After this period, they were removed from their pot, their roots gently cleaned from excess soil, then the leaves were excised with a scalpel at the height of the radicle. Thereafter the plants were oven-dried at 60°C for 48h before measuring above- and below-ground biomass with a high-precision scale (Sartorius

CHAPTER 4

TE214S). The number of germinated seeds that survived until harvesting was used to calculate seedlings survival rate for petri dish, population, treatment and country.

Final germination rate (%) and mean germination time (days) were used to evaluate the results of the germination experiment, whereas above- and below-ground dry biomass (mg) were used to compare differences in early growth. All data were checked for normality and homoscedasticity using the Shapiro-Wilk and Levene's test, respectively. If necessary, data were log-transformed in order to meet statistical model assumptions. Parametric data were analysed using a two-way Analysis of Variance (ANOVA), with population nested within country, to test for differences between treatments, countries, and their interaction. When *P*-values were significant (<0.05), Tukey's HSD test with Bonferroni correction was applied for multiple comparisons. All statistical analyses were conducted using the packages *GerminaR*, *car*, *onewaytests*, *dplyr*, *tidyverse*, *ggpubr*, *rstatix*, *ggplot2* and *multcomp* in R version 4.3.1 (R Core Team, 2023).

2.2. IMPACT ASSESSMENT

2.2.1. Impact on growth and development of different plant life-stages

Two experiments were conducted to evaluate the impact of *A. nonstriata* on the growth and development of different life-stages of *I. pseudacorus*.

In the first experiment, vegetatively grown *I. pseudacorus* plants were purchased from a local nursery in Belgium and transferred to the greenhouse facilities of the Vrije Universiteit Brussel (VUB). Here they were re-planted in medium pots (25 × 20cm) with a 1:1 mix of sand and potting soil. During the transfer, the number and length of all leaves of each plant was recorded and the rhizome washed and measured. Plants of similar size were set aside accordingly. These were left to establish in the greenhouse for a period of two weeks, after which they were individually placed within mesh netted cages (60 × 60 × 90cm), where they were

CHAPTER 4

left to acclimatize for another two weeks. During this period, a new generation of *A. nonstriata* was reared on *I. pseudacorus* plants previously grown within the greenhouse. Upon emergence from the pupal stage, naïve adult insects were moved onto the test plants following three treatments: i) control (insect-free); ii) low density (10 adult insects per plant); and iii) high density (20 adult insects per plant).

In the second experiment, the plants used were grown from seeds collected in Belgium during the previous fruiting season and germinated following the same protocol detailed in the previous section. At 6 weeks development, the plants were transferred to the greenhouse facility of the Vrije Universiteit Brussel, Belgium. Here they were transferred into bigger pots (10 × 10cm) filled with a 1:1 mix of sand and potting soil, individually moved to mesh netted cages (40 × 40 × 40 cm) and left to acclimatize for one week. During the transfer, the number and length of all leaves of each plant was measured. As the rhizome is not yet formed at this stage, this could not be included in the measurements. After one week, newly emerged adult flea beetles were introduced following three different treatments: i) control (insect-free); ii) low density (4 insects per plant); and iii) high density (8 insects per plant).

For both experiments, each treatment was replicated 8 times, and the 24 test plants were arranged in a Latin square design within the greenhouse. Care was taken to distribute the test plants among treatments to minimize variability in initial conditions (e.g. number of fully formed leaves, length of the tallest leaves, etc.). The insects were left on the plants for a total of 6 weeks, during which the plants were watered and monitored at 2-3 days intervals. At the end of the experiment, adult beetles were removed from all cages and the plants were harvested. These were removed from their pots and gently washed off the sand/soil mix to preserve the rhizomatic system intact. During harvesting, the number and size of all leaves, as well as the number of intact, damaged and dead

CHAPTER 4

leaves (i.e. a leaf was considered dead if all of its parts were dried) were measured. Damage caused by the insect was assessed visually and scored as a percentage of the total leaf area. Finally, the plants were dried in an oven at 60 °C for 48h, following which their above- and below-ground biomass were weighted separately on a high precision scale (Sartorius TE214S).

2.2.2. Impact on photosystems in the invaded range

This experiment took place within the quarantine facilities of the Centre for Biological Control (CBC), Rhodes University, Makhanda. The environmental conditions in the quarantine facility were held constant at a temperature of 25 ± 2 °C, a relative humidity of 70 ± 10 %, and a 16/8h photoperiod. Eighteen individual *I. pseudacorus* plants were harvested from a nearby invaded site (-33°19'27", 26°36'39"), transported to the quarantine facilities of the Centre for Biological Control and planted in pots (25 × 20 cm) placed inside individual netted cages (60 × 60 × 90 cm). Here they were left to establish for a period of two weeks, after which newly emerged *A. nonstriata* adults were released onto each plant following three treatments: i) control (insect-free); ii) low density (10 adult insects per plant); and iii) high density (40 adult insects per plant). The plants were monitored and watered every 2 to 3 days for a period of 10 weeks. Regular measurements of plant photosynthetic efficiency and chlorophyll content were taken at the beginning of the experiment, after a period of 4 weeks, and every week thereafter until its conclusion. Photosynthetic efficiency represents the fraction of light energy converted into chemical energy during photosynthesis. This is calculated as the ratio of variable to maximum fluorescence (Fv/Fm) obtained from a chlorophyll fluorescence induction curve, which was measured by means of a portable fluorometer (Handy PEA⁺, Hansatech Instruments). This measure has been proven useful in determining the effectiveness of biological control agents in post-release evaluations (Miller et al., 2019). Herbivory can also affect the uptake and concentrations of

CHAPTER 4

nutrients needed by a plant to attend optimum growth. Nutrient deficiencies can manifest, in addition to other symptoms, as a reduction in leaf chlorophyll content. This reduces the amount of solar radiation that can be absorbed, which in turn limits the plant primary production. Since chlorophyll-rich leaves will bounce away more radiation in the near-infrared spectrum to avoid overheating, chlorophyll content is linearly correlated with the difference between the reciprocal reflectance of the green and near-infrared bands. This metric, referred to as chlorophyll content index (CCI), was measured using a hand-held chlorophyll content meter (CCM-200, ADC Bioscientific).

2.2.3. Statistical analyses

The following parameters were used to assess differences between treatments across the three experiments: i) foliar growth, expressed as the accrued growth of all leaves of each plant between the beginning and the end of the experiment; ii) dead leaves ratio, measured as the number of dead leaves over the total number of leaves for each plant; iii) above- and below-ground dry biomass, used as proxies of plant health and vigour iv) photosynthetic efficiency (F_v/F_m), calculated as the as the variable fluorescence (F_v) over the maximum fluorescence (F_m); v) chlorophyll content index (CCI); and vi) leaf area damaged, as a percentage of the total leaf area.

Shapiro-Wilk's and Levene's test were used to test whether the data conformed to the assumptions of normality and homoscedasticity, respectively. Where this was the case, an analysis of variance (ANOVA) was used to determine whether any significant differences existed between treatments. This was followed by post-hoc pairwise t-tests to detect where the significant differences occurred. In case of non-parametrical data, a Kruskal-Wallis rank sum test was used instead, followed by a Dunn's test for multiple comparisons. In both cases, the Bonferroni correction was applied to post-hoc testing. Because the indices of photosynthetic activity (F_v/F_m and CCI) were recorded at fixed intervals during

CHAPTER 4

the experiment, a two-way repeated measures ANOVA (preceded by a Mauchly's test to check for the assumption of sphericity) was performed to test differences between treatments within different times, accounting for sample identity. This was followed by post-hoc pairwise t-tests to detect where the significant differences occurred. Finally, a Kendall rank correlation coefficient was computed to explore relationships between leaf area damaged by the insect and the photosynthetic activity (Fv/Fm and CCI) of the plants after 10 weeks. All statistical analyses were conducted in R version 4.3.1 (R Core Team, 2023).

3. RESULTS

Table 4.1 - ANOVA results of mean germination rate, mean germination time, above-ground dry biomass and below-ground dry biomass for seeds of *I. pseudacorus*. Differences between country (Belgium vs Argentina), treatment (cold-stratification vs control), their interaction (*), and the nested effect of population of origin within each country (/), were tested.

Index	Term	Sum square	F-value	P-value
Mean Germination Rate	Country	13553	65.452	< 0.001 ***
	Treatment	3443	16.625	< 0.001 ***
	Country*Treatment	592	2.857	0.096
	Country/Population	23941	9.635	< 0.001 ***
Mean Germination Time	Country	0.334	2.089	0.153
	Treatment	26.040	162.704	< 0.001 ***
	Country*Treatment	6.294	39.330	< 0.001 ***
	Country/Population	10.929	5.691	< 0.001 ***
Above-ground Biomass	Country	0.00031	1.711	0.191
	Treatment	0.00002	0.098	0.754
	Country*Treatment	0.00375	20.806	<0.001 ***
	Country/Population	0.00744	3.442	<0.001 ***
Below-ground Biomass	Country	0.001975	67.462	< 0.001 ***
	Treatment	0.000765	26.130	< 0.001 ***
	Country*Treatment	0.001197	40.883	< 0.001 ***
	Country/Population	0.001897	5.402	< 0.001 ***

CHAPTER 4

3.1. GERMINATION AND EARLY GROWTH

The germination rate of *I. pseudacorus* seeds showed significant differences between countries, treatments, and populations within each country (Table 4.1). Seeds from Argentina displayed a significantly higher germination rate than seeds from Belgium, under both the cold and control treatment (Figure 4.1A). However, the cold treatment had a significant negative effect on seeds from Argentina, but not on seeds from Belgium. Within Argentina, control seeds showed a significantly higher germination rate ($84.2 \pm 12.2\%$) compared to cold-treated ones ($65.5 \pm 25.7\%$). Whereas in Belgium, germination rates were comparable between the cold treatment ($44.3 \pm 15.6\%$) and the control ($52.1 \pm 28.9\%$). Overall, there was a very high variability in the germination rate across populations within both Argentina (25 – 100%) and Belgium (0 – 95%).

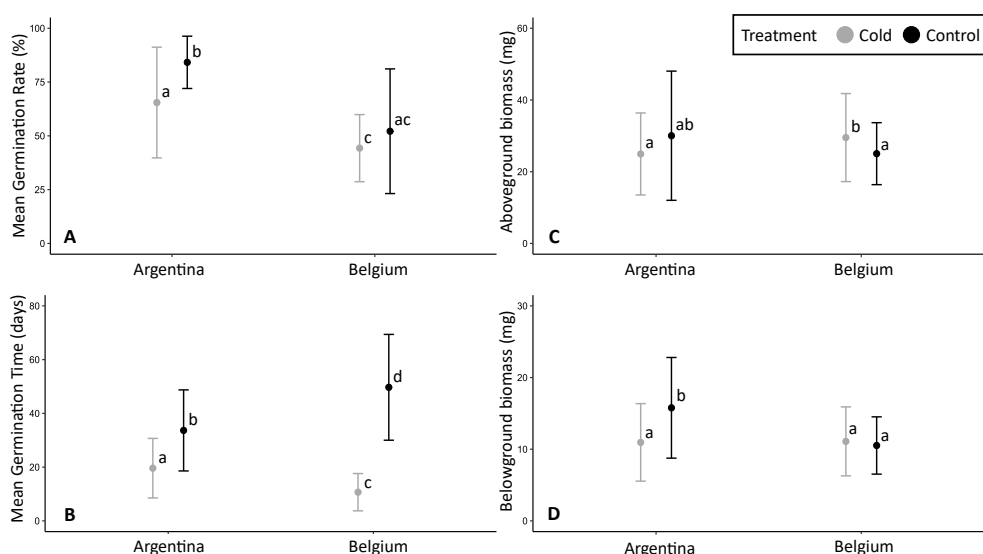


Figure 4.1 – Comparison of germination and early growth of cold-treated (gray) and control (black) seeds of *Iris pseudacorus* from Argentina and Belgium. **A)** Mean germination rate (%); **B)** Mean germination time (days); **C)** Aboveground biomass (mg); **D)** Belowground biomass (mg). Error bars display the mean (large black dot) and the standard deviation of the samples. Low case letters represent the results of post-hoc pairwise comparisons between treatments and countries (same letter indicates no significant difference).

CHAPTER 4

The mean time to germination of seeds from Argentina and Belgium showed significant differences between treatments, populations, and the interaction of country and treatments (Table 4.1). On average, cold-treated seeds germinated significantly faster than the control seeds, regardless of the country (Figure 4.1B). However, this effect was more pronounced in Belgium, where cold-treated seeds germinated 5 times faster (10.7 ± 6.9 days) than control ones (49.7 ± 19.7 days). In comparison, cold-treated seeds from Argentina germinated only 1.7 times faster (19.6 ± 11.1 days) than control seeds (33.7 ± 15.1 days). Accordingly, a lower variability in mean time to germination was observed across populations within Argentina (8 – 61.2 days) than within Belgium (2.3 – 103 days), where germination was delayed in the absence of cold-stratification (Supplementary Figure S4.2).

A significant inverse effect of the interaction term between country and treatment was observed on the biomass accumulation of early stages of *I. pseudacorus* (Table 4.1). In Argentina, there was no significant effect of the treatment on the aboveground biomass (Figure 4.1C), whereas control seeds produced significantly more belowground biomass (15.8 ± 7 mg) than control seeds (11 ± 5.4 mg) (Figure 4.1D). On the other hand, in Belgium cold-treated seeds produced significantly more aboveground biomass (29.5 ± 12.3 mg) than the control (25 ± 8.6 mg) (Figure 4.1C), but the belowground biomass accumulation was comparable (Figure 4.1D). Overall, there was a significant variability in biomass accumulation between *I. pseudacorus* populations, especially within Argentina (± 88.3 mg).

Finally, seedling survival was strongly linked to the stratification treatment. While both countries showed around 80% survival rate in the control group, cold-stratification increased seedlings mortality by 3.7% in Belgium and by 24.1% in Argentina, on average.

CHAPTER 4

3.2. IMPACT ASSESSMENT

3.2.1. Impact on growth and development of different plant life-stages

Herbivory by *A. nonstriata* caused a significant impact on the foliar growth, dead leaves ratio, above- and below-ground biomass accumulation of *I. pseudacorus* test plants (Table 4.2). In plants grown from rhizomes, a significant difference was observed in foliar growth and biomass accumulation between the control and the two herbivory treatments, but not between the low and high insect density (Figure 4.2). Compared to the control, test plants subjected to herbivory by the insect showed, on average, 23% less foliar growth and 32% to 58% fewer biomass accumulation in their above- and belowground organs, respectively. The ratio of dead leaves was significantly different between all three treatments, with test-plants subject to herbivory losing between 50% and 100% of their leaves (Figure 4.2).

Impact on plants grown from seeds was even more noticeable, with significant differences across all variables between control and test plants, but not between the two insect densities (Figure 4.2). During the 6 weeks of the experiment, control plants grew 4 to 5 times taller and accumulated 5 to 30 times more biomass, on average, than test plants subjected to herbivory, irrespective of insect density. Accordingly, test plants in both density treatments showed a 75-100% dead leaves ratio, compared to 0-20% observed in the control plants (Figure 4.2). As expected, leaf damage accumulated significantly faster in the high insect density treatment, reaching lethal levels within 3 to 4 weeks. Nonetheless, plant mortality was observed also within the low-density treatment within 5 to 6 weeks (Supplementary Figures S4.3 and S4.4).

CHAPTER 4

Table 4.2 - ANOVA and Kruskal-Wallis test comparisons of the impact of different *A. nonstriata* densities on *I. pseudacorus* in terms of foliar growth, dead leaves ratio, above- and below-ground biomass accumulation.

Experiment	Response	Sum square	Test Statistic	P-value
Impact on plants grown from rhizomes	Foliar growth	14767.2	F-value = 16.217	< 0.001 ***
	Dead leaves ratio	0.75366	F-value = 62.194	< 0.001 ***
	Aboveground biomass	10.4226	F-value = 14.422	< 0.001 ***
	Belowground biomass	305.35	F-value = 15.247	< 0.001 ***
Impact on plants grown from seeds	Foliar growth	2558.77	F-value = 89.119	< 0.001 ***
	Dead leaves ratio	-	H-value = 19.761	< 0.001 ***
	Aboveground biomass	1.13902	F-value = 80.835	< 0.001 ***
	Belowground biomass	0.44837	F-value = 163.33	< 0.001 ***

CHAPTER 4

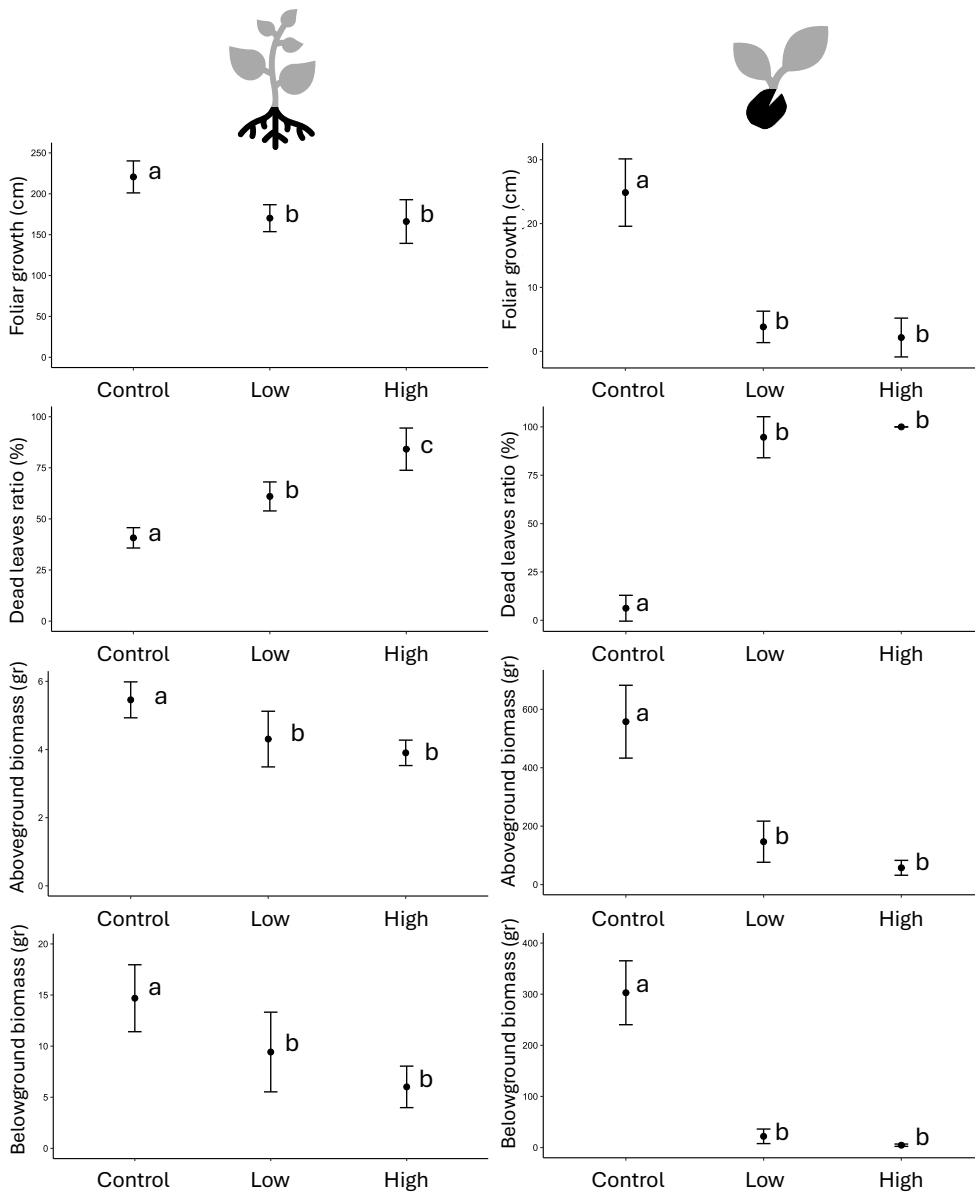


Figure 4.2 – Foliar growth, dead leaves ratio, above- and below-ground biomass accumulation of *Iris pseudacorus* plants subjected to 6 weeks of herbivory by *Aphis nonstriata*. Left panels indicate the response of plants grown vegetatively by rhizome fragmentation, whereas the right panels show the response of plants germinated from seeds. Error bars display the mean and standard deviation of the samples. Low case letters represent the results of post-hoc pairwise comparisons (same letter indicates no significant difference) between treatments within the same experiment. As insect density on plants grown from rhizomes and plants grown from seeds differed, no statistical comparisons were calculated between the two experiments.

CHAPTER 4

3.2.2. Impact on photosystems in the invaded range

There was a significant impact of herbivory on the photosynthetic efficiency and chlorophyll content of *I. pseudacorus* plants between treatments and over time (Table 4.3). Photosynthetic efficiency showed a significant decline only after 8 weeks of herbivory, with no difference between density treatments (Figure 4.3A). Accordingly, a significant negative correlation was observed between photosynthetic efficiency and leaf area damage as recorded the end of the experiment (Kendall's $R = -0.47$, $p\text{-value} = 0.011$). The chlorophyll content index showed a significant decrease compared to the control already after 4 weeks of herbivory, with no difference between density treatments (Figure 4.3B). Again, the chlorophyll content of the test plants at the end of the experiment was negatively correlated with the leaf area damaged by the insect (Kendall's $R = -0.55$, $p\text{-value} = 0.002$).

Table 4.3 – Repeated measures ANOVA comparing the effects on the photosynthetic efficiency (Fv/Fm) and chlorophyll content index (CCI) of *Iris pseudacorus* plants exposed to the feeding of *Aphthona nonstriata* at different densities (treatment) over time. The generalized effect size (GES) indicates the amount of variability attributable to the within-subjects factor.

Index	Effect	F-value	P-value	GES
Fv/Fm	Treatment	7.092	< 0.01 **	0.143
	Time	2.121	0.125	0.104
	Treatment * Time	2.492	< 0.05 *	0.215
CCI	Treatment	196.156	< 0.001 ***	0.766
	Time	1.659	< 0.001 ***	0.088
	Treatment * Time	6.651	< 0.001 ***	0.437

CHAPTER 4

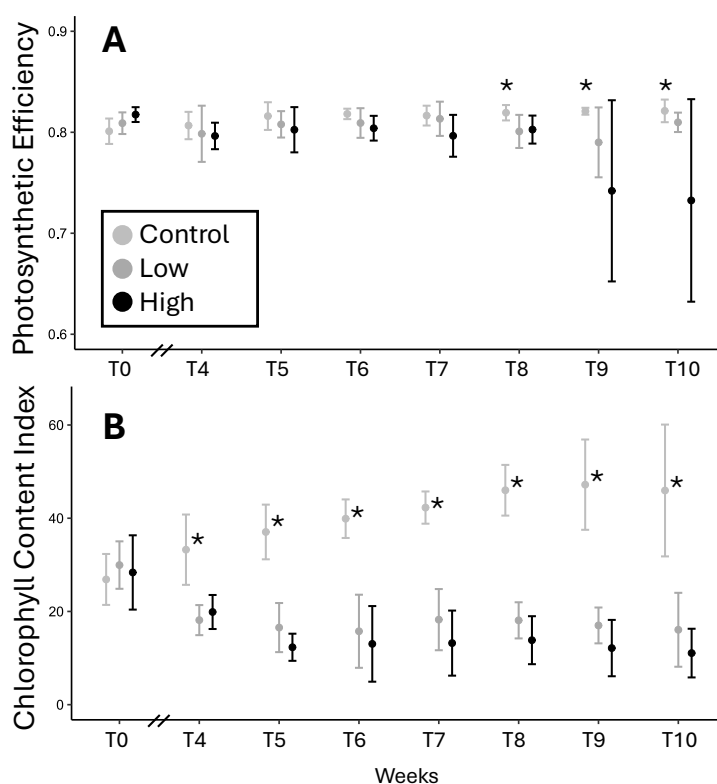


Figure 4.3 – Response over time in **A)** photosynthetic efficiency (Fv/Fm) and **B)** chlorophyll content index (CCI) of *I. pseudacorus* plants subjected to various densities of *A. nonstriata*. Treatments are Control: insect-free; Low: 10 insects per plant; High: 40 insects per plant. Error bars display the mean (large black dot) and the standard deviation of the samples. Asterisks (*) represent significant differences in post-hoc comparisons (for better visual representation, only significant differences are reported).

4. DISCUSSION

4.1. GERMINATION AND EARLY GROWTH

Our results highlighted some major differences in the germination and early growth of *I. pseudacorus*, both between cold- and warm-stratified seeds and between native and invaded range. First, the germination rate of Argentinian seeds was 1.5-fold higher compared to seeds from Belgium, irrespective of treatment. The mean germination reached by Argentinian populations was $74.1 \pm 22.4\%$, in line with what was observed for populations of *I. pseudacorus* in

CHAPTER 4

South Africa (83.3%) and North America (69.6 - 89.0%) under similar alternating temperature regimes (Sandenbergh, 2021; Gillard et al., 2021; Gillard et al., 2022; Thomson et al., 2023). This relatively high germination rate supports the hypothesis of sexual reproduction driving the high genetic differentiation observed among invasive *I. pseudacorus* populations (Gaskin et al., 2017). On the other hand, Belgian populations achieved a mean germination rate of $48.2 \pm 23.3\%$, which is substantially higher than the 22-25% reported by Sutherland (1990) and Coops & Van Der Velde (1995) for the native range. It is noteworthy however that the abovementioned results were obtained at constant temperatures of 20 to 25°C. As observed by Gillard and colleagues (2022), seeds of *I. pseudacorus* tend to reach low germination (< 25%) at constant temperature, whereas exposure to alternating temperatures can increase germinability. In Sutherland (1990), a series of germination rates ranging from 20% to 85% are listed, however these are mostly drawn from personal accounts and unpublished reports, where methodology is not provided. To our knowledge, this is the first study testing the germination potential of *I. pseudacorus* in the native range under controlled alternating temperature regimes.

Secondly, cold stratification had a negative impact on germination, decreasing the mean germination rate of Argentinian and Belgian seeds by 18.7% and 7.8%, respectively. This was unexpected, as cold stratification is often reported to have a positive effect on germination (Baskin & Baskin, 2014). This holds especially true for plants inhabiting regions experiencing short growing seasons, low annual temperatures and frequent winter frosts (Chen et al., 2015; Yang et al., 2020; Cheng et al., 2022). In these habitats, plants might control the timing of germination via seed dormancy, delaying germination until conditions are optimal for seedlings growth and survival. Dormancy can be broken both by physiological (e.g. gibberellic acid) and environmental factors, the most common being temperature and light (Baskin & Baskin, 2014). In seasonal climates, two main groups are distinguished: plants whose seeds break

CHAPTER 4

dormancy through periods of wet, cold winter exposure (i.e. cold stratification) and those that respond to dry, hot summer conditions (Baskin & Baskin, 2014). For the former, cold stratification allows seeds to react to early spring germination cues such as daily fluctuating temperatures and longer photoperiod (Liu et al., 2013). The latter, which includes most Mediterranean lowland wetland species, are characterized by cold-cued germination, allowing seedlings to take advantage of the mild winter season before the onset of summer drought (Carta, 2016; Mattana et al., 2021). In addition, many aquatic plant species exhibit a plastic germination response that is typical of neither group (Carta, 2016). This could be the case for *I. pseudacorus*. In fact, little evidence of a stratification requirement was found in invasive *I. pseudacorus* populations from North America, suggesting a lack of generalized primary dormancy (Gillard et al., 2022). These results are strengthened by records of vivipary in *I. pseudacorus* across its invaded range (Gillard et al., 2021; Sandenbergh, 2021); a phenomenon not observed, at least to our knowledge, in its native habitat.

As suggested by some authors, it is conceivable that invasive *I. pseudacorus* populations might be undergoing a shift in the timing of germination, with a portion of the seeds germinating in late summer and early autumn of the year of production, and another portion subject to winter dormancy (Gillard et al., 2022). In our experiment, the germination of Argentinian seeds was affected significantly more by cold stratification compared to the germination of seeds from Belgium. We argue that this phenomenon could be linked to the mortality of the non-dormant portion of seeds produced by the plant. In this scenario, Belgian plants, being continuously exposed to cold winter temperatures, would be selected to produce a small portion of non-dormant seeds. Contrarily, plants from Argentina might be experiencing a release from such environmental selection and a shift towards a more plastic germination strategy (Mattana et al., 2021). This hypothesis could also explain the significant difference we observed in germination speed between cold-stratified and control seeds across the two

CHAPTER 4

countries. During our experiment, seeds from Belgium germinated 5 times faster after cold stratification. This would be explained by a higher reliance on cold stratification to break seed dormancy and thus a faster response to fluctuating temperatures as germination cue (Liu et al., 2013). As a result, Belgian seeds were both the fastest – 10.7 days when cold-stratified – and the slowest – 49.7 days for the control – to germinate in our experiment. On the other hand, seeds from Argentina were less affected by cold stratification, which sped up germination from 33.7 to 19.6 days, on average. We also observed a minor, albeit significant, contrasting effect of cold stratification on the early growth of *I. pseudacorus* seedlings based on their origin. Belgian seeds that underwent cold-stratification produced heavier plants than warm-stratified ones, whereas the opposite was observed in seeds from Argentina. Specifically, cold-treated Belgian seeds invested more in aboveground organs (i.e. foliar tissue to maximize photosynthesis), whereas control seeds from Argentina accumulated a larger belowground biomass (i.e. rhizomatic system to increase nutrients uptake and storage). Although little information is available on the effect of stratification on early biomass accumulation, similar results were observed in Douglas fir (*Pseudotsuga menziesii* (Mirbel) Franco) from Europe and eastern North America (Houšková et al., 2023). A lower dependence on stratification could allow non-native *I. pseudacorus* populations to distribute germination in time, filling different phenological niches (Dong et al., 2023). Early germination may allow seedlings to establish before winter, offering a competitive advantage over plant species germinating in spring (Gioria & Pyšek, 2017).

On the other hand, the production of a portion of dormant seeds would ensure survival over rigid winters and germination in favorable conditions (Gioria et al., 2018). Invasive plants are known to exhibit broader germination cues in their non-native range as a result of phenotypic plasticity or rapid adaptive evolution in seed traits (Molina-Montenegro et al., 2018). In the case of *I. pseudacorus*, germination has been observed over a wide range of temperatures (18 – 40°C),

CHAPTER 4

inundation regimes, disturbance, and even salinity (Coops & Van Der Velde, 1995; Grewell et al., 2021; Gillard et al., 2021; Gillard et al., 2022). This has several implications for weed management, as most control strategies currently target vegetative stages of the plant. Only recently some forms of germination control have been suggested (Castillo et al., 2023). However, available options (e.g. flower cutting, monitoring of seedlings emergence, wrack burial) can be laborious, and the large-scale efficacy has yet to be determined. The release of a biocontrol agent targeting reproductive structures of the plant remains a viable alternative. The primary candidate for this function so far is the seed weevil *Mononychus punctumalbum* Herbst (Coleoptera: Curculionidae), selected during early native range surveys of the plant (Minuti et al., 2021; Chapter 1). Adult weevils bore small holes at the base of the plant flowers and on the fruits, where oviposition occurs. During the late summer months, the larvae of this weevil can be found within the fruits of the plant, usually in groups of 2 or 3 per fruit, each consuming 2 to 5 seeds before pupating (Authors, unpublished data). However, there are some concerns regarding the host-specificity of this species, which is known to attack and develop on a variety of *Iris* species (Gültekin & Korotyaev, 2012; Skuhrovec et al., 2017). Additionally, field observations in Argentina and South Africa have revealed the presence of native ecological analogues feeding on *I. pseudacorus* seeds (Authors, pers. obs.). Agents that possess ecological analogues are more prone to apparent competition and accumulation of parasites and parasitoids, which could potentially reduce their efficacy (Paynter et al., 2010).

4.2. IMPACT ASSESSMENT

Our study shows that under controlled conditions, *A. nonstriata* can have a substantial effect on *I. pseudacorus* foliar growth, biomass accumulation and photosynthetic activity. This effect was observed within a period of 6 weeks, even at low insect densities. Compared to the control, plants grown from

CHAPTER 4

rhizomes attacked by *A. nonstriata* suffered a 22-23% stunted leaf growth, a 50-100% higher leaf mortality, and accumulated 22-29% and 32-58% less above- and below-ground biomass, respectively. Additionally, their photosynthetic efficiency and chlorophyll content were reduced in direct proportion to the amount of leaf area damaged by the insect. This impact could be attributed to the direct feeding of adult insects on the foliar tissue of the plant, but also – and maybe more importantly – to the damage caused by the larvae on its rhizomatic system. As underground organs constitute a significant portion of a plant total biomass (35 to 70% in our experiments), root herbivores have a great capacity to affect growth and biomass allocation (Nötzold et al., 1998). Root damage does not only deplete the plant's energy reserves, but can also induce water stress, loss of reproductive output, and increased susceptibility to secondary infections by soil-borne pathogens (Caesar, 2003; Simelane, 2010). Unfortunately, it is complicated to untangle the direct and indirect effects of root damage on plant growth due to the inherent difficulties in observing and manipulating root feeding insect stages under laboratory conditions (Blossey & Hunt-Joshi, 2003).

The growth of plants subjected to herbivory by *A. nonstriata* in our experiment in the native range was significantly affected, however no mortality was observed by the end of the 6 weeks period. Additionally, the rate of photosynthetic efficiency of the invasive plants tested in South Africa was only relatively reduced by herbivory, decreasing from 0.82% to 0.74%, on average. As a reference, the typical photosynthetic efficiency of healthy plants ranges from 0.1 to 1%, rising up to 2% for some high-yielding crops (Zhu et al., 2010). We argue that the presence of an already formed rhizome in these plants granted them higher resilience to the damage inflicted by *A. nonstriata*, especially due to the limited duration of the experiment. Additional testing involving multiple insect generations will need to be performed in order to assess the impact of sustained damage on adult plants. Similarly, although root feeders have often been

CHAPTER 4

associated with reduction in plants reproductive output (Sheppard et al., 1995; Simelane, 2010), this response could not be included in our experiments.

Field exclusion trials should be conducted to evaluate the impact of *A. nonstriata* on the flower and seed production of sexually mature *I. pseudacorus* individuals. Early life stages of *I. pseudacorus* were affected significantly more by *A. nonstriata* than plants grown from rhizomes. Test plants showed 85-91% less foliar growth, 74-90% fewer above-ground biomass and 93-98% fewer below-ground biomass compared to controls. Furthermore, *A. nonstriata* adults caused 94-100% leaf morality, leading to the death of over 95% of test plants by the end of the experiment. Interestingly, whereas *A. nonstriata* larvae and pupae were recovered from plants grown from rhizomes, this was not the case on plants grown from seeds, which did not support a second generation of the insect. It is unclear if this was due to failed oviposition (active copulation was observed during the experiment) or to unsuccessful larval development. If the former, the direct damage observed on the roots of the test plants would have to be attributed to adult insects which, upon depleting the available foliar tissue, switched to feeding on the belowground organs. Although some adult insects were indeed recovered from the soil at the bottom of the growing pots, this would be a behavior not yet observed for this species in natural conditions. In the latter scenario, the test plants would have been subjected to direct feeding by larvae, which were then not able to complete development due to unfavorable environmental conditions. Larval development in flea beetles is influenced by both biotic (e.g. lack of sufficient nutrients to complete pupation) and abiotic (e.g. insufficient or excessive soil moisture) factors (Simelane, 2014). This is unlikely to be the case in our experiments, as moisture levels and other conditions were kept constant, and the same growing medium was used across both trials.

CHAPTER 4

Further research is needed to shed light on the reasons behind the inability of *A. nonstriata* to produce a second generation on *I. pseudacorus* plants grown from seeds. This would not be a major issue in the field, where seedlings are often recruited at the periphery or in proximity of existing *I. pseudacorus* colonies. The lethal effect of *A. nonstriata* on early stages of *I. pseudacorus* is rather promising in this regard, as timely attack by the insect could eradicate seedlings without the need of a second generation. Of course, it is important to highlight that the plants used in the experiment were grown from native-range parents. Invasive weeds in the non-native range are known to grow larger and faster, but also to show lower chemical defences against specialized herbivores (Hinz & Schwarzländer, 2004). Thus, it is difficult to speculate on the effect that would be observed on invasive seedlings. This would be further influenced by climate change, which as we observed could have various effects on *I. pseudacorus* germination time and early growth biomass allocation.

Finally, although *I. pseudacorus* disperses and establishes primarily by seeds (Gaskin et al., 2016), it retains the ability to adjust to biotic and abiotic pressures by switching between sexual and vegetative reproduction (Mopper et al., 2016). Population-level differences in phenology and reproductive strategy may affect the relevance of some biocontrol agents (West et al., 2023). However, being a multivoltine species attacking multiple plant structures across different seasons, makes *A. nonstriata* a flexible and reliable candidate to control the spread of this aquatic invader.

5. CONCLUSION

The present study a) addressed the importance of cold stratification on the germination of *I. pseudacorus* in its native range in Belgium and in the invaded Argentinian range; and b) assessed the impact caused by various density of *A. nonstriata* on different life-stages of the plant. The results obtained suggest that seeds from Argentina possess a higher germination potential than native seeds,

CHAPTER 4

similarly to what has been observed in other regions invaded by the weed (Gillard et al., 2022; Sandenbergh et al., 2024). Furthermore, invasive seeds showed a net positive response (i.e. higher germination rate and increased seedling biomass) in the absence of cold stratification, suggesting an adaptation to warmer winter temperatures. The implications of these results on the invasion ecology and management of this species will have to be considered, especially in light of current climate change.

The impact assessment proved that *A. nonstriata* can have a significant negative effect on *I. pseudacorus*' growth and photosynthetic activity. Herbivory led to a significant decrease in biomass accumulation, even at low insect densities. These results provide sufficient evidence of the damaging potential of *A. nonstriata* to warrant its release. Furthermore, plants grown by seed germination were significantly more affected by herbivory, which resulted in over 90% mortality rate in a short period of time. Although this do not preclude the need to research a biocontrol agent attacking the sexual reproductive structure of the plant, it does suggest that *A. nonstriata* could be able to control seedling recruitment.

ACKNOWLEDGEMENTS

The PhD project of G.M. is funded by a strategic basic research fellowship of the Research Foundation – Flanders (FWO). We thank the Vrije Universiteit Brussel (BAS 53 and BAS 42) and the Centre for Biological Control (Rhodes University) for logistic support. Furthermore, the authors would like to thank the collaborators from the National Scientific and Technical Research Council of Argentina and the Fundación para el Estudio de Especies Invasivas, who helped in the collection and testing of samples from the invaded range.

CHAPTER 4

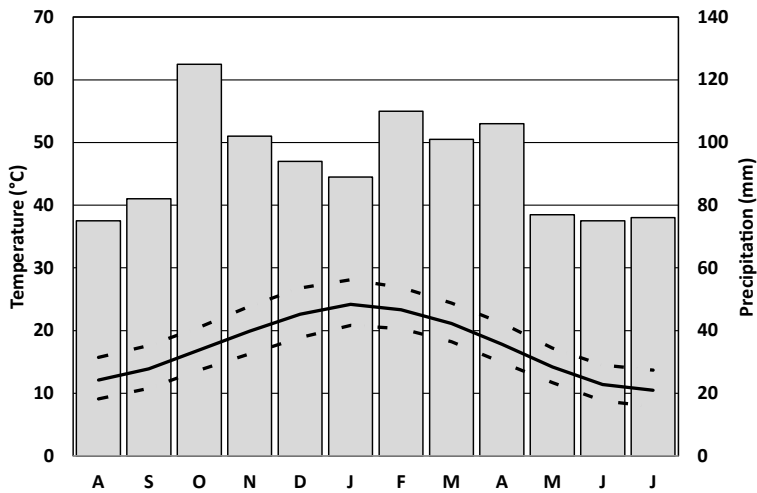
SUPPLEMENTARY INFORMATION

Supplementary Table S4.1 – Locations sampled for collecting *I. pseudacorus* seeds for germination. All populations were sampled when fruits were mature.

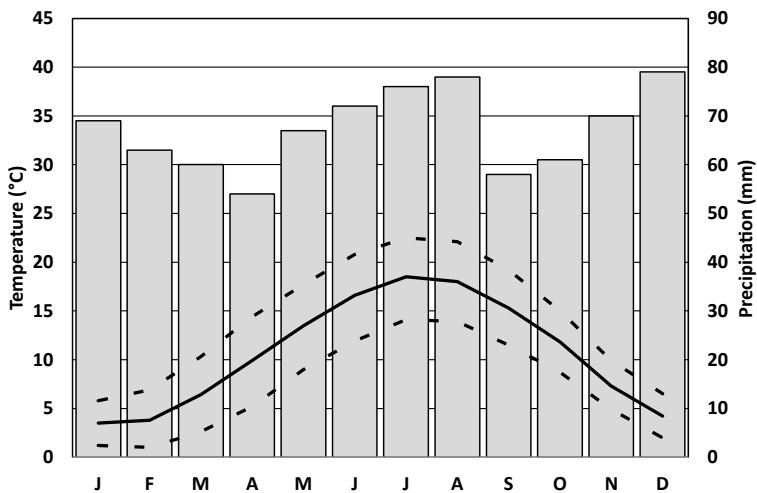
Country	Province	Population Code	Decimal Latitude	Decimal Longitude
Belgium	Tervuren	KFT	50.801795	4.494340
	Waasmunster	HAM	51.099953	4.025459
	Zaventem	ELZ	50.878037	4.466164
	Sint-Peters-Woluwe	PDS	50.835818	4.435555
	Watermael-Boitsfort	TNR	50.804548	4.428212
	Auderghem	SEN	50.810311	4.428780
Argentina	Buenos Aires	BAA	-34.664325	-58.319879
	Cordoba	CBA	-31.430804	-64.179254
	Salta	SAL	-24.709544	-65.479609
	Jujuy	JU1	-24.08753	-65.406683
	Jujuy	JU2	-24.211920	-65.286430
	Misiones	MIS	-27.467519	-55.037280

CHAPTER 4

Buenos Aires, Argentina

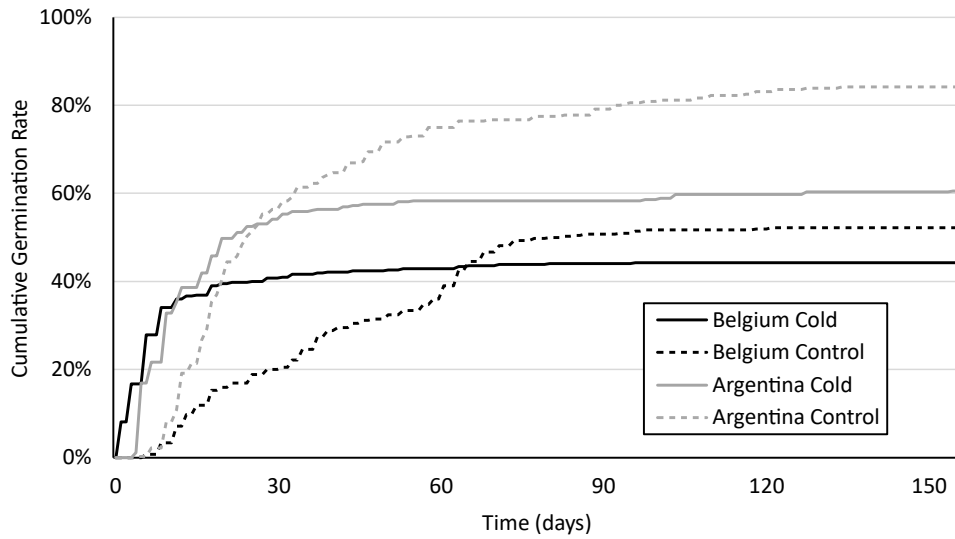


Brussels, Belgium



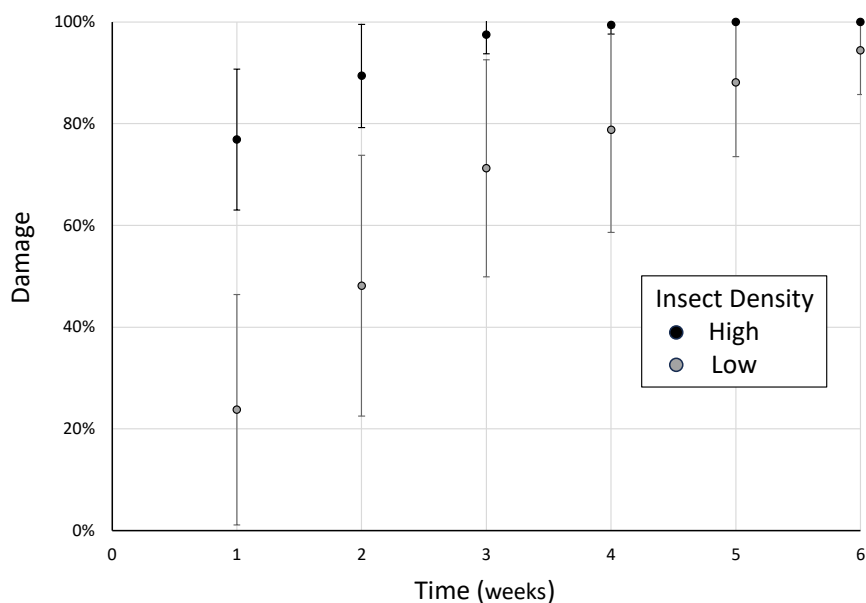
Supplementary Figure S4.1 – Climate patterns for Buenos Aires (Argentina) and Brussels (Belgium). Grey bars represent the monthly average rainfall, black lines indicate average, maximum and minimum monthly temperatures. Note that the two diagrams start from different months in order to more easily compare seasons between Northern and Southern Hemisphere. Data source: <https://weatherandclimate.com/>.

CHAPTER 4



Supplementary Figure S4.2 - Cumulative germination rate (%) of cold-stratified (full lines) and non-stratified (dotted lines) *Iris pseudacorus* seeds from Belgium (black lines) and Argentina (grey lines) over a period of five months.

CHAPTER 4



Supplementary Figure S4.3 – Cumulative damage over time (mean $\pm$ SD), expressed as percentage of total leaf area, recorded during the impact assessment on plants grown by seed germination. Low insect density = 4 insects per plant; high insect density = 8 insects per plant.

CHAPTER 4



Control



Low herbivory

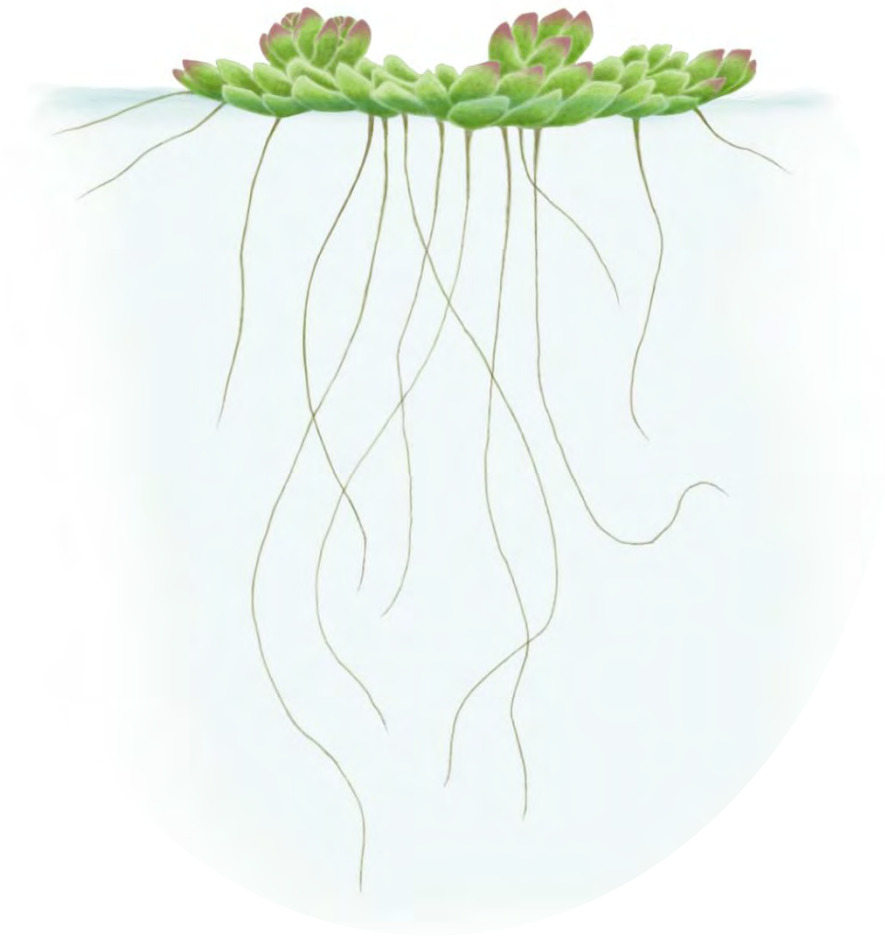


High herbivory

Supplementary Figure S4.4 – Pictures of *Iris pseudacorus* plants after 6 weeks of exposure to herbivory by *Aphthona nonstriata*. Top: plants grown from seeds, exposed either to no herbivory (control, left), low herbivory (4 insects per plant, centre), or high herbivory (8 insects per plant, right). Bottom: plants grown from rhizomes, exposed either to no herbivory (control, left), low herbivory (10 insects per plant, centre), or high herbivory (20 insects per plant, right).

PART II

POST-RELEASE STUDIES



WATER FERN

CHAPTER 5

From honeymoon to debt: the intricacies of evaluating ecosystem recovery - A case study on the biological control of water fern in Belgium

Minuti, G., Staniszewski, M.S., Singh, K., Cantarelli, M., Coetzee, J.C. & Stiers, I. [Manuscript in preparation].

Native to the Americas, the water fern *Azolla filiculoides* Lam. (Salviniaceae) has been exported worldwide and is considered invasive in various countries across Europe, Asia and Africa (Hill, 2014). Dense infestations of this plant can cover the entire water surface, inhibiting light penetration and gas exchanges, suppressing the growth of floating and submerged vegetation, decreasing dissolved oxygen levels, and altering nutrient availability (Janes et al., 1996; Paolacci et al., 2018). This in turn can affect aquatic biodiversity through complex interactions (Gratwicke & Marshall 2001; Pinero-Rodríguez et al., 2021).

The deleterious effects of floating aquatic weeds on freshwater ecosystems are well known (Coetzee et al., 2014; Pinero-Rodríguez et al., 2021; Wahl et al., 2021), and so is the efficacy of biological control against these invaders (Hill & Coetzee, 2017). However, the recovery of freshwater ecosystems after successful biocontrol has been, until recently, left unexplored (Coetzee et al., 2020; Motitsoe et al., 2020, Motitsoe et al., 2022; Woodley et al., 2023; Paterson et al., 2024). Classical biological control of water fern using the weevil *Stenopelmus rufinasus* Gyllenhal (Coleoptera: Curculionidae) has proven to be extremely effective in managing invasions across Africa and Asia (McConnachie et al., 2003; Hill, 2014). Although the weevil has never been intentionally released in Europe, it was recorded in several countries of the union (Carrapiço et al., 2011; Stejskal, 2012; Pratt et al., 2013; Wanat, 2018), including Belgium.

CHAPTER 5

Classical weed biological control programs can be divided into two main phases. The pre-release phase follows a series of defined stages from selection of candidate biocontrol agents, through host-specificity testing and impact assessments, up until rearing and release (Schaffner et al., 2020). These steps have been progressively refined over time, leading to significant improvements in agents prioritization, effectiveness, and safety (Schaffner et al., 2018; Schwarzländer et al., 2018; Hinz et al., 2021). The post-release phase consists of monitoring the biocontrol agents after their introduction, evaluating their establishment, the impact on the target plant, possible non-target effects, and the response of the native ecosystem (Schaffner et al., 2020). This helps detect unforeseen factors that might be affecting agent's performance and ensure their safety and long-term success. As such, post-release evaluations are a cornerstone of responsible and adaptive management (Schaffner et al., 2020).

However, despite raising awareness, dedicated post-release studies are often lacking and, when conducted, they are limited in time and extent (Blossey, 1999; Schaffner et al., 2020; Paterson et al., 2024). This has been attributed to a lack of funding invested in the monitoring of programs that appear effective in controlling their target (Maron et al., 2010; Schaffner et al., 2020). In fact, in countries where funding agencies are mandated to validate the success of management interventions, more resources are directed towards post-release evaluations (Paterson et al., 2024).

In recent decades, advances have been made in tracking the demography of biocontrol agents and quantifying their impact on weed populations and non-target species (Clewley et al., 2012; Blossey et al., 2018; Schaffner et al., 2020). This has brought an increase in studies evaluating the effectiveness of biocontrol at various scales, from plant physiological responses, weed growth, population dynamics, to landscape-level impacts (see Paterson et al., 2024 for details). In most cases, the results of these studies have confirmed that biological control

CHAPTER 5

can reduce the density and performance of invasive alien plant populations (Clewley et al., 2012; Schwarzländer et al., 2018). As these measures are directly correlated with negative ecological and socio-economic impacts of an invasion, it is often assumed – sometimes erroneously – that their reduction would alleviate such issues (Reid et al., 2009). Unfortunately, this is usually not directly quantified (but see Stephens et al., 2009; Setter & Lym, 2013; Moran et al., 2017). In fact, studies measuring the benefits generated from weed biological control are often limited to a few major invaders and focusing on economic rather than ecological responses (Arp et al., 2017; Wainger et al., 2018; van Wilgen et al., 2020; Maluleke et al., 2021).

Only recently research has tried to appraise the recovery of native ecosystem following biological control of invasive aquatic weeds (Paterson et al., 2024). These studies – focusing on community-level indices and ecosystem processes, structure, and functioning – all suggest that biological control is able to elicit, to some extent, a recovery of the invaded ecosystem (Coetzee et al., 2020; Motitsoe et al., 2020, 2022). This marks a step in the right direction, but constitutes only the proverbial tip of the iceberg. The paucity of long-term research focusing on ecosystem recovery leaves a gap to be filled within the field. Along with effectiveness (Schwarzländer et al., 2018) and safety (Hinz et al., 2020), the contribution of biological control towards the restoration of native ecosystems constitutes a fundamental aspect of sustainable management. Including ecosystem recovery in post-release evaluations is vital if we are to ensure that the suppression of the target weed leads to the rehabilitation of native biodiversity and ecosystem functions, rather than unintended ecological imbalances (Schaffner et al., 2020). Further research in this direction is instrumental to mend public perceptions and increase the uptake of biocontrol in more risk-averse regions, such as Europe (Marchante et al., 2023).

CHAPTER 5

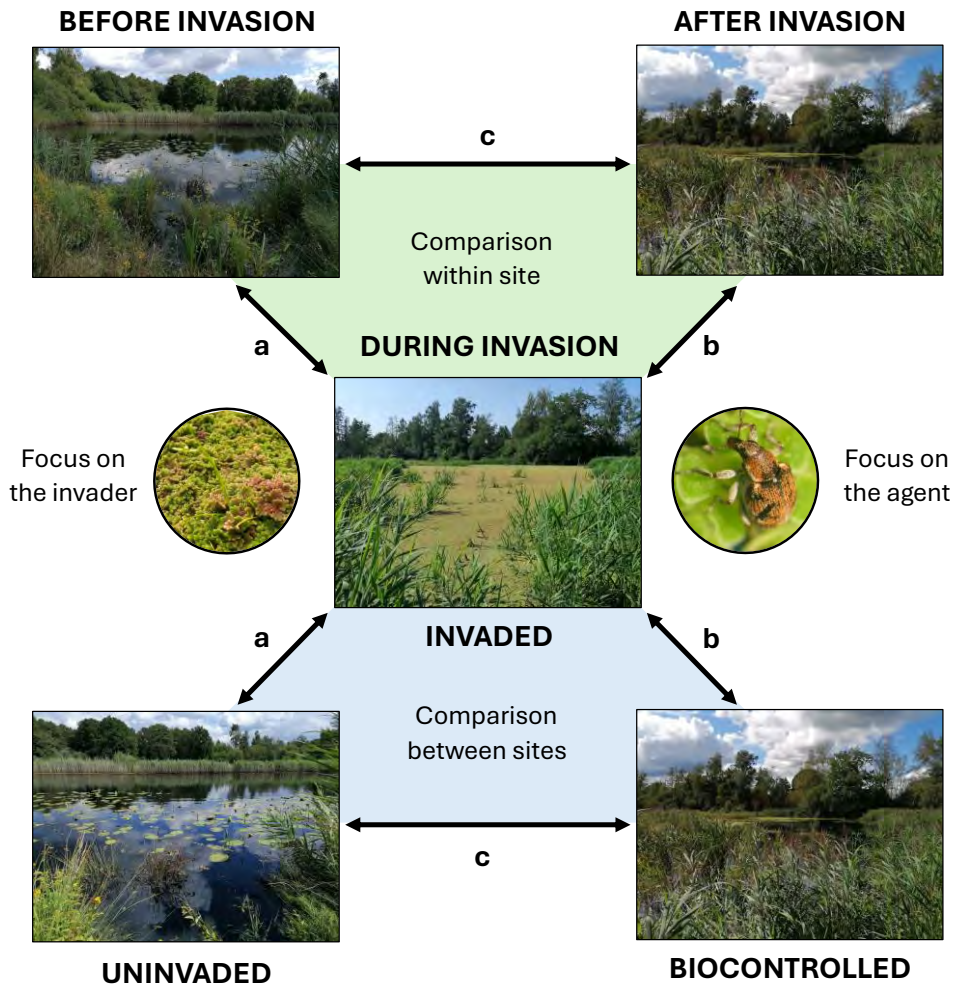


Figure 5.1 – Empirical design followed during the case study of *A. filiculoides* biocontrol in Belgium. Water physicochemical parameters, macroinvertebrates and zooplankton communities were sampled in the field, where comparisons were made between invaded sites, closely located uninvaded sites, and biocontrolled sites (blue triangle). At the same time, a mesocosm experiment was set up at one of the study sites, where water fern and its biocontrol agent were sequentially introduced in order to compare the state of the ecosystem before, during, and after invasion (green triangle). This design allows to obtain independent evidence for a) the impact of the weed; b) the effectiveness of the agent; and c) the recovery of the ecosystem following biological control.

CHAPTER 5

In this case study, we monitored changes in water physicochemical parameters, zooplankton communities and macroinvertebrate assemblages during the invasion of water fern across a pond system in Belgium. Observations were compared between invaded and closely located uninvaded ponds, and mesocosms were set up in the field to compare the state of the ecosystems before water fern introduction, during invasion, and after its control (Figure 5.1). Our field observations confirmed the presence of *S. rufinasus* in Belgium, although the weevil started exerting a significant level of control on the plant late in the season, with water fern cover (100% in July) dropping towards late August. Furthermore, the response observed in water parameters in the field and in constructed mesocosms led to contrasting results, providing an opportunity to discuss the intricacies of evaluating ecosystem recovery.

For instance, water fern introduction in our mesocosms was correlated with low dissolved oxygen levels and increased nitrogen and phosphorus concentrations. These observations mirror those of Pinero-Rodriguez and colleagues (2021), who investigated the impacts of *A. filiculoides* invasion on water quality and aquatic vegetation using a similar setup. However, our field samplings highlighted a different response, with nutrient levels being negatively correlated with plant density and increasing with its control, a phenomenon also observed following the introduction and biological control of giant salvinia, *Salvinia molesta* (D.S. Mitchell), in large constructed pools in South Africa (Motitsoe et al., 2020).

One obvious factor that could explain this difference is the small size and therefore lower water volume contained in our mesocosms compared to large pools and natural ponds. This would not only affect water temperature, but also the amount of sediment and inoculum present, and therefore the concentration of dissolved nutrients. Additionally, small constructed mesocosms cannot adequately recreate the complex ecological interactions observed in natural ecosystems (Schaffner et al., 2020).

CHAPTER 5

This is further complicated by the unique properties of different invaders. Water fern, for instance, is known for its phosphorus uptake and nitrogen-fixing capacity, making it a natural fertilizer (Mandal et al., 1999; Forni et al., 2001; Lepori & Keck, 2012). Accordingly, its introduction would explain the increase nutrients concentration observed in our mesocosms. This would be magnified by the accumulation of dead water fern over time – a process further driven by the presence of *S. rufinasus* – both in the field and in our mesocosms.

The high input of organic material would also exacerbate the hypoxic conditions in the water column (Janes et al., 1996; Peeters et al., 2016; Pinero-Rodríguez et al., 2021). Hypoxia is a common response to floating aquatic plant invasions, as they tend to inhibit gas exchange and limit light penetration, increasing phytoplankton abundance while reducing its photosynthetic activity (Coetzee et al., 2020; Pinero-Rodríguez et al., 2021; Motitsoe et al., 2022). As dissolved oxygen is an essential component for obligate aerobic lifeforms such as zooplankton (Marcus, 2001), hypoxic conditions are known to trigger changes in freshwater community composition (Breitburg et al., 1997), which can have a knock-on effect on higher trophic levels (Coetzee et al., 2014).

However, these dynamics are highly complex and it can be difficult to disentangle the direct impact of biological invasions from time- and context-dependent effects (Soto et al., 2024). In our case study, we observed that Azolla-invaded ecosystems scored higher in community abundance and richness compared to uninvaded ones, yet the reduction in water fern cover and density resulted in increased community evenness and diversity (Table 5.1).

CHAPTER 5

Table 5.1 – Simplified community responses (i.e. zooplankton and macroinvertebrate taxa abundance) to the introduction and subsequent biological control of water fern within constructed mesocosms. Differences between invaded and control (uninvaded) mesocosms are indicated by mathematical operators (+ higher; - lower; = similar; / absent; ! new observation). Arrows are used to display time-comparisons within the invaded mesocosms (↗ increasing; ↘ decreasing; → stable). Functional feeding groups (FFG) according to Cummings et al., (2005).

	Taxa	FFG	Before invasion	During invasion		After invasion	
	ARTHROPODA						
	Cladocera	Filterers	-	↗	+	↗	-
	Copepoda	Filterers	+	↗	+	↘	=
	Ostracoda	Filterers	-	↗	+	→	+
	Malacostraca						
	Asellidae	Shredder	=	↗	+	↗	+
	Gammaridae	Shredder	=	↗	+	↗	-
	Ephemeroptera						
	Baetidae	Scrapers	+	↘	-	↘	-
	Diptera						
	Chaoboridae	Predator	+	↗	+	↘	+
	Chironomidae	Gatherer	=	↗	+	↗	+
	Culicidae	Predator	=	↗	+	↘	=
	Thaumaleidae	Grazers	=	↗	+	↘	=
	Coleoptera						
	Dytiscidae	Predators	=	↘	-	↗	+
	Hydrophilidae	Scavengers	/	→	/	↗	!
	Hemiptera						
	Corixidae	Omnivores	=	→	-	↘	-
	Notonectidae	Predators	+	↗	+	↗	+
	Lepidoptera						
	Crambidae	Herbivores	/	↗	+	↘	+
	Odonata						
	Odonata	Predators	/	→	/	↗	-!
	ANNELIDA						
	Clitellata						
	Hirudinea	Predators	/	↗	+	↗	+
	MOLLUSCA						
	Gastropoda						
	Lymnaeidae	Scrapers	+	↗	+	↘	=
	Physidae	Scrapers	/	↗	+	↗	+
	Sphaeriidae	Filterers	=	→	-	→	-
	ROTIFERA	Gatherers	=	↗	+	↘	-
	Abundance (N)		=	↗	+	↗	+
	Richness (S)		=	↗	+	↗	=
	Diversity (H)		=	↘	-	↗	=
	Evenness (J)		=	↘	-	↗	-

CHAPTER 5

This seemingly positive early response – sometimes referred to as ‘*invasion honeymoon*’ (Soto et al., 2024) – may be attributed to the increase in resource availability and habitat complexity provided by the invader itself (Rodriguez, 2006). In the case of water fern, an increase in phytoplankton abundance is believed to have benefited filter-feeders, such as ostracods, copepodites and small cladocerans (Pinero-Rodriguez et al., 2021), which in turn favored larger copepods, predatory cladocerans and glassworms (Chaoboridae).

At the same time, the new substrate provided by the plant facilitated the abundance of grazers – e.g. bladder snails (Physidae) and trickle midges (Thaumaleidae) – and specialized herbivores – i.e. the small china-mark *Cataclysta lemnata* L. (Lepidoptera: Crambidae) and, of course, the Azolla weevil *S. rufinasus* (Farahpour-Haghani et al., 2019). The increased input of organic matter and low oxygen conditions also played a role, benefiting stress-tolerant species such as non-biting midges (Chironomidae) and mosquitoes (Culicidae), to the detriment of more susceptible species such as mayflies (Baetidae) (Mandaville, 2002). The abundance of primary consumers, in turn, was associated with a boost of larger predators (Figure 5.2).

Nonetheless, the honeymoon phase is usually short-lived, as the long-term impacts of invasion tend to gradually impoverish community composition and function – a phenomenon referred to as ‘*invasion debt*’ (Soto et al., 2024). Indeed, even though water fern invasion elicited an increase in the abundance of zooplankton and macroinvertebrates compared to the open-water control, this resulted in the dominance of a few adaptable taxa – namely copepods, gastropods and chironomids – and an overall decrease in community evenness. On the other hand, the reduction in cover and density of the plant following biological control yielded more diverse and balanced communities (Table 5.1).

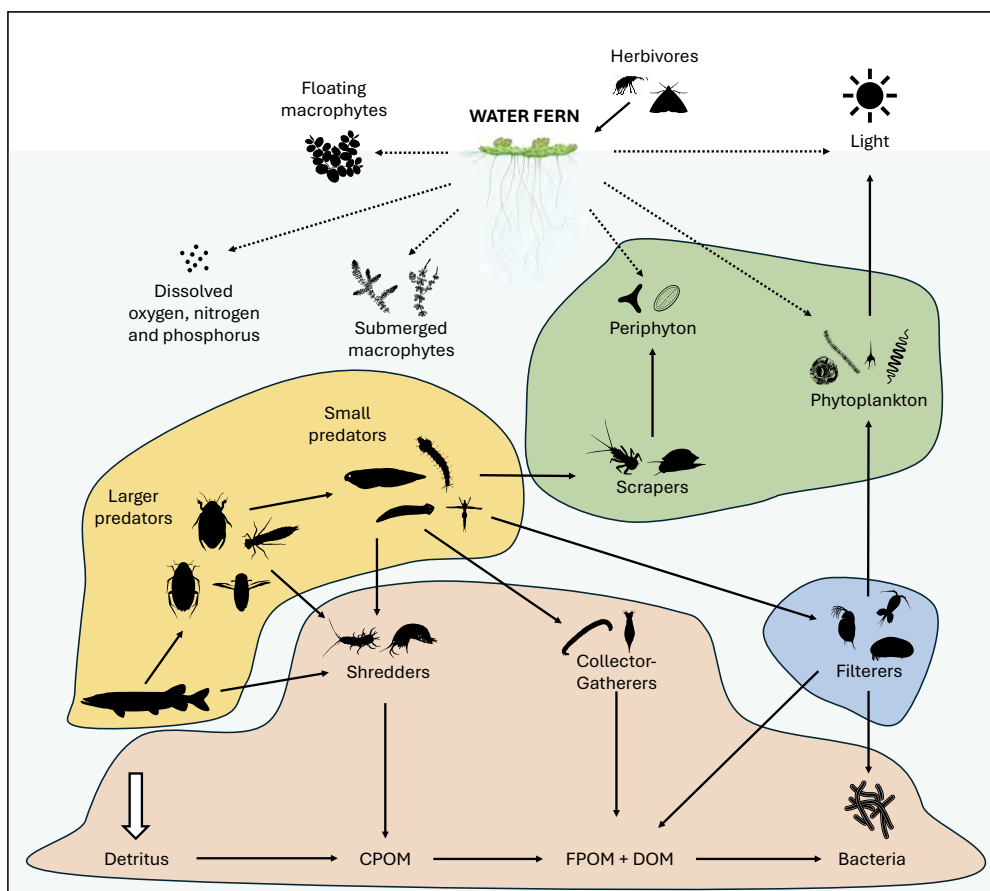


Figure 5.2 – Simplified visual of the effects of water fern invasion (dotted arrows) on a representative pond ecosystem food web (full arrows). Functional feeding groups (according to Cummings et al., 2005) have been populated with taxa found during our samplings of invaded ecosystems in Belgium. Different coloured areas represent different bases for the food web (orange: detritus; green: primary producers; blue: mixed; yellow: other consumers; adapted from Snoeks et al., 2021). The larger white arrow indicates the increased accumulation of detritus following the decay of the weed and other organisms (CPOM: coarse particulate organic matter; FPOM: fine particulate organic matter; DOM: dissolved organic matter).

This was marked by the increase of crustacean shredders (Asellidae and Gammaridae), as well as by the previously unrecorded colonization – in biocontrolled mesocosms – of scavenger beetles (Hydrophilidae) and dragonfly larvae (Odonata), all indicators of improved water quality (Gerhardt et al., 2011).

CHAPTER 5

This highlights the importance of comparing not only invaded and uninvaded ecosystems, but also monitoring the conditions within the invaded ecosystems before and after control (Motitsoe et al., 2020; 2022). Over the course of our experiment, the shift observed in water quality and community composition due to water fern invasion was not reversed. Although we did observe an increase in dissolved oxygen and community evenness following biological control, several taxa failed to recover to levels observed in the control ponds, and others – that had benefited from the presence of the weed – persisted (Table 5.1).

This is undoubtedly exacerbated by the small time scale of our observations, which spanned only one year and did not allow to clearly distinguish causal effects from seasonal fluctuations. Unfortunately, a large number of post-release studies, especially focusing on ecosystem recovery, are conducted on small scales and over short time periods (Paterson et al., 2024). Future research will need to address community responses over longer time frames, gathering accurate data on the state of the ecosystem before, during, and after the invasion has occurred (Schaffner et al., 2020).

Correcting for the numerous factors that might undermine restoration can be particularly challenging. Due to a series of logistical, financial, and stochastic elements, pre-release monitoring of invaded sites is rarely conducted, leading to a lack of baseline data. This makes it impossible to accurately predict how the ecosystem appeared before invasion, and therefore to define the goal of restoration (Schaffner et al., 2020). Nonetheless, some approaches such as including time-since-first-invasion and comparing sites along a gradient of alien species abundance have been suggested to reliably approximate the state of a desirable ecosystem before invasion, and therefore evaluate its recovery (Kumschick et al., 2015). In doing so, one should consider how the biotic and abiotic conditions that promoted invasion in the first place might have influenced the response of the ecosystem to its control (Schaffner et al., 2020).

CHAPTER 5

Factors such as disturbance, land-use change, and increased resource availability (i.e. *eutrophication*) are known to promote invasion and influence the effectiveness of biological control (Catford et al., 2009; Coetzee et al., 2012). It is evident that, if these bottom-up effects are not dealt with, they could alter the direction of ecosystem recovery (Strange et al., 2019). In some other cases, a lack of recovery might be owing to environmental changes elicited by the long-term impacts of the weed (i.e. *legacy effects*), such as depletion of native propagules, alteration of edaphic conditions, and degradations of the microbiota (Nsikani et al., 2018; Schaffner et al., 2020).

These, in turn, generate a positive feedback loop facilitating re-invasion by the same species and/or the recruitment of other invaders (i.e. *invasional meltdown*; Simberloff & Holle, 1999), acting as barriers to restoration. For these reasons, attention needs to be drawn towards understanding and dealing with the underlying causes of invasion, if we want management actions, including biological control, to result in the recovery of natural freshwater ecosystems.

Overall, the results presented in this case study highlight the issues behind evaluating ecosystem recovery following biological control. In light of global change, legacy effects, and a lack of baseline data, this work advocates for the use of quantifiable data, observational and experimental designs to gather independent evidence of recovery and disentangle the complex dynamics observed in freshwater ecosystems.

CHAPTER 5

GENERAL DISCUSSION

SYNTHESIS

Classical biological control of weeds is a dynamic science that continues to evolve in response to new challenges, technological advancements, and changing environmental conditions. By embracing innovation, collaboration, and adaptive management, researchers and practitioners can enhance the effectiveness and sustainability of biocontrol strategies for managing invasive weeds.

The main goal of the present thesis was to advance the biological control program initiated against the invasive wetland plant *I. pseudacorus*. This was accomplished by following the standard steps of a classical biocontrol program. First, using results from bibliographic research and field surveys, a suite of candidate biocontrol agents was identified. Then, based on present climate and future scenarios, the global bioclimatic suitability of these agents was modelled in relation to that of their target weed. This allowed to i) identify areas of climatic mismatch between the agents and the plant; ii) prioritize the most promising agent(s); and iii) inform future control measures against the weed across different geographic regions.

As a third step, the host-specificity of the main candidate agent, the flea beetle *A. nonstriata*, was tested upon various plant species within the family *Iridaceae*. Albeit not yet complete, the results presented in this thesis are used to draw a first assessment of the level of risk associated with the release of this agent across different invaded regions. Furthermore, the results allowed discussion of the difficulty encountered in selecting the correct plants species to use in host-specificity testing when faced with a worldwide invader belonging to such a species-rich and phylogenetically complex genus.

GENERAL DISCUSSION

Finally, the last step compared the germination potential of *I. pseudacorus* between its native and invaded range, and the ability of *A. nonstriata* to control different life stages of the plant, at various insect densities. This aimed at testing the hypothesis that the plant shows increased sexual output across its invaded range, which might hinder the ability of *A. nonstriata* to control it.

An additional objective of the thesis was to evaluate the recovery of native aquatic ecosystems following the biological control of the aquatic fern *A. filiculoides* by its natural enemy, the weevil *Stenopelmus rufinasus*, which is considered naturalized in Belgium. Combining results from a field study and a mesocosm experiment, this section assessed i) the effects of *A. filiculoides* on native aquatic communities; ii) the potential of *S. rufinasus* to control water fern invasion; and iii) the recovery of the aquatic ecosystems in terms of abiotic conditions, zooplanktonic communities and macroinvertebrate assemblages.

The following synthesis will start with a brief summary of the results obtained in this thesis. It will then delve into the key messages emerging from these results, discussing the newly acquired knowledge and how it may advance the biocontrol program. Finally, the limitations of the study as well as the main recommendations for follow-up research and management are discussed.

THESIS FRAMEWORK AND RESULTS

The current thesis followed the main steps of weed biological control, advancing the program against *I. pseudacorus* while touching on some of the major issues facing biocontrol practitioners in the era of global change.

The literature reported a variety of organisms attacking *I. pseudacorus* across its native range. However, only a handful were recorded during the surveys carried out in the context of the current thesis. Among them, three were prioritized based on their abundance, life-history and alleged host-specificity. Furthermore, these candidate biocontrol agents cause complementary damage

GENERAL DISCUSSION

to the plant, which was deemed necessary to hinder its growth and competitiveness (**Chapter 1**; Minuti et al., 2021). The larvae of the sawfly *Rhadinoceraea micans* Klug (Hymenoptera: Tenthredinidae) are voracious leaf feeders that can completely defoliate yellow flag plants growing near water. The seed weevil *Mononychus punctumalbum* Herbst (Coleoptera: Curculionidae) damages the flowers and fruits of the plant, while its larvae develop by feeding on its seeds. Finally, the larvae of the flea beetle *Aphthona nonstriata* Goetze (Coleoptera: Chrysomelidae) feed and develop within the rhizomes of yellow flag, whereas the adults attack its leaves.

In order to further prioritize suitable candidate biocontrol agents for *I. pseudacorus*, and to prevent potential mismatches that could hinder their effectiveness upon release, we modelled their present and future climatic niches in relation to that of their target. The current bioclimatic niche of the weed encompasses humid temperate, continental, oceanic and Mediterranean climates, highlighting a risk of invasion across various regions of the world. However, our results predicted a contrasting scenario between the Northern and Southern Hemisphere under climate change. In North America and eastern Asia, the area climatically available for *I. pseudacorus* is expected to increase and shift northwards, whereas its biocontrol agents show little and decreasing suitability under future climatic conditions. On the other hand, a small reduction in the climatically suitable areas for the plant in South America, southern Africa and Australasia is predicted. A similar decrease in suitability is expected for the candidate biocontrol agents which, however, would still maintain a significant range overlap over their host. In particular, *A. nonstriata* and *M. punctumalbum* appear to be the most suitable candidates for release across the southern Hemisphere (**Chapter 2**; Minuti et al., 2023).

Once the candidate biocontrol agent(s) had been selected and prioritized, the overarching issue of determining their safety for release had to be tackled. Host-

GENERAL DISCUSSION

specificity testing is the cornerstone of every biocontrol program, and the testing of *A. nonstriata* provided the perfect stage to discuss the numerous decisions required during this pivotal step. Firstly, the criteria to be used to select plants to test are seldom considered. Building on a modernized version of the centrifugal phylogenetic method, we delineated an evaluation score based on solid ecological, economic and conservation criteria. Secondly, host-specificity lists are often built for particular regions or countries, with little regard to invasion history and shared flora. In this thesis, a consolidated list of taxa to test for host-specificity was drafted taking into account the phylogeographic patterns in the evolution of the family Iridaceae and the *Iris* genus, and encompassing most phylogenetic clades and all ranges invaded by *I. pseudacorus*. Finally, the risk associated with the release of a particular agent is often context dependent. The preliminary results obtained on the host-specificity of *A. nonstriata* demonstrate that, while the agent is clearly able to feed on a various range of Iridaceae, reproduction is limited to a few species within the *Iris* genus. Although not ideal, these results beg the question of which level of trade-off would be acceptable in order to minimize the invasion of *I. pseudacorus* in certain regions (**Chapter 3**).

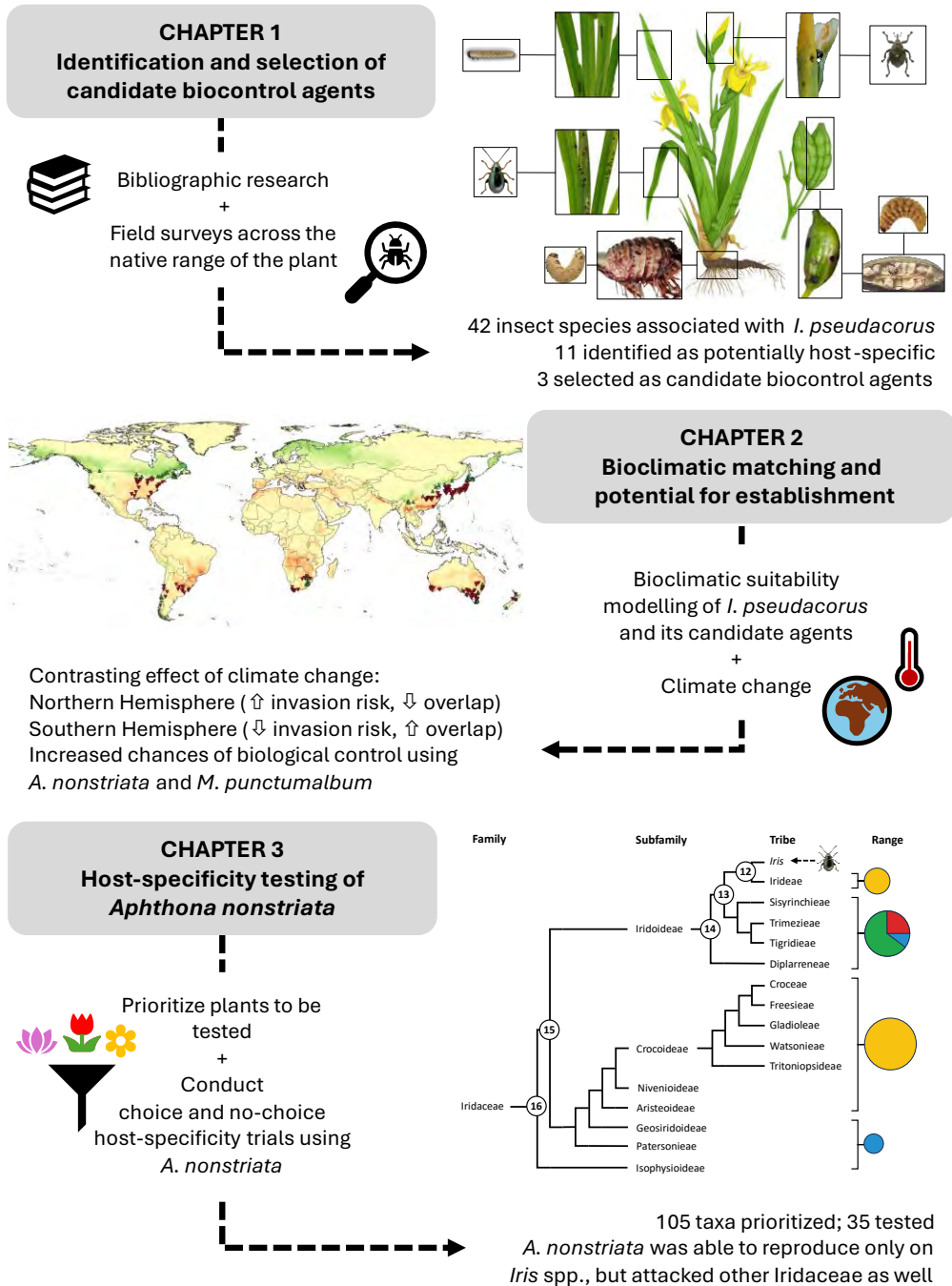
Invasive alien plants are known to display plasticity in reproductive strategies between their native and invaded range. Climate change can influence plant phenology and propagule pressure, modifying the outcomes of biological invasions and, eventually, their biological control. In this context, our results highlighted a significant increase in germination rate across the invaded Argentinian range compared to native plants in Belgium. Furthermore, seeds from Argentina performed better in absence of cold stratification, suggesting an adaptation to warmer winter temperatures (**Chapter 4**). At the same time, the impact of *A. nonstriata* on *I. pseudacorus* was shown to be able to stunt the growth of the plant, even at low insect densities. What's more, the impact on juvenile plants was significantly higher, consistently leading to plant mortality

GENERAL DISCUSSION

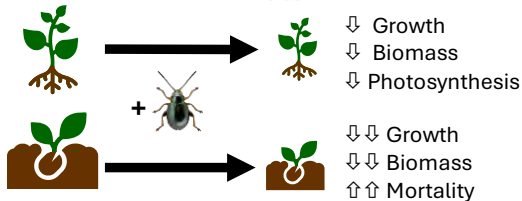
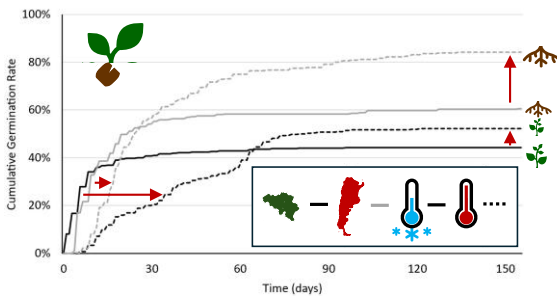
within a short period. These results highlight the importance of including climate change in studies of plant invasion, and support the prioritization of *A. nonstriata* as the main candidate biocontrol agent for *I. pseudacorus*.

Finally, the thesis moves away from the yellow flag in order to tackle a fundamental, and often overseen aspect in the post-release evaluation of biological control agents, namely ecosystem recovery. For this, we investigated the biological control of *A. filiculoides* in Belgium through field observations and a mesocosm experiment (**Chapter 5**). Our results indicate that the invasion of *A. filiculoides* triggered a cascade effect on aquatic ecosystems, affecting water quality, macroinvertebrates assemblage and zooplankton community. Effects were more pronounced in experimental mesocosms, possibly due to the small dimensions and absence of other macrophytes. In the field, *S. rufinasus* successfully controlled water fern cover and density over a period of 6 weeks, although control was achieved late in the season (end of August). Invaded mesocosms showed initial signs of recovery following biological control. However, changes in community composition were long-lived, and full recovery was not observed. Although *S. rufinasus* is already present in Belgium, it is advisable to facilitate its timely redistribution in order to accelerate water fern control and minimize its impacts on native aquatic ecosystems.

GENERAL DISCUSSION



GENERAL DISCUSSION



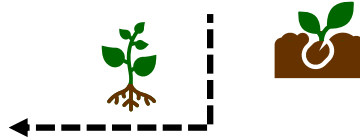
Germination rates are higher in ARG than in BE;
Cold-stratification increases germination speed, but
decreases overall germination rates;
Increased winter temperatures have a positive effect
in ARG but negative in BE;
Herbivory by *A. nonstriata* affects seedlings
significantly more than plants grown from rhizomes

CHAPTER 4 Germination potential and impact assessment



Comparing germination and early
growth of *I. pseudacorus* between
a) Argentina and Belgium; and
b) Cold stratified and control seeds

+
Quantifying the impact of different
densities of *A. nonstriata* on *I.*
pseudacorus grown by rhizome
fragmentation or seed germination

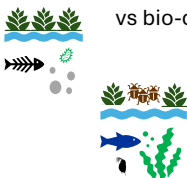


CHAPTER 5 Post-release evaluation and recovery of native ecosystems

Water parameters,
macroinvertebrates and
zooplankton communities

+
Before, during and after
water fern invasion

+
Invaded vs uninvaded
vs bio-controlled



+
↓ Dissolved oxygen
↑ Nitrogen and phosphorus
↓ Species diversity

↑ Water quality
↑ Species diversity
≠ Communities

Water fern invasion affected
water quality and community composition;
S. rufinasus successfully controlled water fern,
eliciting signs of ecosystem recovery;
However, changes in communities were persistent

GENERAL DISCUSSION

KEY MESSAGES

RUSSIAN ROULETTE, SILVER BULLETS AND TASK FORCES

The selection of biocontrol agents has always been the focus of debate amongst biocontrol practitioners (Harris, 1973; Lawton, 1984; Wapshere, 1985; Brieese et al., 2003). Some of the first historical weed biocontrol programs, dating back over 100 years, have followed a somewhat indiscriminate method of introduction (Heimpel & Cock, 2018). This *Russian roulette* approach – the so-called Lottery Model (Myers, 1985) – followed the principle that the more agents released, the greater the chances that at least one would establish and be successful. Indeed, studies have shown that in programs involving multiple introduction, often a single agent is considered responsible for the control of the target weed (Denoth et al., 2002). However, in the early days of weed biocontrol some programs have been criticized for the lack of adequate pre-assessment of agents' safety and efficacy, advocating restraints against the introduction of multiple agents (Denoth et al., 2002).

Fortunately, weed biological control has come a long way in the past century, evolving evermore responsible and evidence-based practices to ensure that introductions are targeted, effective, and environmentally safe (Heimpel & Cock, 2018). These involve careful selection of biocontrol agents based on field surveys, host-specificity testing, pre-release studies, and monitoring of ecological impacts (**Chapter 1, 2, 3 and 4**). Nonetheless, the criteria upon which biocontrol agents are selected remain somewhat outdated and open to discussion (e.g. Stiling & Cornelissen, 2005; van Klinken & Raghu, 2006). Should we search for the winning lottery ticket – a single, highly effective biocontrol agent – to combat an invasive species? Or should we rather assemble an array of complementary agents, imposing cumulative stress on the plant while increasing the chances that the right agent will be amongst them?

GENERAL DISCUSSION

Researchers and policymakers often seek a *silver bullet* solution as an ideal outcome because it offers a straightforward and definitive answer to pest management challenges. The successes obtained on *Salvinia molesta* D.S. Mitch. (Salviniaceae), *Pistia stratiotes* L. (Araceae), *Myriophyllum aquaticum* (Vell.) Verdc. (Haloragaceae) and *Azolla filiculoides* Lam. (Azollaceae) in South Africa provide great examples of how a single agent might provide a simple solution to an already widespread biological invasion (Coetzee et al., 2011). However, finding – and releasing on a first try – the right agent to successfully control an invasive weed is exceedingly rare. Instead, successful biological control programs typically involve the introduction of multiple agents, and even the integrated use of various management strategies. For instance, the control of *Pontederia crassipes* (C. Mart.) Solms (Pontederiaceae) in South Africa follows an integrated approach, that includes herbicide application and augmentative biological control (Coetzee et al., 2011).

The issue of agent selection has come up multiple times across this thesis (**Chapter 1, 2 and 4**). The native range surveys of *I. pseudacorus* presented an opportunity to evaluate various agents to be used against this incipient weed. The three candidates prioritized – the flea beetle *A. nonstriata*, the seed weevil *M. punctumalbum*, and the sawfly *R. micans* – all possessed promising characteristics and were therefore initially considered against yellow flag iris (**Chapter 1**; Minuti et al., 2021). However, there were concerns regarding their host-specificity, as all three organisms were reported to feed and/or reproduce on species other than *I. pseudacorus* (although all within the *Iris* genus). In the case of *R. micans*, these concerns were exacerbated by its low mobility, short activity season, and potential biotoxicity (Boevé et al., 2018), all traits that could undermine its suitability as a biocontrol agent. Furthermore, climate matching showed *A. nonstriata* and *M. punctumalbum* to be better suited for release across the invaded range, especially considering their resilience to climate change (**Chapter 2**; Minuti et al., 2023). Climatic mismatches are reported to

GENERAL DISCUSSION

affect, at least to some degree, about one in three biocontrol programs, resulting in considerable financial and temporal losses (Harms et al., 2020; Harms et al., 2021). For example, regional control of the invasive cactus *Opuntia stricta* Haw. in South Africa, albeit successful, is believed to have been slowed down significantly by the heavy rainfalls dislodging the sessile females and nymphs of its biocontrol agents, the cochineal insect *Dactylopius opuntiae* Cockerell (Paterson et al., 2023). Similarly, *Agasicles hygrophila* Selman & Vogt, a flea beetle released against alligator weed invasions in the USA, is restricted to warm coastal areas in the southeastern USA by cold winter temperatures, leaving areas north of its overwintering range largely uncontrolled (Harms & Shearer, 2017). This is likely also the case for *S. rufinasus*, which great success in South Africa was largely attributed to increased voltinism due to high temperatures (Hill, 1998; Mvandaba et al. 2018). In Europe, the low winter temperatures induce a lag in the emergence of the weevil in spring, which allows the water fern to build up serious invasions before the weevil is able to control it, as observed in the field in Belgium (**Chapter 5**). It is therefore fundamental to include bioclimatic modelling, as well as other pre-release evaluations, into the selection and prioritization procedure for candidate biocontrol agents.

As previously mentioned, finding the *silver bullet* to use against an invasive weed is a difficult task, and often multiple agents are released before control is deemed successful (Denoth et al., 2002; Schwarzländer et al., 2018). This holds especially true for plant species with particular traits, such as the ability to reproduce both sexually and vegetatively (Paynter et al., 2012). Accordingly, complementing the control exerted by *A. nonstriata* on *I. pseudacorus* leaves and rhizomes with that exerted by *M. punctumalbum* on its sexual organs could be considered the best course of action against this target weed (**Chapter 1**). Nonetheless, due to difficulties in sourcing and rearing the seed weevil, host-specificity testing of this candidate has not yet commenced. Although rarely mentioned in selection procedures, difficulty to rear agents – due to vulnerable

GENERAL DISCUSSION

life-history traits, population collapses or plant-herbivore asynchrony (e.g. Gupta et al., 2016) - can cause significant delays and increased costs for a biocontrol program, independently of their host-specificity or predicted effectiveness (Goolsby et al., 2006). Another underestimated issue when selecting biocontrol agents is that of conflict of interests. These are usually associated with the target plant or with other non-target species having beneficial attributes (e.g. economic or cultural value) in particular regions or countries (Stanley & Fowler, 2003). In the case of *I. pseudacorus*, both its use for phytoremediation and the presence of numerous and prolific Iris Societies could raise conflicts within one or more regions. The damage caused by *M. punctumalbum* on the flowers of other horticultural irises, for instance, has recently generated some concerns regarding its potential economic impact (Chris McGrannachan, pers. comm.). Consequently, further native range surveys are planned, across climatically suitable regions of Europe (Minuti et al., 2022), to search for additional natural enemies to be used against yellow flag (e.g., in New Zealand).

At the same time, impact assessments have shown the capability of *A. nonstriata* to eradicate *I. pseudacorus* seedlings and early life stages (**Chapter 4**), which could significantly contribute towards the control of its sexual dispersal. While the concept of a silver bullet remains an aspirational goal in biological control, it is important to recognize the complexities and uncertainties involved. Achieving sustainable and environmentally sound weed control often requires a multifaceted approach that considers ecological dynamics, the coordination of different control methods, restoration and ongoing involvement with local stakeholders (e.g. Moffat et al., 2024).

FUTURE-PROOFING BIOLOGICAL CONTROL

Acknowledging the dynamic nature of ecosystems and the ongoing challenges posed by environmental change – including climate change, habitat degradation

GENERAL DISCUSSION

and the introduction of new invasive species – is crucial in order to bend the curve of global freshwater biodiversity loss (Britton et al., 2023; Lynch et al., 2023). One key aspect to future-proof the biological control of weeds involves considering the potential impacts of environmental change on both target species and their natural enemies (Robinson et al., 2020; Sun et al., 2020). Climate change, for example, may alter the distribution and abundance of weeds and their natural enemies, affecting the outcome of various management measures, including biological control (Harms et al., 2021; Sun et al., 2022). The relative increase in temperatures and atmospheric CO₂ levels is also predicted to affect the nutritional quality of plant tissues, a central factor in regulating plant-herbivore interaction (Reeves, 2017). This in turn is predicted to affect the susceptibility of invasive plants to herbivory by their natural enemies, with contrasting effects for biological control (Baso et al., 2021; Venter et al., 2022; Paper et al., 2023). Future efforts should involve conducting predictive modeling and scenario planning to anticipate how environmental changes could influence the dynamics of weed populations and their interactions with natural enemies. In this context, the results obtained throughout this project are a step in the right direction. Including climate change scenarios within species distribution models, as done here for *I. pseudacorus* (**Chapter 2**), constitutes a powerful addition if we want to predict future invasions and reinforce pre-release evaluations of biocontrol agents. Although some recent studies have already embraced this approach (Sun et al., 2017; Sánchez-Restrepo et al., 2023), a vast majority focuses solely on target weeds (Taylor et al., 2012; Rodríguez-Merino et al., 2019; Araújo et al., 2022) or on already established agents (e.g. De Simone et al., 2023), rather than their interaction. Similarly, the influence of climate change on the germination of invasive plant species has been thoroughly investigated (**Chapter 4**; Gillard et al., 2017; Huebner, 2022 and references therein). However, the adoption of such knowledge by management, especially

GENERAL DISCUSSION

when it comes to biological control, is still lagging behind (Amare, 2016; Gerard & Popay, 2017).

Future-proofing weed biocontrol should also entail diversifying management strategies and increasing the resilience of ecosystems to invasions (Britton et al., 2023; Lynch et al., 2023). This may involve incorporating a broader range of biological control agents (i.e. *task force approach*), manipulating the recipient environment (i.e. increasing *biotic resistance*) and potentially including native species to enhance the effectiveness and longevity of control efforts (i.e. *conservation biological control*; Barbosa, 1998). Furthermore, ongoing monitoring efforts are required to stay ahead of emerging threats and adapt management strategies accordingly. In particular, increased emphasis needs to be put in post-release monitoring, which is often neglected and underfunded in many biocontrol studies, to increase evaluations both quantitatively and qualitatively (Schaffner et al., 2020). This includes tracking the development of resistance in weed populations, as well as assessing the efficacy and ecological impacts of biocontrol agents over time, and evaluating the need for integrated pest management (Schaffner et al., 2020; Faltlhauser et al., 2023). Although only preliminary, the results provided by our case study have provided valuable insight into the benefits and shortcomings of water fern biological control in Belgium (**Chapter 5**). Populations of the naturalized biocontrol agents *S. rufinasus*, where present, have been observed effectively controlling *A. filiculoides*. Nonetheless, the level of control achieved is not equivalent to that observed in South Africa (Hill et al., 2008). This instance, likely due to climatic constraints of the weevil (Mvanbada et al., 2018), mirrors observations from Great Britain (Pratt et al., 2022) and warrants the implementation of timely and augmentative releases to control this invasive weed in Belgium (Reeder et al., 2018). By continuously evaluating and updating control strategies based on new scientific knowledge and changing environmental conditions, efforts to manage invasive weeds can be more effective and sustainable in the long term.

GENERAL DISCUSSION

Finally, future-proofing biological control requires increasing awareness and changing the perception around this method. Public (and scientific) support is crucial for the implementation of environmental policies, especially in democratic settings such as the EU (Messing & Brodeur, 2018). Despite its rich history (Winston et al., 2024), recorded success rates (Schwarländer et al., 2018), quantified economic benefits (e.g. Mouttet et al., 2018; Pratt et al., 2022), and certified safety records (Suckling & Sforza, 2014; Hinz et al., 2020), weed biological control has only recently started to be adopted in Europe, and only within a few member states (Shaw et al., 2018). A recent survey highlighted that, compared to non-EU respondents, EU professionals (i.e. researchers, practitioners and policy makers) still perceive weed biocontrol as a risky method, despite showing a good understanding of its potential (Marchante et al., 2023). The main factor identified by the authors for this diffidence is a lack of successful examples of weed biological control in Europe, leading to a bias towards commonly known failures. It is important to note that the most quoted examples of unsuccessful biological control given by EU respondents (i.e. *Rhinella marina* released in Australia to control cane beetles, and *Harmonia axyridis* released in Europe against aphids) did not constitute, in fact, instances of weed biocontrol (Marchante et al., 2023). Interestingly, the most mentioned example of successful weed biocontrol was that of *S. rufinasus* against *A. filiculoides*. Despite being an accidental introduction to Europe, the azolla weevil is well perceived mostly due to the advertised success and economic value generated in Great Britain through continuous introduction and redistribution campaigns (Pratt et al., 2022). This goes to show the importance of using positive, recent and tangible examples to increase public awareness on weed biological control. The results obtained on *S. rufinasus* and *A. filiculoides* as part of the present thesis constitute a valuable baseline to disseminate knowledge about weed biological control in Belgium. Furthermore, as the weevil already occurs in the country, studies focusing on this system require significantly fewer

GENERAL DISCUSSION

permits compared to novel introductions, for which clear administrative procedures are not yet in place. Indeed, another crucial issue hampering the adoption of weed biological control in Europe lies in the absence of regulatory frameworks, both at regional and national levels, for the study and release of weed biocontrol agents (Marchante et al., 2023). These take notoriously long to be implemented, but the process can be significantly expedited after the introduction of the first agent is deemed successful (Shaw et al., 2018). For this reason, *S. rufinasus* constitutes a golden opportunity to accelerate the uptake of weed biological control in Europe.

THE IMPORTANCE OF CONTEXT

The potential of classical biological control as a key component of weed management programs has been extensively covered (Schwarzländer et al., 2018; Hill et al., 2020; Hinz et al., 2020; Sun et al., 2022). Nonetheless, success rates show large geographic and temporal variations, and concerns remain over unpredictable impacts, especially in the face of climate change (Paynter et al., 2020; Sun et al., 2020; Paper et al., 2023). It thus becomes crucial to factor context-dependency in weed biocontrol programs (Lommen & Beukeboom, 2023). This requires both pre- and post-release studies to predict and validate the biotic and abiotic interactions (e.g. apparent competition) within a biocontrol system (Müller-Schärer & Schaffner, 2020; He et al., 2021). The versatility of climate compatibility models (Sutton, 2019) to avoid mismatches (Harms et al., 2021) and direct native range surveys tailored to various invaded contexts has previously been discussed in this thesis (**Chapter 2**; Minuti et al., 2022, 2023) and thus will not be readdressed here. Genetic matching can also be a useful tool to guide the search for natural enemies according to the origin of the target weed (e.g. Paterson et al., 2009; Sutton et al., 2017). Impact studies have demonstrated that the level of control achieved can be dependent on the right combination of genotypes and phenotypes between agents and target

GENERAL DISCUSSION

plants (Sun et al., 2020). The results presented in this thesis showed the potential of *A. nonstriata* to control different *I. pseudacorus* life-stages, at different insect densities (**Chapter 4**). The experiments have been conducted on both native and invasive test plants in South Africa, and constitute a reliable pre-release assessment for that region. Nonetheless, the phylogenetic and phenotypic relationships at play have yet to be fully explored, and the results should not be overgeneralized. Research is already underway to determine the genetic origin of *I. pseudacorus* invasions across different continents, which should inform future surveys and impact studies.

Together with literature records and native range observations, host-specificity testing constitutes the main tool to evaluate the safety of a biocontrol agent. This typically relies on choice and no-choice feeding and development trials in laboratory conditions (Hinz et al., 2020). Given the highly conservative nature of these trials, the incidence of false positives may lead to an overestimation of risk, resulting in the exclusion of potentially safe biocontrol agents (Müller-Schärer et al., 2023). That is, due to behavioral or ecological interactions, changing environmental conditions, genetic mismatches, and even management practices, the realized host range of some biocontrol agents in the field may be narrower than the fundamental host range determined during traditional host-specificity trials (Paynter et al., 2020; Franceschini et al., 2023). This prompts the need to modernize host-specificity testing protocols, acknowledging context-dependency to better evaluate the risks to non-target species. The results presented in this thesis were still produced via the traditional, conservative host-specificity testing method (**Chapter 3**). However, the selection of plants for testing raised some issues regarding the need for clear and flexible criteria when dealing with widespread targets such as *I. pseudacorus*, which invasion influences various phylogenetic and geographic contexts. Accordingly, the risks associated with the use of *A. nonstriata* are contingent to the region considered, and further host-specificity testing should

GENERAL DISCUSSION

reflect this dependency. The incidence of non-target attacks on native South African Iridaceae, for instance, could be evaluated analyzing the beetle host-finding behavior (Fung et al., 2021; Subedi et al., 2023) through multigenerational studies, before drawing a final risk assessment.

Finally, not all context-dependent interactions can be predicted beforehand, that is why post-release evaluations should be conducted more often and more rigorously as part of every biocontrol program (Hoffman et al., 2019; Schaffner et al., 2020). These studies not only allow us to evaluate the success and safety of biocontrol, but also to assess its transferability across different geographic and environmental gradients. For instance, one of the most problematic weeds, water hyacinth, was recently deemed under complete control in a 50-years post-release study in Argentina (Faltlhauser et al., 2023). This is not an isolated case of a biocontrol program being widely successful in some parts of the world while falling behind somewhere else. Our observations on *A. filiculoides* in Belgium depict a similar scenario (**Chapter 5**), with the weevil *S. rufinasus* being unable to exert the same level of control that it achieved in South Africa and other countries (Hill et al., 2008; Pratt et al., 2022). Furthermore, our research tackled an often-overlooked aspect in post-release studies, that of ecosystem recovery (Reid et al., 2009). The aim of weed biocontrol is to reduce the impact of invasive species on native ecosystems and the economic and social costs linked to biological invasions. However, control is only half the game. To ensure that management efforts align with broader conservation goals, post-release evaluations must take into consideration not only the level of control achieved, but also the recovery displayed by native ecosystems (Paterson et al., 2023). This is achieved by directly measuring native communities, ecosystem functions and habitat quality (Motitsoe et al., 2022). Only with reliable, quantitative data at hand, can conservation practitioners and policy makers correctly evaluate and endorse biological control as a sustainable management strategies.

GENERAL DISCUSSION

LIMITATIONS OF THE STUDY

SAMPLE SIZE, SAMPLE SIZE, SAMPLE SIZE

The importance of sample size in ecological research cannot be overstated, as it directly influences the reliability, accuracy, and generalizability of findings. Unfortunately, due to the intrinsic difficulties and unpredictability of field work and experimental designs, no ecological study is flawless when it comes to sample size. And the present research is no exception. For instance, the native range surveys for natural enemies of *I. pseudacorus* took place in Belgium and Northern Italy over the course of two following years. Aside from the small geographical extent relative to the entire native range *I. pseudacorus* (which spans several other countries and climate zones), the opportunistic and seasonal nature of the surveys could be the reason for the low number of natural enemies collected. Indeed, of over 42 arthropod species known to attack *I. pseudacorus*, only five were observed during our study (**Chapter 1**; Minuti et al., 2021). As insect populations are known to fluctuate between years and seasons, and some species can be rare and geographically isolated, it goes without saying that a larger sample size would have increased the likelihood of detection. Nonetheless, effort went into trying to minimize this sampling bias by conducting a thorough literature review beforehand and computing species accumulation curves afterwards. Furthermore, follow-up climatic compatibility models have been computed in order to guide future native range surveys for *I. pseudacorus* across different regions and climatic zones (Minuti et al., 2022).

Sample size could have also affected, to some extent, the results of the host-specificity testing. Difficulties sourcing test plants, budget limitations, time and space constraints play a major role in the sample size available for testing within a biocontrol program (Van Driesche & Reardon 2004). Although due diligence was used to ensure a critical minimum number of replicates and test subjects, the host-specificity trials conducted on *A. nonstriata* were not unaffected to the

GENERAL DISCUSSION

aforementioned issues. Studies using few plant replicates and/or a small number of insects can yield inconclusive or at times even misleading results, as they lack the statistical power to detect true patterns in the data (Whiters et al., 2013). Cage choice tests, in particular, can be affected by Type I (false positive) and Type II (false negative) errors (Mahorsay, 1998; Whiters et al., 2013). These can be due to various erratic behavioral phenomena such as sensory inhibitory/excitatory response, eggs ‘dumping’, habituation, and so on (Marohasy, 1998). No-choice host-specificity trials, on the other hand, are known for their robustness to false negatives, even in the face of relatively small sample sizes (Marohasy, 1998). Nonetheless, increasing sample size and testing time – including multi-generational testing – remains essential in order to provide greater resilience to confounding factors and improve the reliability of study outcomes. These issues have been acknowledged and changes are already taking place going forward with the host-specificity of *A. nonstriata* and other candidate biocontrol agents against *I. pseudacorus*.

ALL MODELS ARE WRONG, BUT SOME ARE USEFUL

Ecological niche models (ENMs) are simplifications or abstractions of complex systems and processes observed in the natural world. The user must omit certain details and make several assumptions to make the model more manageable and the output understandable. As a result, no model can fully capture every aspect of reality, and all models are inherently flawed to some extent. Nonetheless, some models can still provide valuable insights, predictions, or explanations of natural phenomena.

The objective of ENMs is to identify the limits to a species distribution, extrapolate them and use them to forecast its suitability in space and time (Barbosa et al., 2009; Werkowska et al., 2017; Yates et al., 2018). The increasing availability of species occurrence databases (e.g. GBIF, IUCN, NatureServe, etc.) and spatial environmental repositories (e.g. WorldClim; Fick & Hijmans,

GENERAL DISCUSSION

2017) made correlative ENMs very prominent in recent literature. The issue with correlative niche models is that they can be extremely prone to errors in data filtering, background selection, spatial resolution, variables correlation, and user-defined settings (see Sillero et al., 2023 for details). For instance, occurrence records are often influenced by sampling bias (i.e. data are collected more often from sites with easier access) and therefore clustering of records might represent accessibility rather than a real environmental preference of the species (Barbosa et al., 2013). In order to minimize clustering, modelers usually resort to spatial (Aiello-Lammens et al. 2015) and environmental filtering of the data (Varela et al., 2014). However, if the underlying bias is not properly addressed, filtering might provide even worse results (Gábor et al., 2019). In our modelling approach, care was taken to clean and filter the data based on robust methodologies and a thorough understanding of the bias present (**Chapter 2**; Minuti et al., 2023). Still, one of the decisions taken was to minimize spatial autocorrelation among occurrence data within each geographical region (Boria et al., 2014). This could raise some eyebrows, as spatial autocorrelation is considered an important and necessary property in geography, and models can suffer from the underestimation of environmental gradients (Sillero et al., 2023). Although we agree with this concept, the choice was taken after computing hundreds of dummy models, using various filtering methods and settings, and evaluating the statistical and ecological robustness of their results. Accordingly, we believe this to be the best approach to our model species, which worldwide distribution was significantly affected by sampling bias, leading to a strong imbalance between native and non-native occurrences (Minuti et al., 2022). Nonetheless, this and many other limitations arise when modelling range-shifting species (Elith et al., 2010). It is by openly acknowledging these limitations, considering factors such as prior assumptions, uncertainties, and potential biases, that researchers and practitioners can make informed decisions. Despite their imperfections, well-

GENERAL DISCUSSION

designed and rigorously evaluated ENMs can still be useful tools for predicting invasion dynamics and forecasting future changes.

DEFINING AND MEASURING ECOSYSTEM RECOVERY

Invasive plant management, even when successful, does not always result in the restoration of natural ecosystems, and many studies that report recovery of native communities are based on observations rather than empirical data (Reid et al., 2009). Unfortunately, quantifying ecosystem recovery is no small feat. Although empirical approaches have been developed to study the impacts of invasive alien species under management (see for example Kumschick et al., 2015), field conditions are seldom ideal for these frameworks to apply. Aside from the intrinsic political difficulties related to working with invasive species in the field, oftentimes an ecosystem becomes the subject of research only after it has been invaded. In this case, the lack of baseline data regarding the conditions of the ecosystem before invasion makes it difficult to draw reliable before-after comparisons. One must thus rely on proxies, such as comparing invaded, uninvaded and managed ecosystems while considering time since invasion (Kumschick et al., 2015). Although this can help discover significant differences between treatments, building a causal link between invasion and/or management and the observed changes can be tricky.

In our field study, we tried to quantify the effects of *A. filiculoides* on water quality, macroinvertebrates and zooplankton communities in the presence and absence of its biocontrol agent, the weevil *S. rufinasus*. Besides finding hints of recovery in macroinvertebrate diversity in biocontrolled plots compared to uncontrolled ones, our results could not disentangle the direct changes caused by water fern invasion and its management. Field studies involving open ecological systems, even at small scales, are subject to a plethora of stochastic and confounding effects, making results difficult to interpret. Heavy rains, periods of drought, paucity or heterogeneity between sampling sites within each

GENERAL DISCUSSION

pond, differences in water depth, sediment and vegetation between treatment and control sites; these and many other factors can “*muddy the waters*” – excuse the pun – when it comes to drawing conclusions. To minimize these issues, an alternative approach is conducting semi-controlled studies through the use of exclusion cages or constructed mesocosms. These setups offer various advantages, from gathering baseline data to being able to control the sample size and manipulate particular variables within each ecosystem. However, they are not without limitations (Stewart et al., 2013). Observations in constructed mesocosms are bound by spatial and temporal constraints, and often rely on assumptions regarding the initial – or *desirable* – state of the ecosystem. Nonetheless, small-scale experiments can be useful to help explain large-scale patterns (Schaffner et al., 2020).

In our mesocosm experiment, the impact of *A. filiculoides* on the controlled ecosystems was highlighted by direct changes in water quality and aquatic communities. This was expected, as similar results were obtained by Pinero-Rodríguez and colleagues (2021) using comparable methods. Although a partial recovery was observed in water quality after biocontrol, this was not the case for the aquatic communities, which diversity and composition was strongly affected by the plant. However, it is important to highlight that this lack of recovery could be due to the restraints imposed by the controlled design (e.g. small water quantity, absence of macrophytes, sensitive dependence on initial conditions, lack of interconnectivity, etc.), influencing the resilience and trajectory of these ecosystems. Although useful tools to isolate direct relationships between few, controlled variables, these setups cannot be the only source of empirical data when it comes to quantifying the impacts and ecosystem recovery. It is thus crucial for post-release evaluations to rely on robust methodologies and baseline data (if available) and integrate both field and controlled studies in order to reliably account for temporal variability and other confounding factors.

GENERAL DISCUSSION

PERSPECTIVES

A basic principle of applied research is that its results should not only increase our fundamental knowledge of a particular field or topic, but also lead to practical applications. In the field of weed biocontrol, theoretical results can be extrapolated to inform future research and guide management practices. In this thesis, we advanced the biocontrol program of *I. pseudacorus* and conducted a post-release assessment of the control exerted by *S. rufinasus* on *A. filiculoides* in Belgium. While we were able to fill some knowledge gaps, many questions still remain. In these final paragraphs we highlight the practical applications of our results, and how these can inform future research and management going forward.

In **Chapter 1**, native range surveys were conducted across Belgium and Northern Italy to identify and prioritize candidate biocontrol agents for *I. pseudacorus* (Minuti et al., 2021). Furthermore, a list was compiled of the organisms reported to attack *I. pseudacorus* in the literature. Aside from their direct application, which permitted to further investigate the prioritized agents, the results obtained in the chapter open the way to future native range surveys of the weed. Further research is needed to identify the genetic origin of *I. pseudacorus* invasions (Gallego-Tevar et al., 2024), which together with climatic suitability models (Minuti et al., 2022) will be used to guide future surveys for new candidate biocontrol agents and/or more compatible populations of the prioritized agents (Mukherjee et al., 2021; Andersen & Elkinton, 2023). This is already underway, with researchers from New Zealand planning to carry surveys in Europe in the near future (McGrannachan, pers. comm.).

The bioclimatic suitability models developed in **Chapter 2** form a solid foundation to predict areas at risk of invasion by *I. pseudacorus* and prioritize amongst the already known candidate agents. Nonetheless, these models are intended as a first, conservative approach, and could undoubtedly benefit from

GENERAL DISCUSSION

further refining and validation. Great advances have been made in the field of species distribution modelling, especially with regards to alien and range-shifting species (Elith et al., 2011; Verbruggen et al., 2013). Several algorithms are available for the purpose, and many can be applied to presence-only data (Sillero et al., 2023). The use of ensemble modelling, as well as the inclusion of non-climatic variables, could significantly increase the accuracy and predictive capacity of our models (Shabani et al., 2016; Omer et al., 2023). Similarly, insights into the biology and the life-history of yellow flag and its candidate agents could help develop mechanistic models to be used for comparison and validation (Kearney et al., 2010; Harms et al., 2021). Altogether, these results will be able to guide biological control and integrated management actions to combat the invasion of *I. pseudacorus* in the present, and tailor them to future climatic scenarios (Deere et al., 2023).

Chapter 3 presented a consolidated list of plant species to be considered for host-specificity testing across different regions invaded by *I. pseudacorus*, while displaying the first results obtained with *A. nonstriata* on a subset of these species. Further research is needed to fully evaluate the safety of this agent by testing more plant species, including multi-generational testing, field-cage experiments, and behavioral trials (Van Klinken, 1999). Additionally, future studies should aim to assess the accuracy and completeness of the plant list, and by extension the validity of the scoring system used to produce it.

In **Chapter 4** two separate but equally important sets of results were produced. First, the germination rate and timing of *I. pseudacorus* varies between native and invaded range and is dependent on cold stratification. Future research should investigate the importance of this phenomenon across different regions and climate types and explore the effects of other factors on germination (e.g. Gillard et al., 2022; Sandenbergh et al., 2024). This in turn will help guide management practices (e.g. flower clipping, biocontrol releases, etc.) to

GENERAL DISCUSSION

counter the sexual reproduction of the weed and hamper its dispersal. Secondly, the impact of *A. nonstriata* was deemed sufficient, even at low densities, to stunt *I. pseudacorus* growth and photosynthetic activity. However, it is often difficult to extrapolate the results obtained in laboratory settings and predict what will happen in field conditions (Morin et al., 2009). Future studies are needed to quantify the control potential of *A. nonstriata* under variable biotic and abiotic factors (Augustinus et al., 2019; Klötzli et al., 2023).

Finally, **Chapter 5** reported the results of a case study on the biological control of *A. filiculoides* in Belgium, and used them to discuss the importance of including ecosystem recovery in post-release evaluations. Whilst confirming the capability of this agent to seek and control its host, our results raised some concerns regarding the timing of control, and the subsequent recovery of the aquatic ecosystems. Further research is needed to explore the factors limiting *S. rufinasus* populations from timely controlling *A. filiculoides* infestations in central Europe (e.g. Mvandaba et al., 2019). This could help implement the active relocation of the weevil, and even the rearing of adaptive genotypes (Sun et al., 2020). Finally, more attention needs to be drawn towards evaluating the state of the ecosystems before and during invasion, and after biological control. This will help developing sustainable management, aimed not only at controlling invasive aquatic plants, but at eliciting the recovery of native ecosystems.

CONCLUSION

This thesis follows the main steps of a weed biocontrol program, from agent selection to post-release evaluations, while advancing our knowledge on the invasion of *I. pseudacorus* and *A. filiculoides*. Each chapter offers a platform to discuss the merits and pitfalls of current methodologies, and how these can be adapted to the challenges posed by global change. Altogether, the results and thoughts presented will serve to guide management actions and inspire future research on the invasion ecology and biological control of these two weeds.

GENERAL DISCUSSION

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Gianmarco Minuti

After obtaining his BSc in Natural Sciences *summa cum laude* from the Milan State University (UNIMI), Gianmarco went on to enrol in the MSc in Biology – Ecology & Biodiversity program at the VUB, from which he graduated with greatest distinction. He was then awarded a PhD Fellowship for Strategic Basic Research by the Research Foundation – Flanders (FWO) to pursue a joint doctorate between the VUB and the Rhodes University (RU) of Makhanda, in South Africa.

The main objective of his PhD is to explore the use of biological control as a sustainable management method against invasive alien aquatic plants in freshwater ecosystems. This research fits within an international network that extends beyond Belgium to include research institutes in Argentina, South Africa and New Zealand.

He is also a member of the Centre for Biological Control (CBC) and the International Organization for Biological Control (IOBC).

Education

PhD in Biology & Entomology	2019 - 2024
Vrije Universiteit Brussel (VUB)	
Joint with Rhodes University (RU)	
MSc in Biology	2016 - 2018
Vrije Universiteit Brussel (VUB)	
Graduated with Greatest Distinction	
BSc in Natural Sciences	2012 - 2015
Università Statale di Milano (UNIMI)	
Graduated <i>summa cum laude</i>	



ORCiD

<https://orcid.org/0000-0002-1632-8612>

ResearchGate

<https://www.researchgate.net/profile/Gianmarco-Minuti>

Fundings & Awards

Travel grant (FWO), 2023

PhD Fellowship Renewal (FWO), 2021

Travel grant for University Development Cooperation (Global Minds), 2020

Joint PhD (VUB), 2020

PhD Fellowship - Strategic Basic Research (FWO), 2019

Travel grant (VUB), 2019

Travel grant (VLIR), 2018

Languages

Italian	Native
English	C1-C2
French	B2-C1
Spanish	B1-B2
Dutch	A1-A2

International peer-reviewed publications

Minuti, G., Coetzee, J.A., Ngxande-Koza, S., Hill, M. & Stiers, I. (2021). Prospects for the biological control of *Iris pseudacorus* L. (Iridaceae). *Biocontrol Science & Technology*, 31(3): 314-335.

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Gervazoni, P., **Minuti, G.**, Fuentes-Rodriguez, D., Coetzee, J.A., Sosa, A., Sabater, L. & Franceschini, C. (2023). Citizen science improves the known and potential distribution of a strong wetland invader: implications for niche modeling and invasion management. *Environmental Management*, 71, 1176–1187.

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Sandenbergh, E., Gervazoni, P., Grewell, B., Franceschini, C., **Minuti, G.**, McGrannachan, C., Stiers, I. & Coetzee, J.A. Biology of Invasive Plants: *Iris pseudacorus* L. *Invasive Plant Science and Management*, in press.

Zilio, M.I., Andrés, F., Gervazoni, P., **Minuti, G.**, Muzón, J., Djeddour, D., Mc Kay, F. & Sosa, A.J. Economic loss of ecosystem services caused by yellow flag iris and white ginger lily, two invasive alien plants in natural ecosystems of Argentina. *Journal for Nature Conservation*, in review.

Contributions to conferences

Minuti, G., Gervazoni, P., Nicoló, R., Delange, O., Cantarelli, M., Boon, E., Franceschini, C., Sosa, A., Stiers, I., & Coetzee, J.A. (2024). Stick to your grubs: a flea beetle to combat the seedling recruitment of *Iris pseudacorus* (Iridaceae), an invasive wetland plant in the Southern Hemisphere. International Congress of Biological Control (ICBC3), San José, Costa Rica.

Minuti G., Staniszewski M.S., Singh K., Coetzee J.A. & Stiers I. (2023). Assessing the recovery of aquatic ecosystems after biological control of the invasive water fern *Azolla filiculoides*: A case study from Belgium. International Symposium on Aquatic Plants (ISAP16), Antwerp, Belgium.

Stiers, I., Meert, P., **Minuti, G.** & De Boeck, S. (2023). Bugs 2 the Rescue: A Citizen Science Project. International Symposium on Aquatic Plants (ISAP16), 13-17 Nov 2023, Antwerp (Belgium).

Minuti G., Coetzee J.A. & Stiers I. (2023). Contrasting effects of climate change on the invasion risk and biocontrol potential of the invasive *Iris pseudacorus* L.

between Northern and Southern Hemisphere. International Symposium on Biological Control of Weeds (ISBCW16), Puerto Iguazu, Argentina.

Zilio, M.I., Andrés, F., Gervazoni, P., **Minuti, G.**, Muzón, J., Djeddour, D., Mc Kay, F. & Sosa, A.J. (2023). Economic loss of ecosystem services caused by yellow flag iris and white ginger lily, two invasive alien plants in natural ecosystems of Argentina. International Symposium on Biological Control of Weeds (ISBCW16), Puerto Iguazu, Argentina.

Minuti, G., Coetzee, J.A., & Stiers, I. (2022). Present and future climatic suitability of the invasive aquatic plant *Iris pseudacorus* L. and its candidate biocontrol agents. International Conference on Aquatic Invasive Species (ICAIS22), Oostende, Belgium.

Minuti, G., Coetzee, J.A., & Stiers, I. (2021). Climatic suitability of *Iris pseudacorus* and *Aphthona nonstriata* in the Southern Hemisphere. Symposium in Insect-Plant interactions (SIP17), Universiteit Leiden, Netherlands.

Minuti, G. (2021). Invasive Aquatic Plants (IAPs): A Growing Threat to Sustainable Development. VUB PhD Day: Global Challenges translated into Local and Research Projects (GCLRP), Vrije Universiteit Brussel, Belgium.

Minuti, G., Stiers, I., Ngxande-Koza, S. & Coetzee, J. A. (2019). A first step towards the biological control of *Iris pseudacorus* L. (Iridaceae). International Conference on Ecology and Management of Alien Plant invasions (EMAPI15), Prague, Czech Republic.