Evaluation of *Megabruchidius tonkineus* (Coleoptera: Chrysomelidae: Bruchinae), a candidate biological control agent for *Gleditsia triacanthos* L. (Fabaceae) in South Africa

THESIS

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Abstract

Gleditsia triacanthos L. (Fabaceae) (honey locust) is a fast-growing, deciduous tree indigenous to the United States of America. Introduced around the world as an ornamental tree, it has become invasive in a number of countries. Where it is invasive, *G. triacanthos* competes and replaces indigenous species; it creates dense stands along watercourses, posing a significant environmental threat. In South Africa, *G. triacanthos* is regarded as one of the country's fastest spreading weeds. *Gleditsia triacanthos* produces numerous seeds contained in large hanging pods. Once dislodged from the pods, the seeds are dispersed by birds and mammals, including livestock, which eat the pods. It has been suggested that the seeds should be the target for biological control programme.

Some invasive alien plant species are characterised by their ability to spread and establish in new ecosystems because they tolerate a wide range of environmental conditions. In order to predict areas of likely invasion, species distribution models (SDMs) are used to identify areas climatically suitable for their invasion, so enabling better targeted control of the plant species. *Gleditsia triacanthos* adapts to a wide range of climates and soil types, and tolerates salinity, drought and frost. Currently primarily restricted to the Grassland Biome of South Africa, *G. triacanthos* has doubled its distribution area in the past 15 years, and it is not known how far the species will spread. In this study we used two different modelling programmes, CLIMEX and MaxEnt, to predict areas where *G. triacanthos* could find favourable growing conditions; both SDMs showed that most of the country is suitable for *G. triacanthos* and that it will probably continue to spread, if left unmanaged, into new bioregions, such as the Karoo.

In South Africa, the Asian seed-feeding bruchid, *Megabruchidius tonkineus* (Pic*,* 1914) (Coleoptera: Chrysomelidae: Bruchinae) has been recorded in the plant's seed pods and has been considered as a biological control agent. The insect was not released as part of a formal biological control programme and neither host-specificity nor impact studies were conducted on the species prior to its discovery. In 2017 a decision was made to re-consider its status as a biological control agent until further details of its biology, host specificity, and impact on the seeds of *G. triacanthos* in South Africa were available. This study shows that *Megabruchidius tonkineus* has established across the entire *G. triacanthos* population in South Africa damaging approximately 9% of seeds. Laboratory studies show that, *Megabruchidius tonkineus* completes its larval development in the seeds of *G. triacanthos* in about 66.80 ± 0.6880 SE days before eclosing. In addition, the adult females oviposit on the following Fabaceae species: *Arachis hypogaea, Albizia, julibrissin, Cicer arietinum, Pisum sativum, Dipogon lignosus, Peltophorum africanum, Podalyria buxifolia Senegalia burkei, Umtiza listerina* and *Vachellia sieberiana*. However, larval development was limited to *G. triacanthos*.

It is concluded that the seed-feeding beetle is not a threat to native Fabaceae species in South Africa, however, it does not damage enough *G. triacanthos* seeds to be considered a valuable biological control agent at this stage, and additional seed-feeding biological control agents should be considered to reduce the number of *G. triacanthos* seeds entering the environment.

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Chapter One: General introduction

1.1 Introduction

1.1.1 Problem statement

Gleditsia triacanthos L. (Fabaceae), or honey locust, is a fast-growing, deciduous tree native to the United States of America. Introduced around the world as an ornamental tree, it has become invasive in a number of countries, including South Africa (Isely, 1975; Thompson *et al.,* 1998; ILDIS, 2019; CABI, 2021). Honey locust comes from a wide climate range in the native range where it grows in a variety of soil types; features that have allowed it to establish in many novel ecosystems (CABI, 2021). It is currently spreading rapidly in South Africa and is regarded as one of the nine fastest spreading weeds in the country, thus posing a significant environmental threat (Henderson & Wilson, 2017). It is not known whether the tree has reached it full distribution in South Africa, or if it will continue to spread into new environments.

Wherever the tree establishes out if its native range it competes with and replaces indigenous species, and dense stands along watercourses can significantly reduce stream flow (CABI, 2021). In South Africa, it is regarded as Category 1b under the National Environmental Management: Biodiversity Act (NEMBA) regulations, which means honey locust requires an invasive control programme owing to its high invasive potential; it should be eradicated where possible, and is prohibited on any land. As of 2017, the tree has been considered as an option for biological control in South Africa (Martin *et al.*, 2020).

The tree is assumed to be spread primarily by seeds that are readily dispersed by stream flow, birds as well as mammals, suggesting the seeds should be a target for biological control. In South Africa, the beetle, *Megabruchidius tonkineus* (Pic, 1914) (Coleoptera: Chrysomelidae: Bruchinae), a seed- feeding beetle, has been recorded in the seedpods of the plant. The insect was not released as part of a formal biological control programme, and it is not known how it was introduced, although it has been considered as a biological control agent (Klein, 2011). However, no host-specificity nor impact studies were conducted on the species prior to its appearance in South Africa (Klein, 2011). The beetle is native to Asia, and therefore, *G. triacanthos* is a novel host. The impact of the beetle on the seeds of the tree may influence future management options; if the seed-feeding beetle is not affecting the plant seeds sufficiently, additional seed-feeding biological control agents may need to be considered. The aim of this thesis was, firstly, to determine if *G. triacanthos* had reached its full distribution in South Africa or would continue to spread, and secondly, to investigate the host range and impact of *Megabruchidius tonkineus* on *G. triacanthos.*

1.1.2 Biological invasions

Invasive alien species are organisms that are new to an ecosystem, which cause ecological harm, affect human and/or animal health (Pimentel *et al.,* 2001; Diagne *et al.,* 2021). Globally, biological invasions are considered one of the leading factors driving biodiversity loss, causing changes in the community structure of native species and disturbing local ecosystems and ecosystem functioning. These disruptions are intensified by human disturbance, climate change and pollution (Levine *et al.,* 2003; Tylianakis *et al.,* 2008; Srivastava *et al.,* 2014). The negative consequences of invasive species cost billions of dollars in damages every year. A study in 2001 (Pimentel *et al.,* 2001) showed the economic damage associated with the effects of invasive alien species and their control amounted to approximately US $306 billion in USA, UK, India, South Africa, Australia and Brazil combined. Invasive alien plants are considered to be the most widespread and damaging of all the invasive biota. These invasive species have severe impacts on the environment; they modify the quantity and quality of water, and nutrient cycling, for example, the carbon, nitrogen, oxygen cycle (Powell *et al.,* 2013; Hanley & Roberts, 2019).

Biological invasions in South Africa

South Africa has a rich native biodiversity and boasts a rich floral diversity, hosting 21 643 native plant taxa including 16 507 (76.3%) that are endemic to southern Africa (SANBI, 2016). However it is also host to several thousand invasive or naturalised alien species, which threaten native biodiversity (Moran *et al.,* 2013). The coverage of the invasive or naturalised alien species includes terrestrial plants (759), freshwater aquatic plants (19), terrestrial vertebrates (30), freshwater fauna (77), terrestrial invertebrates (466), plants on offshore islands (17), fauna on offshore islands (18) and coastal marine species (56), giving a total of all taxa 1422 (van Wilgen *et al.,* 2020). Within the high number of invasive or naturalised alien species, 559 taxa are under some sort of regulation in the country (van Wilgen *et al.,* 2020). The success of invasive species establishing and spreading in South Africa means it is one of the most invaded countries in the world, with both natural and semi-natural ecosystems seriously affected (Richardson *et al.,* 1991, 1997; Moran *et al.,* 2013). South Africa has more invasive alien trees per square kilometre than any other country (Henderson, 2001). These invasive plants negatively affect biodiversity conservation (Mooney 2005; Vellend *et al.,* 2007) and therefore need to be managed (van Wilgen *et al.,* 2001; Richardson & Kluge 2008). In order to manage these species, South Africa spends about around ZAR2 billion (US $142 million) per year, primarily through the Working for Water Programme, a programme in the Department of Environment, Forestry and Fisheries (van Wilgen *et al.,* 2020). Owing to sustained funding and growing human capacity, South Africa has become a world leader in the management of invasive species, particularly plant species, through the use of weed biological control (Schwarzländer *et al.,* 2018; van Wilgen *et al.,* 2020).

Invasive alien plants in South Africa

Invasive alien plants were introduced into South Africa in the 1600s, and since then a high number of intentional (i.e., timber, ornamental, horticulture and agriculture) and unintentional introductions of exotic species have continued to be reported (Zimmermann &

Olckers, 2003; van Wilgen *et al.,* 2012). There are an estimated 9000 alien plant species in South Africa (Mucina & Rutherford, 2006; Willis, 2017; Richardson *et al.,* 2020), and of the high number of alien plant species present in the country, about 2000 are alien tree species (Glen & van Wyk, 2016; Richardson *et al.,* 2020). Owing to the diverse nature of the South African biomes, many of these alien species have escaped and become invasive. Currently, 10 million ha (around 8% of the country) have been invaded (van Wilgen *et al.,* 2020). Of the alien plant species in South Africa, 759 are currently considered invasive, 240 of them are trees (dominated by Australian *Acacia* and *Eucalyptus* species and the European *Pinus* species, but also including a number of northern temperate weeds such as *Robinia pseudoacacia* L. and *G. triacanthos* (Nel *et al.,* 2004; Martin *et al.,* 2020). Trees are particularly problematic in South Africa as a number of the country's biomes (such as the fynbos and grassland biomes) are particularly vulnerable to invasive trees and shrubs (van Wilgen *et al.,* 2001; Musil & Macdonald, 2007; van Wilgen *et al.,* 2020).

Invasive alien plant legislation in South Africa

Owing to the number, distribution and severe impact of invasive alien species in South Africa, legislation is required to systematically and effectively attempt to manage them. Presently, South Africa has two legislative acts directed at biodiversity and alien species management. These acts are the National Environmental Management Act (Government of the Republic of South Africa, hereafter referenced as: NEMA 1998) and the National Environmental Management: Biodiversity Act (Government of the Republic of South Africa, hereafter referenced as: NEMBA, 2004). Together the two acts dictate how South Africa deals with invasive alien species issues.

The principal Act regarding the control of Invasive Alien Species (IAS) in South Africa was the Conservation of Agricultural Resources Act (Government of the Republic of South Africa, 1983, hereafter referenced as: CARA 1983). This Act falls under the Department of Environment, Fisheries and Forestry (DEFF) and is predominantly focused on addressing those species that pose a threat to agricultural resources (van Wilgen *et al.,* 2012). NEMA was developed under the Department of Environmental Affairs (DEA) and was aimed at the preservation of biodiversity and ecosystem functioning. After NEMA, NEMBA was promulgated to deal directly with the control of the various IAS in South Africa. One of the mandates of NEMBA was the prioritisation of invasive alien species. This process involved separating IAS into four distinct categories which pertain to different management regulations and legal requirements. These categories and the species belonging to them are contained in the Alien and Invasive Species lists (Government of the Republic of South Africa, 2016, hereafter referenced as: AIS, 2016). This list was officially sanctioned and released in July 2016. Category 1 is split into 1a and 1b. Those IAS falling into Category 1a must be removed on site and have a national management programme dedicated to their eradication. Category 1b AIS are to be removed on site but do not have national management programmes dedicated to their eradication. No species in either 1a or 1b may be propagated on a commercial or agricultural scale; however, harvesting the wild population's fruits or produce is legal. Category 2 species are those that may be propagated and purposefully cultivated provided a permit specific to that use and that individual or organisation has been acquired from the DEA. Category 3 species are those that have been identified as invasive and are prohibited in terms of use or propagation; however, their management is not warranted as a priority, and no agricultural or economic value has been cited. *Gleditsia triacanthos* is one of the invasive alien trees in South Africa classified as follows under existing legislation: Conservation of Agricultural Resources Act (CARA) 2002 – Category 1 NEMBA – Category 1b; consequently, programmes to control it must be prioritised (van Wilgen *et al.,* 2001; Henderson & Wilson, 2017; SAGIC, 2019).

Under these acts there are three main methods of control used in South Africa: mechanical, chemical, biological, and integrated control.

1.1.3 Mechanical control

Mechanical control is the physical activity of managing invasive alien plant populations. The first attempt at controlling invasive alien species starts with physical destruction, or total removal of plants by hand or machine, felling, strip barking, ring barking, hand pulling, and mowing the species from the site, often in combination with fire or herbicides (van Wilgen *et al.,* 1995) mechanical control of this nature is one of the primary methods used within the Working for Water programme, mentioned previously. Fire has extensively been used as a management option for Invasive Alien Species in the South African grassland and fynbos biomes (Holmes *et al.,* 2008; van Wilgen, 2009). Mowing can be appropriate for some of the invasive species, but not all of them. Knowing the biology of the plant is also important, so that mowing takes place when it is about to flower to prevent its spread through the seeds. Ring barking must be combined with herbicide for better results; the herbicide should be applied in the cuts, and the cuts should be beyond the cambium (DNR, 2019). This control method is very labour intensive and costly to use in extensive infestations, but can be used on a small scale. It can cause soil erosion, and it is a short-term solution only (van Wilgen *et al.,* 1995).

1.1.4 Chemical control

Chemical control also extensively used by the Working for Water program, is less intensive than mechanical control, involves the use of herbicides to control the population of invasive plants, and may be effective for a new or small infestation of plants in an area. However, the use of herbicides can affect aquatic environments (Di Marzio *et al.,* 2009). The use of herbicides will vary depending on the target weed species, the crops and plants on site, and environmental considerations. Each country has registered herbicides to use against invasive plant species; using them properly requires a high level of training and is often governed by legislation. On the other hand, the use of chemicals can have unintended, undesirable impacts in the ecosystems, since those herbicides affect non-target plant species and have residual effects. Appropriate use on the target plants is important because plants can build up resistance to herbicides which allows weed species to survive mortality (Powles & Holtum, 2018). The three main components of chemical control are the efficiency of the herbicide, its frequency of use, and the duration of its effect (Coetzee, 2018; Powles &

Holtum, 2018). However like mechanical control chemical control is labour intensive and costly to use in extensive infestations, and it is a short-term solution only (van Wilgen *et al.,* 1995).

In their recent publication van Wilgen *et al*, (2020) state, control measures (other than biological control) have largely failed to check invasions at a national scale, and there have been no documented eradications of plant invasions from continental South Africa.

1.1.5 Biological control

The success of invasive alien species can depend on the absence of natural enemies in the new ecosystem. The Enemy Release Hypothesis predicts that this escape from natural enemies results in the introduced plant having a competitive advantage over native plants in the introduced range (Williamson, 1996; Crawley, 1997). The Enemy Release Hypothesis is based on three fundamental assumptions: 1) natural enemies regulate the abundance of native plant species; 2) natural enemies have a greater impact on native species than on invasive species, and 3) invasive plants benefit from the reduction of regulator natural enemies (Keane & Crawley, 2002). Biological control aims to reverse this enemy release and involves the intentional importation of natural enemies (biological control agents), native to the same geographical region as the target weed species. One of the fundamental aspects of biological control is that the selected biological control agent is host specific to the target plant (McFadyen, 1998).

Biological control offers a management solution which has been successfully implemented by a number of countries worldwide to control invasive alien plants (Schwarzländer *et al.,* 2018; Hinz *et al.,* 2020), however it is not practiced by all countries. Biological control when appropriate is sustainable, less expensive, and more environmentally friendly than mechanical and chemical control. It is worth noting that biological control does not eradicate the invasive species but aims to achieve a situation where an invasive plant species becomes a non-invasive naturalised alien which allows progressive recovery of the natural (van Wilgen *et al.,* 1995; McFadyen, 1998; Coetzee, 2018).

In South Africa, the introduction of classical biological control started in 1913, since when a total of 284 natural enemies has been studied in South Africa. These biological control methods have shown positive results on 74 invasive alien plant species within 23 plant families (Klein, 2011). Of these, 270 entities (95%) were intentionally introduced, while 14 (5%) were found to be present in the country, either as natives or as inadvertently introduced non-natives. Most of these 14 cases involved locally-occurring pathogens that were studied as potential mycoherbicides, two of which have been formulated and distributed for this purpose. The majority (83%) of the 284 natural enemies considered have been phytophagous insects, five (2%) of the agent species are mites (Acari), and 42 (15%) are plant-pathogenic organisms (Klein, 2011). Biological control research is primarily funded by the Working for Water programme in South Africa.

When starting a biological control programme, it is important to determine whether potential agents are not already present in the introduced distribution as this can result is a waste of time and resources. For example, when the biological control programme against *Sesbania punicea* (Cav.) Benth. (Fabaceae) was initiated in South Africa in 1982, four weevil species, including *Trichapion lativentre* Beguin-Bellecocq (Curculionidae), were introduced into quarantine to undergo host-specificity testing (Hoffmann & Moran, 1991). However, retrospective surveys two years later revealed *T. lativentre* was already widely distributed in South Africa (Hoffmann & Moran, 1991). Sometimes an unexpected agent that is not from the species' native range may already be present, for example, the invasive *Schinus terebinthifolia* Raddi (Anacardiaceae) (Brazilian pepper tree) in the USA where pre-release surveys showed the presence of an unintentionally introduced seed-feeding wasp, *Megastigmus transvaalensis* Hussey (Hymenoptera: Torymidae) was damaging the fruit of the tree. In Florida the overall seed mortality caused by this wasp has been reported to be as high as 76% (Wheeler *et al.,* 2001) and is regarded as an important component of the biological control programme against Brazilian pepper in the USA. Interestingly *S. terebinthifolia*, is native to South America and the wasp is native to South Africa where it is associated with plant from the *Rhus* genus (Hussey, 1956; Habeck *et al.,* 1989, Scheffer & Grissell, 2003). This suggests that a that they wasp may have a broader host range than what is usually considered safe in biological control, however, if the country has no native or beneficial plants in the same genus as the IAP; then broader host specificity (such as specificity to the subfamily level) may be possible. In the USA no native members of the Anacardiaceae found within the Florida distribution of Brazilian pepper tree have been attacked by the wasp, despite numerous attempts to rear the insect from the drupes of commercially important and American closely related species and therefore the wasp is considered as a successful biological control agent. Conversely, the wasp has also been recorded from the Brazillian pepper trees native range where it is also damaging the drupes of *S. terebinthifolia* and is thus considered a damaging invasive species. The movement of the Asian seed beetle *Megabruchidius tonkineus* onto *G. triacanthos* in South Africa could provide a similar example. *M. tonkineus* originates from Asia while *G. triacanthos* is from North America. Moreover, in its native habitat, the beetle develops in the seeds of *Gleditsia australis* Forbes & Hemsley, *Dolichos lablab* (L.) Sweet and *Tagetes* sp. (Southgate, 1979; Borowiec, 1984). However, this does not mean it is not sufficiently specific in South Africa to be considered a safe biological control agent.

1.1.6 Integrated control

In order to reduce the population of invasive plant species, a combination of management techniques can be implemented (DiTomaso *et al.,* 2006). Used in combination, chemical and mechanical control of weeds is better, and together, are often essential components of integrated control (van Wilgen & De Lange, 2011). An example of successful integrated control in Australia was against the invasive tree, *Mimosa pigra* L. (Fabaceae); to avoid the spread of the tree, fire, mechanical control, herbicide applications, together with biological control were applied and have been shown to be a successful combination (Buckley *et al.,* 2004).

1.1.7 Control of invasive trees in South Africa

In South Africa it has been shown that invasive trees use more water than the native vegetation, and intercept water in the catchments, extracting water all along the river courses (Versfeld *et al.,* 1998; van Wilgen *et al.,* 2004). To this extent, the 'Working for Water' programme started in South Africa in 1995 as a national programme to control alien plant invasion and its impacts, principally on water resources; its main focus was to remove alien invasive trees from conservation areas and river courses. Since then, the Working for Water Programme has grown to employ more than 20 000 people per year, and at present, operates in all provinces and major biomes in South Africa. In 2004, the Working for Water Programme had already spent more than US $530 million clearing around one million hectares of conservation areas and wetlands, using a combination of mechanical, chemical and biological control (van Wilgen *et al.,* 2001; Macdonald, 2004; Moran *et al.,* 2005). The control of invasive trees is particularly difficult compared to other invasive alien plants because the costs and difficulty of control increases with the amount of biomass that must be removed from the system (Marais *et al.,* 2004). Even though invasive trees are abundant worldwide, their management is still limited, with relatively few examples of success (van Wilgen & Richardson 2014).

As mentioned previously, South Africa has more invasive trees per square kilometer than any other country (Henderson, 2001). It has 17 main invasive tree genera dominated by Australian *Acacia* and *Eucalyptus* species, but also including a number of *Pinus* and *Fabaceae* species from Northern temperate regions of the globe (Nel *et al.,* 2004; Martin *et al.*, 2020). Chemical and mechanical control has been implemented against a number of these species involving substantial investments; in all cases, success has been limited (van Wilgen *et al.,* 2012; van Wilgen & Richardson, 2014). There are, however, 16 species of invasive tree in South Africa that have been subjected to biological control, and in six cases the level of control achieved has been substantial (Klein, 2011; Zachariades *et al.,* 2016). One of the best examples of biological control against trees in the country is the biocontrol of the invasive tree, *S. punicea*. This small tree, native to South America (Argentina, Uruguay and Brazil), was first recorded in South Africa in 1858, and it became a problem after 1974 when it was recognised for its invasive potential, especially along rivers and wetlands (McGibbon, 1858; Taylor, 1974). The biological control used against the tree was a combination of three weevils, *Trichapion lativentre* (Cav.) Benth, *Rhyssomatus marginatus* Fåhraeus and *Neodiplogrammus quadrivittatus* (Olivier). Since these weevils attack different parts of the tree, they were able to reduce the high densities of the tree (Hoffmann & Moran, 1998). The tree populations have been reduced by over 95% (Hoffmann & Moran, 1998; Hill *et al.,* 2020).

1.1.8 Control of Gleditsia triacanthos

There are not many examples of successful control of *G. triacanthos* and currently in South Africa, *G. triacanthos* is not controlled by chemical or mechanical methods (Henderson, 2017). In Queensland, Australia, a successful mechanical control programme to eradicate *G. triacanthos* was implemented, using a methodical "search and destroy" campaign with government and landholders working together for two years in two different stages, 1993–1994 and 1996–1997. The programme continued for years after the campaign in order to destroy the seedlings growing from the seed bank (Csurhes, 2004). The programme cost approximately US $400,000 to clear a relatively small region. In Uruguay, chemical control by injecting systemic herbicides on mature trees was evaluated and shown to be successful against adult *G. triacanthos,* but was not useful for managing seedlings (Sosa *et al.,* 2015). In Argentina, different mechanisms have been applied against *G. triacanthos,* such as different selective and non-selective chemical control options. Selective control in the form of systemic herbicides like picloran, 2.4D among others; these chemicals were applied on the bark of the trees that were cut with a chainsaw or ring-barked (Maranta, 2001). To control high densities of *G. triacanthos* in Argentina, non-selective methods, such as the use of a bulldozer, or fire and area-wide application of agrochemicals have been implemented. However, these methods have significant non-target environmental impacts (Maranta, 2001; Chaneton *et al.,* 2004; Cappello & de la Peña, 2007). Most studies conclude that chemical and mechanical control is not a long-term solutions owing to the high cost and persistence of seed banks (Leggieri, 2010).

The use of natural enemies may be an alternative option to reduce the populations of *G. triacanthos*. In Argentina, Di-Iorio (2005) reported the presence of an Asian Bruchinae, *B. endotubercularis*, attacking the seeds of *G. triacanthos*. However, the damage caused by *B. endotubercularis* has not been quantified (Di-Iorio, 2005). Further, as this insect is novel to Argentina, it may have non-target effects (Di-Iorio 2005). In Europe, the seed-feeding beetle *Megabruchidius tonkineus* (Pic, 1914) and *Dorsalis* Fåhraeus (both Coleoptera: Chrysomelidae: Bruchinae) have been recorded in the seed pods of *Gleditsia triacanthos* (György & Germann, 2012). There are also a number of insects associated with the tree with in the native range. Some of these are considered monospecific and could therefore be considered as potential biological control agents (CABI, 2020).

In South Africa, Klein, (2011) lists *M. tonkineus* as a 'not released biological control agent' for *G. triacanthos,* with an undetermined degree of control. However in 2017 it was decided by the South African biological control community that the insect would no longer be listed a biological control agent as the insects host range in South Africa was undetermined. The insect was therefore categorised as an unintentional introduction with unknown impacts in South Africa. *Gleditsia triacanthos* has been part of a formal biological control programme since 2017, however no agents have yet to be released against the species (Martin *et al.*, 2020).

1.1.9 Objectives of the study

The first objective, reported on in Chapter 2, was to determine if *G. triacanthos* has reached its full ecological niche in South Africa, or if the weed will continue to spread. This would assist in prioritising and refining management options for South Africa. In Chapter 3, the distribution and impact of the unintentionally introduced *M. tonkineus* in South Africa is investigated. In addition the chapter describes a series of laboratory experiments conducted to determine the biology and the fundamental host range of the insect. Finally in Chapter 4 the importance of climate matching in invasive species management is discussed; the potential of *M. tonkineus* as a biological control options for in South Africa is considered, and management priorities are suggested.

1.1.10 Study species

1.1.10.1 Gleditsia triacanthos *L. (Fabaceae)*

The genus *Gleditsia* L. (Fabaceae: Caesalpinioideae) was named for the German botanist Johann Gleditsch. This genus is native to North America and Asia and comprises 15 species: *Gleditsia amorphoides* (Griseb.) Taub., *G. aquatica* Marsh., *G. assamica* Bor, *G. australis* Hemsl., *G. caspica* Desf., *G. delavayi* Franch., *G. fera* (Lour.) Merr., *G. ferox* Desf., *G. japonica* Miq., *G. macracantha* Desf., *G. microphylla* Gordon, *G. pachycarpa* Gagnep., *G. sinensis* Lam., *G. rolfei* Vid., *G. texana* Sarg., and *G. triacanthos* L. (Schnabel & Wendel, 1998; The Plant List, 2013). *Gleditsia triacanthos* L. was named by the botanist, physician and zoologist, Carl Linnaeus (Calisher, 2007).

The specific epithet of *G. triacanthos* is derived from the Greek 'akantha' (thorn) and 'treis' (three) for the three-branched thorns. The 'honey' in the common name 'honey locust' derives from the sweet pulp in the pods, while 'locust' comes from the Christian tradition that maintains John the Baptist fed on 'locusts' when he was in the desert (CABI, 2021). Other common names include sweet locust, sweet bean locust, thorny locust, honey shucks locust, three-thorned acacia, thorn tree (English); Amerikaanse driedoring, soetpeulboom (Afrikaans); acacia negra, algarrobo de miel, robinia de la miel, acacia de tres espinas (Spanish) (Business Queensland, 2019; Glen & van Wyk, 2016; Fernández *et al.,* 2017). In North America and around the world, the preferred common name is 'honey locust'.

1.1.10.2 Varieties

There are a number of varieties of *Gleditsia triacanthos:*

Gleditsia triacanthos var. *brachycarpos* Michx.; *G. triacanthos* var. *bujotii* [Neum] Rehd.); *G. triacanthos* var. *horrida* Aiton; *G. triacanthos* var. *inermis* Willd.; *G. triacanthos* var. *inermis* (L.) Castigl.; *G. triacanthos* var. *Laevis* Koch; *G. triacanthos* var. *Macrocarpos* Michx.; *G. triacanthos* var. *nana* [Loud] A. Henry.; and *G. triacanthos* var. *elegantissima* [Grosdemange] Rehd (Santamour & McArdle*,* 1983; Burns & Honkala, 1990; GBIF, 2021). The varieties present in South Africa are undetermined but it is expected that at least some of the varieties are present in the country (Henderson, 2001).

1.1.10.3 Description

Gleditsia triacanthos is a long lived, medium- to large-sized deciduous tree that can to grow over 25 m in height with a trunk diameter of up to 90 cm (Fig. 1.1). Usually the tree shape has crown form. The trunk and branches are protected by long, strong, red thorns that can grow up to 20 cm length. On young trees the bark is flat, thin, olive-tan to olive-grey in colour; with age the bark becomes fissured, forming grey plates. The thorns are green and soft when young and become red when mature. The leaves are pinnate (mature trees), or bi-pinnate (young trees), with 15–30 leaflets, 2–3 cm long, and change colour from shiny green to golden-yellow in autumn (Nesom, 2003; Seiler *et al.,* 2011; Glen & van Wyk, 2016).

The flowers are small, pale yellow to greenish-yellow in colour. The racemes of flowers are 5–7 cm long and can be male, female or hermaphrodite on the same tree, or can have male and female flowers on separate trees (Orwa *et al.,* 2009; CABI, 2021).

The flat pods turn dark brown to purplish-brown; they are 30–40 cm long when mature and contain 15–35 seeds each pod. Pods mature during late summer and early autumn and fall from the tree unopened. The seeds are oval, green, smooth and beanlike while young, and turn brown and leathery when mature. The seeds are 10 mm long and are divided by a sweet pulpy tissue; they are viable for long periods because of the impermeable seed coat (Nesom, 2003; Funk, 1965; Seiler *et al.,* 2011).

Gleditsia triacanthos has a deep tap-root that can grow between 3–6 m deep. Because it is able to stabilise soils, the tree has been imported unto certain countries to prevent soil erosion, and to improve degraded agricultural lands. It is used for environmental purposes, such as ornamental and agroforestry (Djumaeva *et al.,* 2009; Orwa *et al.,* 2009).

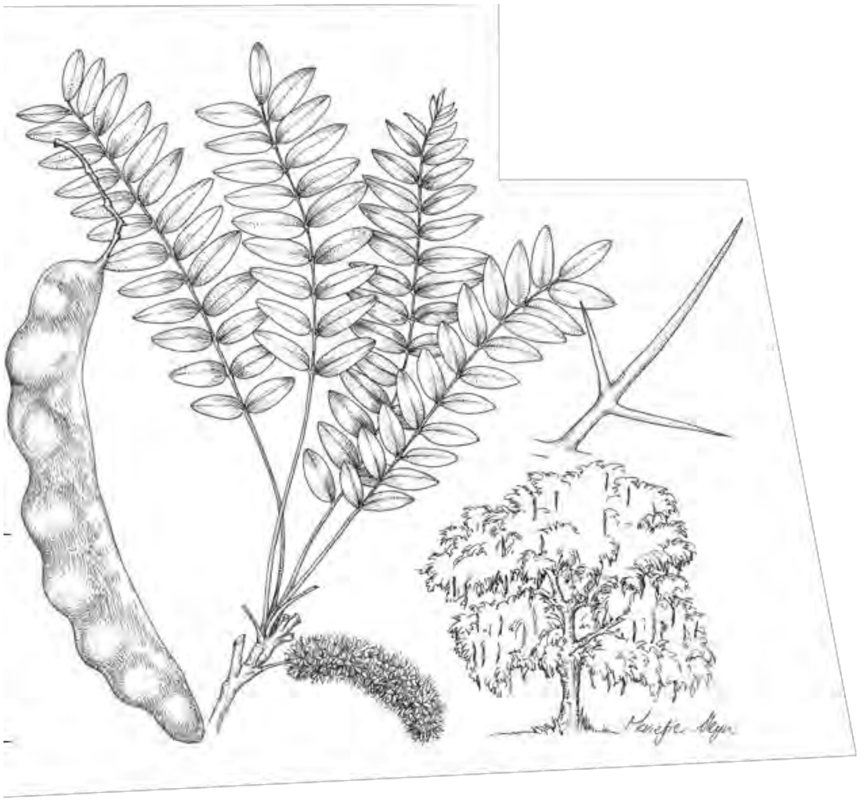

Figure 1.1: The form and distinguishing features of *Gleditsia triacanthos* showing flowers, leaves and pods (drawing by Marietjie Steyn first published in Henderson, 2001).

1.1.10.4 Distribution

Native range

Gleditsia triacanthos is native to central and eastern North America and southern Ontario, Canada; it is native in Alabama, Arkansas, Georgia, Illinois, Indiana, Iowa, Kansas, Kentucky, Louisiana, Michigan, Mississippi, Missouri, Nebraska, Ohio, Oklahoma, Pennsylvania, Tennessee, Texas, Virginia, and Wisconsin, USA (CABI, 2021) (Fig. 1.2). The list of states is evidence of the versatility and adaptability of *G. triacanthos*. Honey locust on its native range occupies a variety of habitats, such as woodlands, rocky upland, and abandoned pastures (Schnabel & Hamrick, 1990).

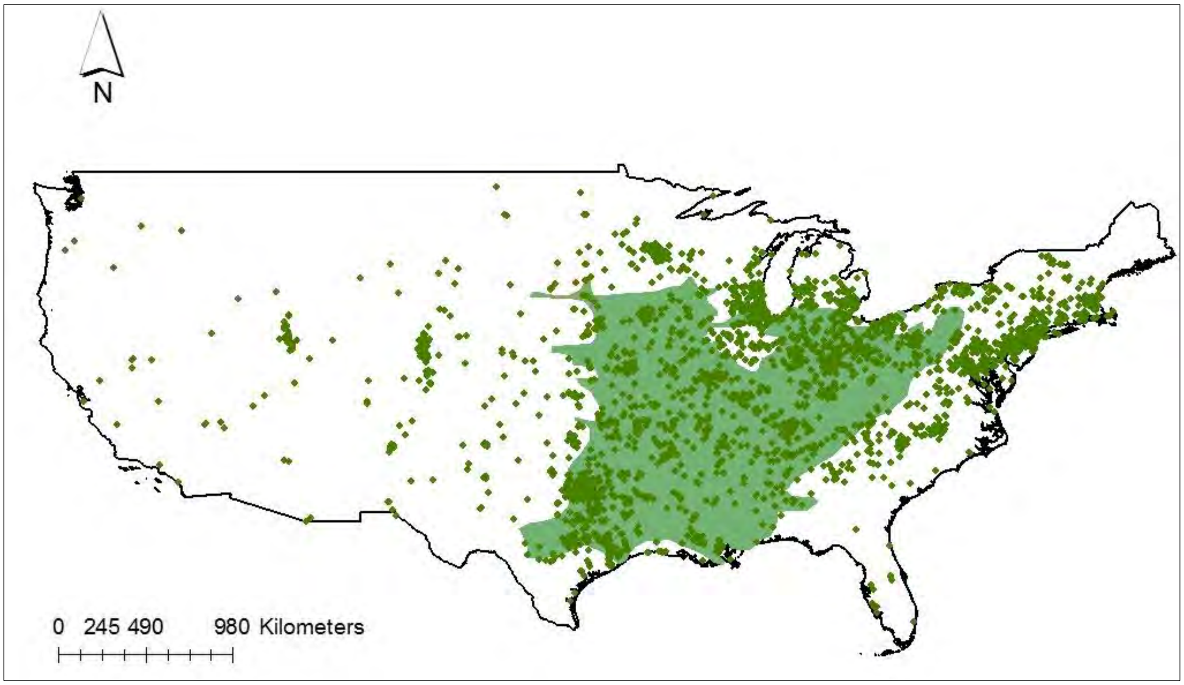

Figure 1.2: Distribution of *Gleditsia triacanthos* in the United States of America, the green colour in the background indicates where the tree is regarded as indigenous. Adapted from GBIF, (2021) and Thompson *et al.,* 1999.

Invaded range

Gleditsia triacanthos is one of the most widely planted woody tree species in the world and is regarded as invasive in a number of Countries (Table 1; Fig. 1.3).

Table 1.1: Showing global invaded distribution of *Gleditsia triacanthos* L (Fabaceae) with associated references.

Continent	Country	Reference
Africa	Ghana, Mauritius, Mozambique, South Africa, Lesotho, Tanzania, Tunisia, Uganda, Zimbabwe	Wells *et al.,* 1986; Henderson, 2016; CABI, 2021;GBIF, 2021;
Asia	Afghanistan, India, Iran, Iraq, Nepal, Pakistan, Turkey	CABI, 2021; GBIF, 2021;

Europe	Austria, Bulgaria, Croatia, Cyprus, Czechia Federal Republic of Yugoslavia, France, Germany, Greece Hungary, Italy, Montenegro, Poland, Portugal, Romania, Russia, Serbia, Slovenia, Spain, Switzerland, Ukraine, United Kingdom	CABI, 2021; National Research Council, 1980; Blanco *et al.,* 2012; Dana *et al.,* 2001; Sanz *et al.,* 2004;
North America	USA, Canada, Mexico	Thompson *et al.,* 1999; Sullivan, 1994; CABI, 2021
Oceania	Australia, New Zealand, Papua New Guinea	GBIF 2021; Csurhes & Kriticos 1994;
South America	Argentina, Uruguay	CABI, 2021; GBIF, 2021; Marco & Páez, 2000: Fernandez *et al.,* 2017; CABI, 2021; GBIF, 2021;

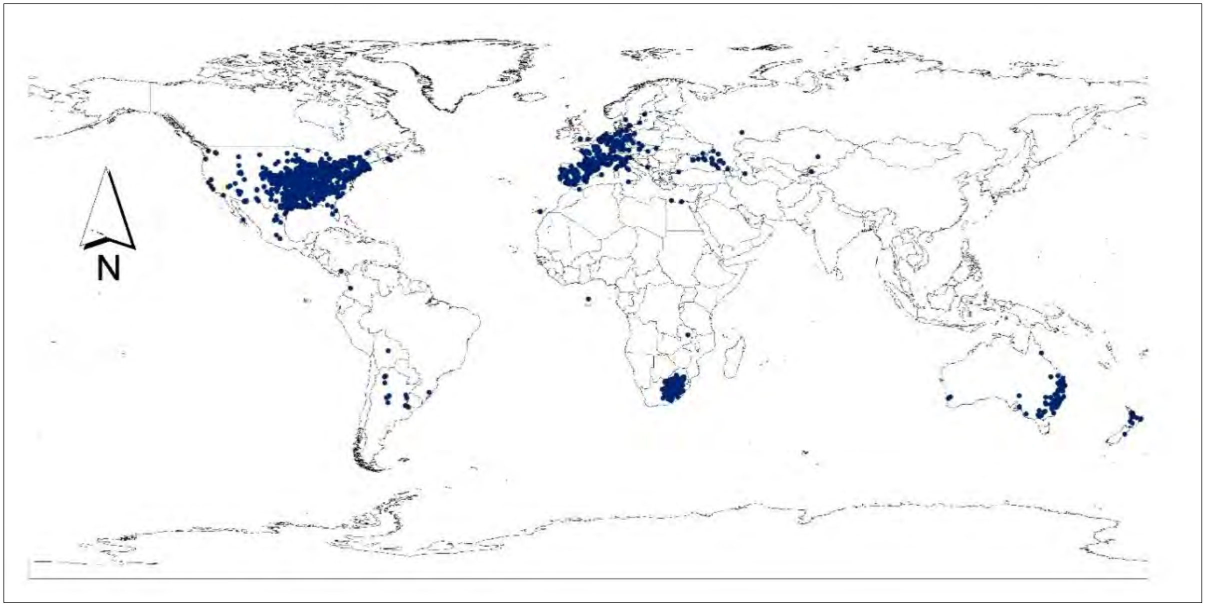

Figure 1.3: Global distribution of *Gleditsia triacanthos.* Adapted from the Global Biodiversity Information Facility (GBIF, 2021).

In South Africa *G. triacanthos* is categorised under the National Environmental Management: Biodiversity Act (NEMBA) as a 1b invasive species (NEM:BA, 2004). *Gleditsia triacanthos* is one of the nine fastest spreading weeds and its control programmes must be prioritised. In South Africa, honey locust is mainly found in the colder interior regions, with sub-zero winter temperatures and a mean annual rainfall of 600–1200 mm, which predominantly falls in the spring and summer months (Schulze & Lynch, 2007) (Fig. 1.4).

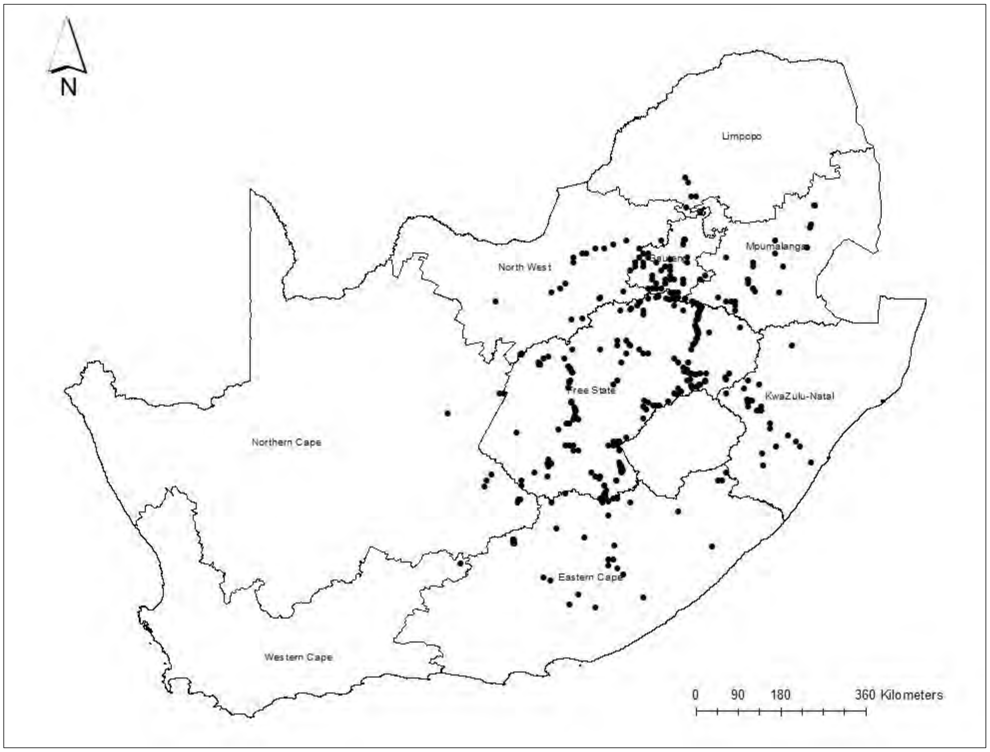

Figure 1.4: Distribution of *Gleditsia triacanthos* in South Africa adapted from SAPIA (2018) and personal observations.

1.1.10.5 Facilitation

The bean family (Fabaceae) consists of many species with the ability to nodulate, which is an important adaptation in this family. The root nodules are induced by a symbiotic relationship with the soil bacteria *Rhizobium*, a symbiosis that increases the level of atmospheric nitrogen fixation (Doyle, 2001). Unlike most leguminous species, honey locust does not form root nodules by *Rhizobium* and is not considered to be nitrogen-fixing (Allen & Allen, 1981). Honey locust does have a mechanism that produces biochemical compounds that affect the development of other plants; this mechanism is called allelopathy, and honey locust has a moderate allopathic effect in the roots, which tends to make it an aggressive invader (Whitcomb, 1972; Whitcomb & Roberts, 1973).

1.1.10.6 Flowering and Fruiting

In its native range, honey locust flowers in late spring to early summer between May and June. In Australia and South Africa, flowers are present from spring to early summer (September to November) (Henderson, 2001). Flowers are produced when the leaves are nearly full grown, which means it is late enough for the seed crop to be safe from frost damage. Honey locust first produces seed at about 10 years of age, but seed production is highest between 25 and 75 years, the longevity of the tree is around 120 years. Usually the trees produce abundant seeds every year or two (Funk, 1965). Each pod encloses 15–35 seeds. The seeds are produced within woody pods that remain on the tree during winter; they can persist for more than a year. Seed production varies annually, with higher densities recorded, on average, every second year. Honey locust plants can yield between 7 to 15 kg of seeds per 45 kg of fruit (pods) (Converse, 1984).

1.1.10.7 Economic Importance and Environmental Impacts

Detrimental

In South Africa, honey locust competes with and replaces indigenous species (Henderson & Wilson, 2017; SAGIC, 2019). Additionally, the plant is a problem weed in pastures because it smothers desirable grass species, thus reducing stocking rates (Business Queensland, 2019). Its sharp spines can inflict serious injuries and infections to humans and livestock, even after the plant is dead. However, tree growth is often suppressed by the native grasses in unburned tussock grasslands, but when these grasslands burn, seeds of honey locust are stimulated to grow and colonise the fields (Chaneton *et al.,* 2004).

Honey locust forms dense thickets that block stock access to waterways, reduce stream flow and create dense monocultures that provide a haven for pests, for example, foxes, cats and rabbits (Blair, 1990). In Australia and Argentina, the tree has been observed growing well and spreading on alluvial soils of floodplains and river and creek banks, replacing the native herbaceous vegetation (Business Queensland, 2019, Flora of Australia, 2020). This probably affects litter decomposition rates and the species composition of detritus-feeding aquatic invertebrates (Gantes *et al.,* 2011).

Beneficial

Honey locust has numerous uses for humans, including landscaping (garden ornamental), timber, livestock fodder and ethnobotanical uses, which contribute to its spread (Shadow, 2008; Fernández *et al.,* 2017). The pods are relished by stock and constitute excellent feed (Flora of Australia, 2020). The name, honey locust, derives from the sweet taste of the legume pulp, which was historically used for food by Native American people, and could also be fermented to make beer (Flowers of India, 2020). The fruits are commonly eaten by cattle and pigs. The beans of some cultivars contain as much as 12–13% protein, and the pods contain up to 42% carbohydrate (Mattoon, 1943; Stoutemyer *et al.,* 1944).

Historically, *G. triacanthos* was used as a local medicine by the Native Americans for pain relief, and for the treatment of measles and smallpox (Duke & Wain, 1981). Biochemical studies have shown that the plant contains phytochemicals such as triterpenes, sterols, flavonoids, alkaloids and phenolic. Mohammed *et al.,* (2014) found that the leaves of *G. triacanthos* have cytotoxic properties that could be used to treatment cancer (liver, cervix, larynx and colon) by inhibiting cell division and destroying tumours, as well as possibly treating rheumatoid arthritis. Furthermore, *G. triacanthos* seeds contain saponin which has analgesic and anti-inflammatory effects (Mohammed *et al.,* 2014; Saleh *et al.,* 2016).

Gleditsia triacanthos has a deep tap-root and is helpful for soil erosion control (Reuben *et al.,* 2007). In agroforestry systems, it may be planted with living mulch (such as clover), an association that provides reliable cover for the soil (FAO, 2012). In Central Asia, the honey locust and *Robinia pseudoacacia* L. (Fabaceae) are both used to improve degraded agricultural land so that dairy production can be introduced, based on foliage trees (Djumaeva *et al.,* 2009). Honey locust trees are often used as windbreaks (FAO, 2012; Orwa *et al.,* 2009). In the USA, both the common honey locust and its thorn-less varieties are widely planted for erosion control and for wind breaks; the thorn-less varieties are widely planted as shade and ornamental trees (Burns & Honkala, 1990). Owing to its ability to stabilise soils, the tree has been imported into several countries to prevent soil erosion, and to improve degraded agricultural lands. It is used for environmental purposes, such as ornamental and agroforestry species (Djumaeva *et al.,* 2009; Orwa *et al.,* 2009).

Chapter Two: The potential distribution of *Gleditsia triacanthos* in South Africa

2.1 Introduction

Invasive alien plant species are characterised by their ability to spread and establish in new ecosystems. There are a number of hypotheses available which attempt to explain the drivers behind these invasions (Dai *et al.,* 2020). Despite what drives the invasion, determining the areas where invasions are likely to occur is vital for management. A number of species distribution Models (SDM) have been developed to try to predict areas of likely invasion (Phillips *et al.,* 2006) in order to have better targeted control of the invasive plant species and to identify areas that are at risk (Gormley *et al.,* 2011; Newbold *et al.,* 2010).

There are fundamentally two different SDMs: mechanistic and correlative (Beerling *et al.,* 1995). Mechanistic SDMs use the biological limits of the species and environmental data to predict potential distribution (Beerling *et al.,* 1995). These mechanistic models form the basis of CLIMEX-Compare Locations, which is one of the most frequently used mechanistic software packages for modelling for invasive species; however, access to data suitable for mechanistic models is very difficult to obtain (Sutherst *et al.,* 1999) or requires extensive knowledge of the target species which can often only be derived from deductions made by an experienced ecologist. By comparison, data for correlative SDM is easier to access. Correlative ecological niche modelling works with environmental parameters and the relationship that the species has to its environment (Robertson *et al.,* 2004). Within correlative models, the data need to be converted into a grid system or raster dataset where each grid cell either has presence-absence data or presence-only data (Yackulic *et al.,* 2013). Presence-only data is the most commonly used data for making predictions in correlative SDM; if this is the case, then pseudo-absence data points are created, usually automatically by the programme (Merow *et al.,* 2013; Yackulic *et al.,* 2013). Of the software used in correlative SDM or environmental niche modelling, MaxEnt has been a popular choice by ecologists and conservation managers over the past 15 years because it is easily accessible and has been shown to outperform similar programmes (Merow *et al.,* 2013; Phillips *et al.,* 2017). Since its introduction in 2006, it has been cited in over 6000 publications (Phillips *et al.,* 2017).

MaxEnt is a general-purpose correlative method of predicting distributions, starting from incomplete information (Phillips *et al.,* 2006; Phillips *et al.,* 2017). MaxEnt software is based in the maximum entropy principle, a statistical mechanism that has been available since the 1950s (Jaynes, 1957; Elith *et al.,* 2006; Phillips & Dudík, 2008; Phillips *et al.,* 2017) and uses the known species distribution (locality data), as well as a set of environmental predictors (e.g., precipitation, temperature) across a user-defined background (or landscape) that is divided into grid cells. From this background, MaxEnt extracts a sample of background locations (absence points) that it contrasts against the presence locations (Phillips *et al.,* 2006). Because the programme was first created to estimate densities of species across landscapes, MaxEnt calculates two probability densities: 1) the relative probability of finding the species with all environmental variables in the model, and 2) the background points that characterise the available environment in the study region where the density of the presence data characterises the probability of the right environment where the species has been found (Phillips *et al.,* 2006; Elith *et al.,* 2011; Merow *et al.,* 2013). Additionally, the programme estimates the ratio between the two probability densities, and as a result, the relative environmental suitability per grid cell for the species (Phillips *et al.,* 2006; Phillips & Dudík, 2008). As a measure of model accuracy, MaxEnt selects out a proportion of the occurance localities to test the model once complete, termed ‘testing’ data compared to ‘training’ data used to run the model.

Apart from mechanistic and correlative models, climate-matching algorithms can be used as a first approximation to assess the climatic similarity between the region that the species currently inhabits to an area of interest, that is, where it has not yet invaded. This method is commonly used in pest risk analyses to estimate the potential geographic distribution of weeds (Panetta & Mitchell, 1991; Csurhes & Kriticos, 1994) as well as a number of other organisms (Boag *et al.,* 1995; Peacock & Worner, 2006) in biological control programmes, both to narrow searches for climatically suitable agents and to define climatically suitable locations for releasing agents (Senaratne *et al.,* 2006; Robertson *et al.,* 2008; Fisher *et al.,* 2011). This method makes it possible to quickly and cost-effectively determine a species distribution by simply matching the climatic conditions of where the species has been recorded to the area of interest. These algorithms differ from correlative models as they generate areas of climatic similarity between different locations without reference to particular species. Therefore, the outcome is not influenced by biotic drivers such as plant competition or enemy release (Kriticos *et al.,* 2014), nor by the subjective development of species absence points such as used by MaxEnt (Kriticos *et al.,* 2014).

Models are only as good as the data that are used to calibrate them (Fielding & Bell, 1997; Hirzel *et al.,* 2001; Wolmarans *et al.,* 2010). It then follows that the more studied the species, the more data are available to calibrate the models, the better the models. To date there have been no correlative models conducted to determine the potential distribution of *G. triacanthos*. However, there is ample literature available for *G. triacanthos* and its known global distribution. The literature provides significant data on the climates in which *G. triacanthos* may survive as well as some of its physiological limits (Blair, 1990; Thompson *et al.,* 1999; Wilson *et al.,* 2011; Fernández *et al.,* 2017) which should facilitate the building of accurate SDM for the species.

The aim in this chapter is to use SDMs to predict the potential distribution of *G. triacanthos* in South Africa. To achieve this aim, three separate models were created in MaxEnt to determine the potential distribution of *G. triacanthos* using: 1) The invasive range in the southern hemisphere, including Australia, New Zealand, South America and South Africa, with backgrounds clipped to the Koppen-Geiger classification zones (named: Southern Hemisphere) (refinement to Koppen-Geiger classification zones are described in 2.2.4 Geographically defined background) (named: Southern Hemisphere SDM); 2) the native range in USA, and the invasive range in the southern hemisphere with backgrounds clipped to the Koppen-Geiger classification zones (named: Native range + Southern Hemisphere) and; 3) the native range in the USA, and invasive range in North America, Europe and the southern hemisphere Australia, New Zealand, South America and South Africa with backgrounds clipped to the Koppen-Geiger classification zones (Named: Global). In order to get an additional perspective, a CLIMEX 'match climates regional' analysis was conducted to determine if it supported the MaxEnt outputs and to ensure the models were not over-predicting the potential distribution of *G. triacanthos* in South Africa.

2.2 Methods and Material

MaxEnt

MaxEnt is software based on presence data only (geo-referenced species locations, where the species were found) and a set of environmental variables data (precipitation, temperature, climate) (Phillips *et al.,* 2006; Merow *et al.,* 2013). Once the model is completed, the software uses a set of occurrence locations of the species in question to test the models. To avoid overfitting in the models, the background or pseudo-absence data (a sample of points from the landscape) must be regularised, that is, the background must be data with the same bias as the occurrence data (Phillips *et al.,* 2009). As the models relies on the species occurrence records, their accuracy determines the accuracy of the model.

2.2.1 Species occurrence locations

The global record of *G. triacanthos* localities was obtained from the Global Biodiversity Information Facility (GBIF) and therefore included both the native range and invaded range distributions of *G. triacanthos.* Invaded range distributions were supplemented with data from the Southern African Plant Invaders Atlas (SAPIA) database and the Living Australia database. The SAPIA database is one of the most comprehensive databases on invasive alien plants in Africa. The majority of these data points are recorded from extensive roadside surveys mainly by Lesley Henderson during road surveys over the last 20 years.

Although the data are from reliable sources, the data were ‘cleaned’ by 1) removing duplicates, 2) correcting co-ordinate errors where possible, 3) excluding coordinates that lacked sufficient fine-scale precision, 4) removing coordinates recorded before 1980 with no new records around the areas where they were recorded, and 5) omitting the records of localities that were ‘artificial’, such as botanical gardens, golf courses, university properties and gardens. In addition, maps of the refined occurrence locations for native and invaded range data of *G. triacanthos* were produced in ArcMap 10.8.1. This was done so that individual occurrence data could be further inspected on an individual basis to make sure there were no errors with the locations, such as points occurring in water bodies.

Spatial filtering of occurrence data has also been found to reduce bias and improve the quality of the model (Boria *et al.,* 2014). Therefore, spatial autocorrelations were conducted within MaxEnt so that location points were refined to one location point to each grid pixel. This limits occurrence points in high density regions to 1 occurrence point per 1 km^2. The occurrence data were refined to the backgrounds used in each of the model runs.

2.2.2 Environmental parameters

Bioclimatic variables or environmental variables are the result of annual trends like annual mean temperature, annual precipitation, isothermality, among others. These bioclimatic variables have been used extensively to study distribution modelling in niche-based plants. All this information is free and easily obtainable from the WorldClim dataset (www.worldclim.org) which provides high-resolution climate averages for the period 1970–2000, and 19 bioclimatic variables (Hijmans *et al.,* 2005; Fick & Hijmans, 2017). In this study, the standard set of 19 bioclimatic variables based on the historical climate data set was obtained from the WorldClim database (data source: https://www.worldclim.

org/data/worldclim21.html) (Hijmans *et al.,* 2005, 2016; Fick & Hijmans, 2017). These grids have a spatial resolution of 30 arc-seconds, which is equivalent to 0.86 km^2 at the equator and is commonly referred as '1-km' spatial resolution (Fick & Hijmans, 2017). In order to make the environmental layer suitable for MaxEnt, the layers were converted from raster file format into an ASCII file in ArcMap 10.8.1 (ESRI, 2020). Suitable environmental layers selected were based on their biological relevance to plant species distributions and other modelling studies of plant species in South Africa (Trethowan *et al.,* 2011, Martin *et al.,* 2020). Following this, the variables were tested for multicollinearity by examining cross-correlations (Pearson correlation coefficient). They were clipped to the suitable geographically defined background (described below) using the 'Extract by Mask' function in ArcMap 10.8.1 (ESRI, 2020) for each of the model runs. Each of the models was processed in the same way. The environmental layers used in each model run are presented in the results.

2.2.3 Geographically defined background

The modelling programme, MaxEnt, produces pseudo-absence data from a defined background, from pixels (grid cells) where the presence of records is absent. It has been reported that the size of the 'background' from which the pseudo-absence data are acquired can influence the results of the models (Phillips & Dudík, 2008; Kramer-Schadt *et al.,* 2013); accordingly, it is important that the background should be restricted or should represent places where the species could potentially occur. Therefore, the backgrounds used in the model runs were carefully considered. For the USA, the background was clipped to match the spatial extent of the native range (Thompson *et al.,* 1999; Sullivan, 1994). For regions in the southern hemisphere areas were used that were climatically representative, that is the Koppen-Geiger climate classifications (Koppen-Geiger classifications, as recommended by Webber *et al.,* (2011) and Hill and Terblanche (2014). To do this, climate data were downloaded from http://koeppen-geiger.vu-wien.ac.at and were placed into ARCMAP 10.8.1. The localities of *G. triacanthos* occurrence were layered over the data layer to determine what climate zones contained at least one record; these climate zones were used as background data in the MaxEnt model.

2.2.4 Modelling parameters in MaxEnt

The software MaxEnt (version 3.4.1) (Phillips *et al.,* 2006) was used to model the climatically suitable areas for *G. triacanthos*. 'Auto features' was used in this study, which creates a continuous, linear means by which the software automatically calculates the complexity of the model based on the quantity of the occurrence locations. In addition, parameters of 500 iterations and 0.00001 convergence thresholds were used when running the programme's 'logistic output', which creates a continuous, linear-scaled map that allows fine distinctions to be made between the modelled probabilities of habitat suitability. Also used in the models were 'create response curves' and 'jackknife measures of variable importance'. 'Clamping', which resets values that are outside the range found in the study area, was not applied to match the upper or lower values found in the study area, in order to avoid overfitting. There can be considerable variation in the performance of models when choosing a particular random selection of points for the training and testing sets, so a bootstrap selection was made, which is good for relatively large datasets as it is best to make several random selections (splits). This process was done six times for each model so that the models could be compared for constancy. The split of 70% training and 30% testing was done randomly and is suggested as one of the best splits for modelling invasive species (Trethowan *et al.,* 2011). The final model produced was the mean of the six models created. The mean minimum training presents the logistic value that was used from the six maps produced.

2.2.5 Model evaluation

In MaxEnt (version 3.4.1), different methods can be used to evaluate the accuracy of the model (Phillips *et al.,* 2006). The models were evaluated with jackknife analysis, cross-validation and response curves, with the Area Under Curve (AUC) statistic for each replicate per each treatment. The AUC gives a realistic prediction of species occupancy potential, and is more important than the accuracy of the model (Lobo *et al.,* 2008). The AUC values generated from the model can fit into the following ranges: 0.5 and 1.0, where 0.5 is no better than a random generated model. Generally, it is accepted that an AUC of <0.8 is a poor model, 0.8–0.9 is a fair model, 0.9–0.995 a good model, and >0.995 an excellent model (Fielding & Bell, 1997; Trethowan *et al.,* 2011). The mean and standard deviation of the six replicate AUC values were provided for each model and were used to determine the quality of the models. In this study, to estimate the model overfitting, the 10 percentile training presence was applied, where the binary threshold excludes values of 10% of the calibration localities from the model with low values of prediction, and where it has an expected omission rate of 0.10 (Boria *et al.,* 2014). Additionally, the mean of the six values of test omission rate (10 percentile training presence) indicates whether the model has an overfit or not, where <0.1 means that the model is more conservative, and >0.1 indicates that the model has an overfit (Boria *et al.,* 2017).

The ASCII files generated by MaxEnt were worked in ArcMap version 10.4 and converted into maps. The ASCII raster layer was changed into classified values, where the minimum training presence logistic threshold was set as the upper limit of one of two categories, so producing the potential distribution map (effectively a presence-absence map). This potential distribution is usually larger that the area suggested as suitable but avoids ignoring any known risky areas (Kriticos, pers comm.).

2.2.6 CLIMEX

In addition to the MaxEnt modelling, a CLIMEX-MCR of CLIMEX Version 4 or ‘Regional Match’ was used to compare climates where *G. triacanthos* has already been recorded with those areas elsewhere in the world, using standard variables of maximum monthly temperature, minimum monthly temperature and monthly rainfall, each with the default weight of one (Sutherst *et al.,* 2007; Kriticos *et al.,* 2015). This weighting for each variable was suggested as a suitable run for *G. triacanthos* in this situation (Kriticos per comm., 2021; Sutherst *et al.,* 2007; Kriticos *et al.,* 2015). These monthly temperature and rainfall records are determined from additional information (Kriticos *et al.,* 2015). The CliMond historical data set (CM10_1975H_V1) was used for these runs (Hearne Software, Melbourne, Australia).

From these standard variables listed above CLIMEX-MCR calculates a 'composite match index' (CMI), which can range from zero (no match) to one (perfect match). Choice of a biologically meaningful level of similarity between the areas of known distributions and unknown distributions is often arbitrary (Senaratne *et al.,* 2006), though it is generally considered that CMIs > 0.7 indicate that a species present in an unknown distribution would also find the climate in one or more parts of the predicted area suitable for establishment (Robertson *et al.,* 2008; Kriticos, 2012). The indicator CMIs > 0.8 were considered suitably significant in this case. Further details of how CMIs are calculated are given in Sutherst *et al.,* (2007), Kriticos *et al.,* (2015) and the supplementary information of Kriticos (2012). The composite index match was calculated for the entire world, even though we were focussing primarily on South Africa. The data were then exported into ARCMAP 10.8.1 and converted into a raster file for easier visualisation.

2.3 Results

In South Africa, *Gleditsia triacanthos* has primarily invaded the Grassland Biome (Henderson, 2001). At a provincial level, the Free State Province has the greatest number of occurrence localities followed by Gauteng, Mpumalanga, KwaZulu-Natal, with occasional records in the North West, Eastern Cape, Northern Cape, Limpopo and Western Cape provinces. All SDMs predicted broad potential distributions in South Africa suggesting the species would continue to spread.

2.3.1 Southern Hemisphere SDM

The Southern Hemisphere model was calibrated for 452 occurrence localities (Fig. 2.1 A) and a background of climatically suitable regions in the southern hemisphere. The environmental parameters used were annual mean temperature, temperature seasonality, maximum temperature of warmest month, mean temperature of driest quarter, mean temperature of coldest quarter, precipitation of driest month, and precipitation of warmest quarter. The model determined that the current distribution of *G. triacanthos* matched the modelled areas of high suitability (Fig. 2.1 B, C), indicating that the Free State, Gauteng and Mpumalanga provinces are highly suitable, and that some areas of the Northern Cape, Western Cape, Limpopo, Kwazulu-Natal and the Eastern Cape provinces are also suitable for *G. triacanthos* (Fig. 2.1 D). The model also indicated that some of the northern regions of South Africa are not suitable but suggested that there is a high probability the species could move down in to the Eastern and Western Cape provinces from its current distributions. The minimum training presence predicted a larger area of South Africa than the area where the tree is currently recorded: an area of 888 280 km^2 (Fig 2.4 C). The minimum training presence potential distribution is usually larger that the area suggested as suitable but avoids ignoring any known risky areas (Kriticos, pers comm). The AUC for the models were 0.904 SE ± 0.009, suggesting is a good model. Omission rate was 0.0995 ± SE 0.0002, indicating a slightly overfitted model (Fig. 2.4 D).

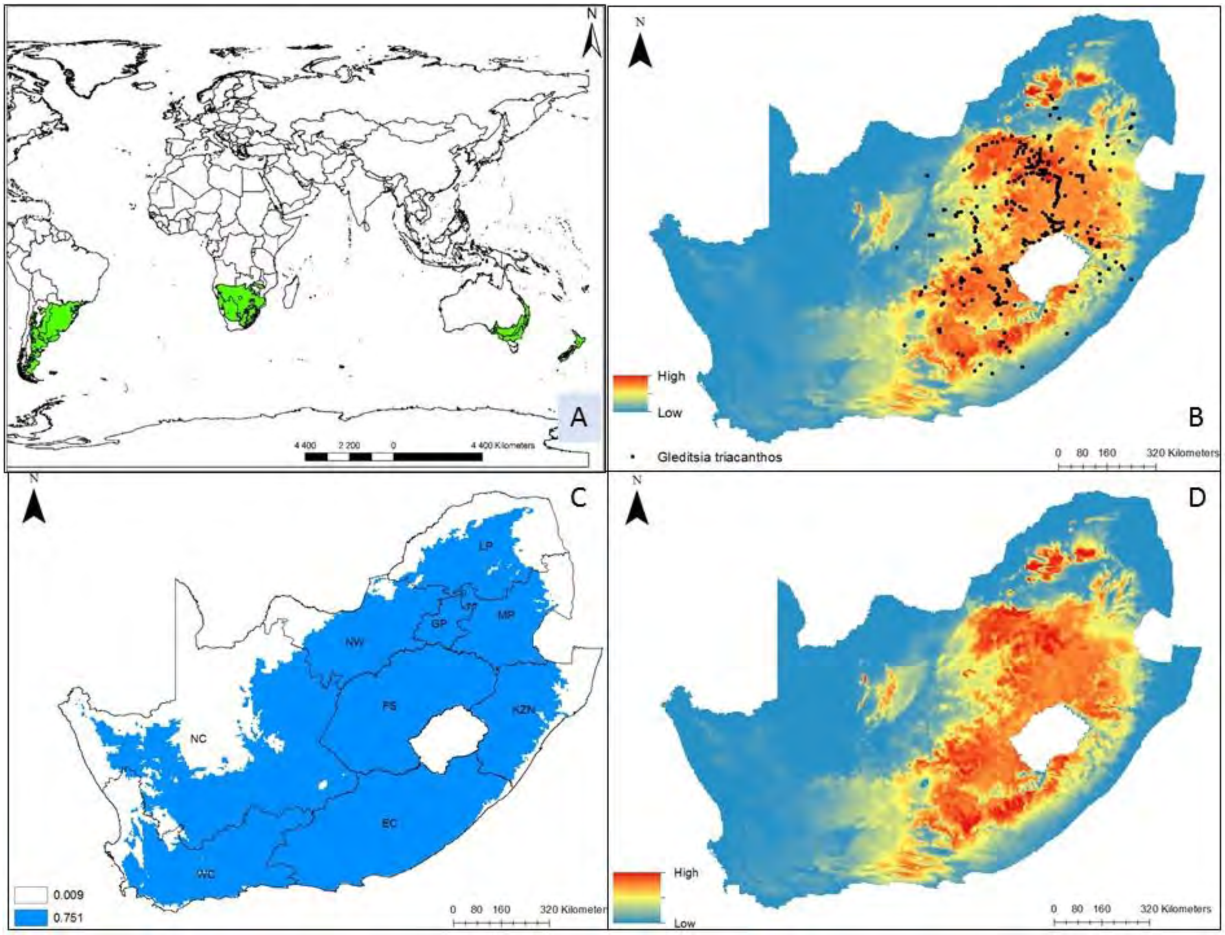

Figure 2.1: The potential distribution of *Gleditsia triacanthos* in South Africa using the invaded range in the southern hemisphere distribution points to calibrate the model with backgrounds clipped to the Koppen-Geiger classification: (A) distribution sites from southern hemisphere (Australia, South America, South Africa and New Zealand) clipped to the Koppen-Geiger classification; (B) predicted distribution with known localities overlaid; (C) presence-absence distribution map of South Africa; (D) predicted distribution in South Africa.

2.3.2 Native Range + Southern Hemisphere SDM

The Native Range + Southern Hemisphere SDM was calibrated with 1300 occurrence localities (Fig. 2.2.A; 2.4 A). The backgrounds were refined to the native range in USA (Thompson *et al.,* 1999; Sullivan, 1994) and the invasive range in the southern hemisphere with backgrounds clipped to the Koppen-Geiger classification zones. The environmental parameters used were annual mean temperature, temperature seasonality, maximum temperature of warmest month, mean temperature of driest quarter, mean temperature of coldest quarter, precipitation of driest month, and the precipitation of warmest quarter. Once again, the model suggested that there is a high probability the species could move down in to the Eastern and Western Cape provinces from its current distributions. In addition, the SDM predicted a narrow region on the east coast of South Africa, primarily in KZN, as well as regions in the furthest northern parts of South Africa as unsuitable for the species (Figs. 2.2 B, C & D). The model was calibrated using the minimum training presence 0.009 SE ± 0.001, where 1 028 185 km^2 are suitable for *G. triacanthos* in South Africa (Fig. 2.4 B). The average training AUC for the replicate runs was 0.848 ± SE 0.003 (Fig. 2.4 C), suggesting a fair model. Omission rate was 0.0995 ± SE 0.0003, indicating a slightly overfitted model (Fig. 2.4D).

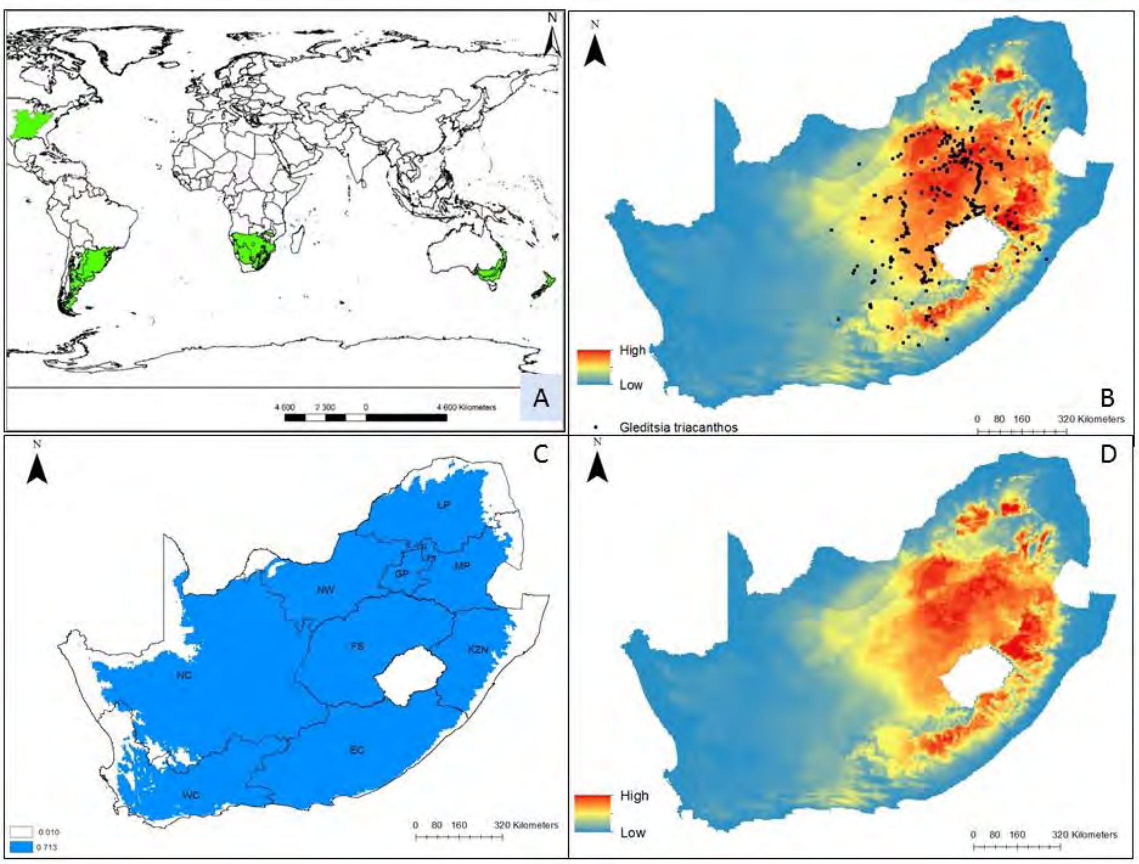

Figure 2.2: The potential distribution of *Gleditsia triacanthos* in South Africa using the native and invasive range in the southern hemisphere distribution points to calibrate the model with backgrounds clipped to the Koppen-Geiger classification: (A) distribution sites from the native range and invasive range southern hemisphere (Australia, South America, South Africa and New Zealand), clipped Koppen-Geiger climate regions for South Africa suitable for the plant; (B) predicted distribution with known localities overlaid; (C) presence-absence distribution map of South Africa; (D) predicted distribution in South Africa.

2.3.3 Global SDM

The occurrence localities used to calibrate this model were taken from the native and invaded range: 2253 occurrence localities of *G. triacanthos* (Fig. 2.3 A; 2.4 A). The background was limited to the native range in the USA, and the invasive range in North America, Europe and the southern hemisphere: Australia, New Zealand, South America and South Africa. With backgrounds clipped to the Koppen-Geiger classification zones, the environmental parameters used were: annual mean temperature, mean temperature of warmest quarter, mean temperature of coldest quarter, annual precipitation, precipitation of driest month and precipitation of driest quarter. The suitable areas for *G. triacanthos* were greater in this model than in the previous models, suggesting areas in the Western Cape and Eastern Cape provinces to be highly suitable; these areas were not predicted by the other SDM. The model had a minimum training presence of 0.002 SE ± 0.00016 (Figs. 2.3 C, D), where 122 million km^2 are suitable for *G. triacanthos* in South Africa (Fig. 2.4 B). However, the model had an AUC 0.764 ±SE 0.008 suggesting a poor model (Fig. 2.3 D; 2.4 C), and the omission rate was 0.1 ± SE 6E-18, indicating an overfitted model (Fig. 2.4 D).

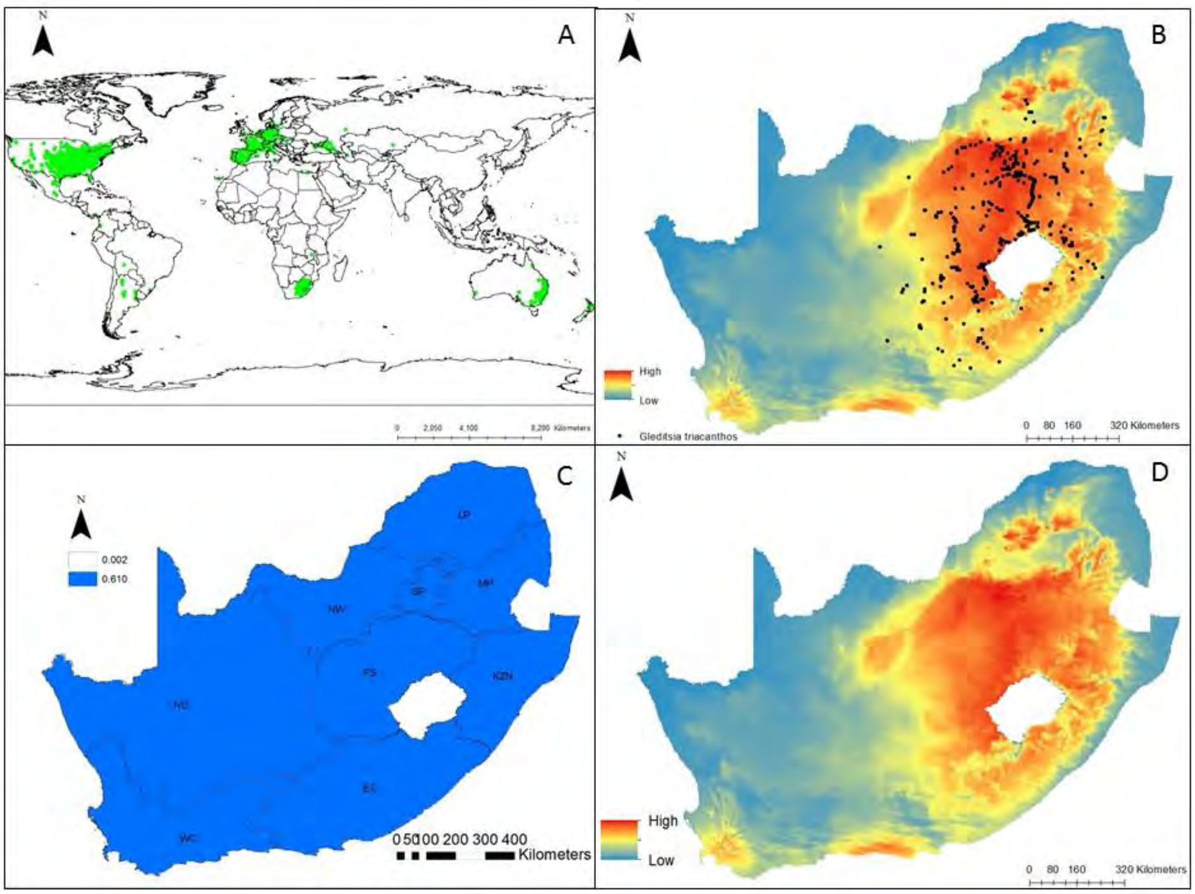

Figure 2.3: The potential distribution of *Gleditsia triacanthos* in South Africa, using the native range in the USA, and invasive range in North America, Europe and the southern hemisphere backgrounds clipped to the Koppen-Geiger classification; (A) distribution sites from the native range in the USA, and invasive range in North America, Europe and the southern hemisphere Australia, New Zealand, South America and South Africa with backgrounds clipped to the Koppen-Geiger classification; (B) predicted distribution in South Africa with known localities overlaid; (C) presence-absence distribution map of South Africa; (D) predicted distribution in South Africa.

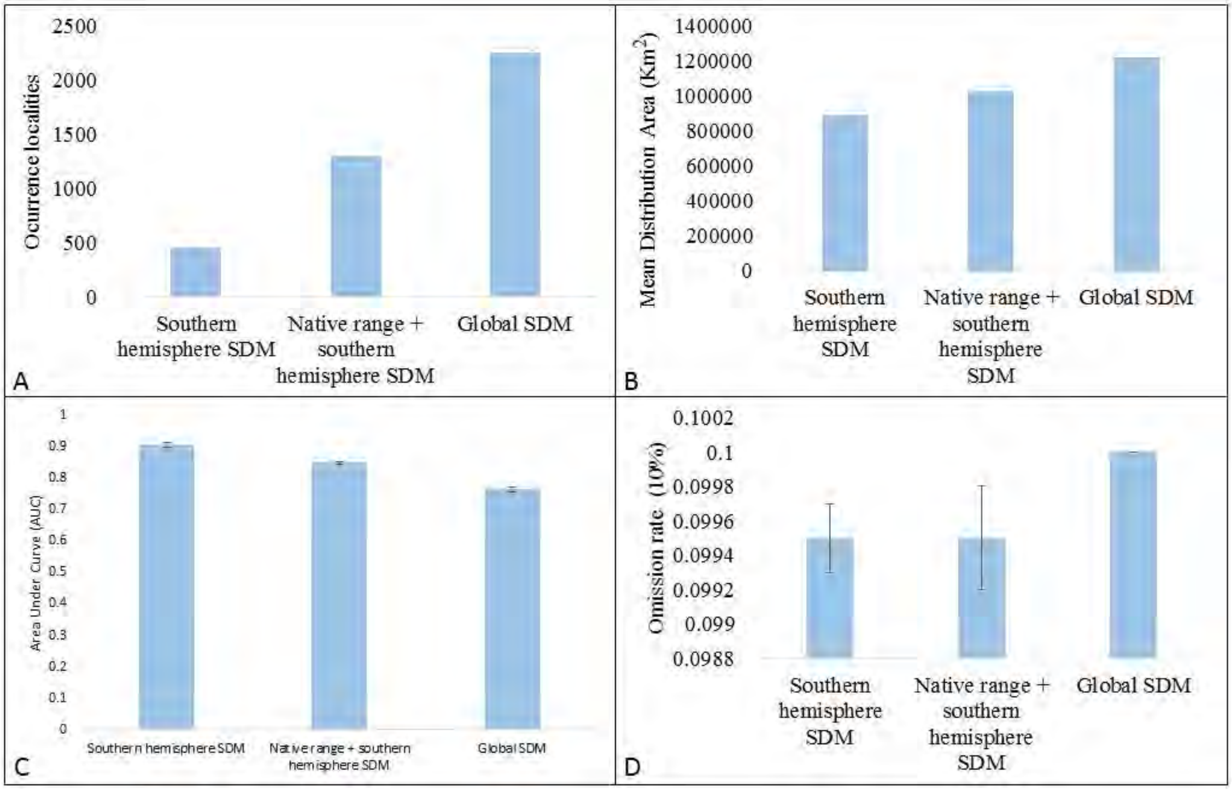

Figure 2.4: (A) number of occurrence localities used to calibrate SDMs; (B) Mean distribution Area from SDM; (C) Area Under Curve (AUC) for SDM (D) 10th Percentile Omission rate of SDM for *Gleditsia triacanthos* in South Africa. Error bars represent standard error.

2.3.4 CLIMEX-MCR SDM

CLIMEX is software most frequently used to determine potential distribution of invasive species. The CLIMEX region match suggested *G. triacanthos* could continue its spread into new countries where it is not yet recorded as an invasive (Fig. 2.5 B). The areas in South Africa predicted as suitable for *G. triacanthos* are similar to the models predicted in MaxEnt suggesting a large portion of the country is suitable for the species (Fig. 2.5 C).

However, not all the suitable areas will became completely invaded; the provinces where *G. triacanthos* would be more problematic are: Free State, North West Province, Gauteng, Mpumalanga, and a portion of KwaZulu-Natal and Eastern Cape provinces (Fig. 2.5 D). Setting the CMI to 0.8 suggested a close climate match location where the species has already been recorded growing suggesting an accurate model.

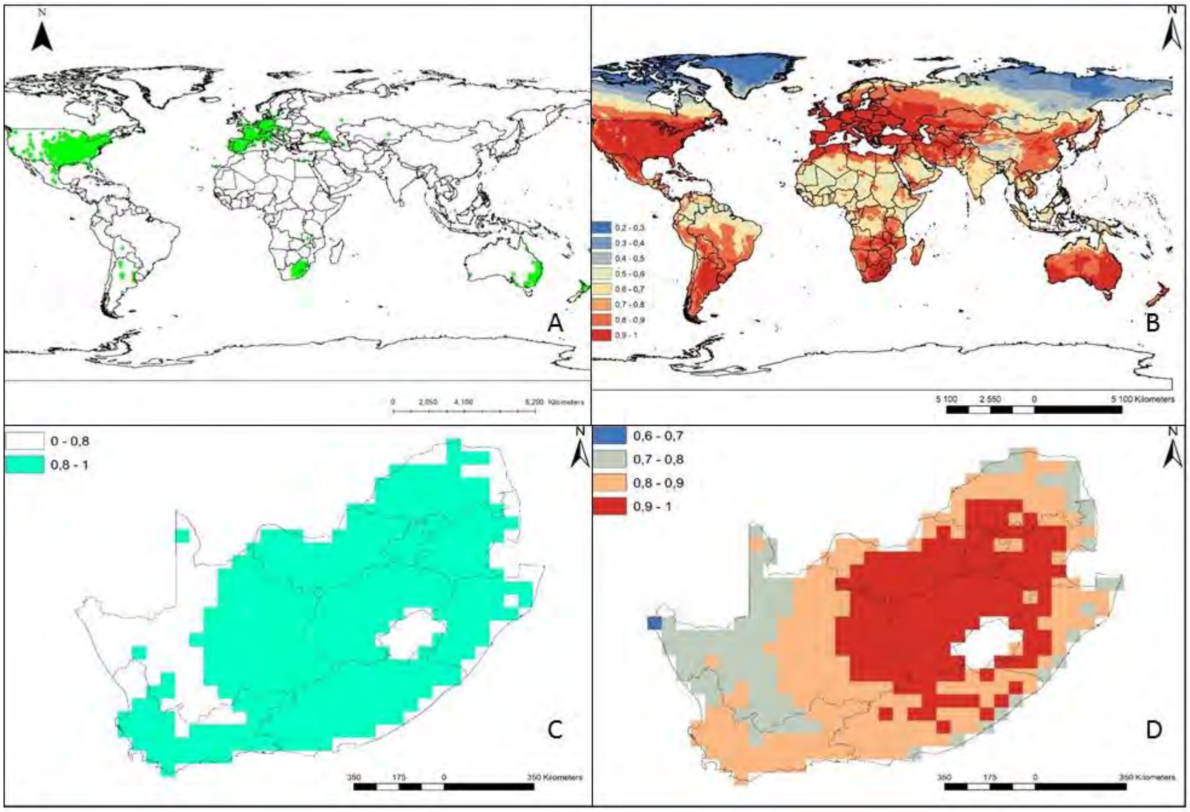

Figure 2.5: Potential distribution of *Gleditsia triacanthos* using CLIMEX-MCR. (A) Global distribution of *Gleditsia triacanthos* used to calibrate model; (B) Global climate suitability map for *Gleditsia triacanthos*; (C) Presence-absence distribution map of *Gleditsia triacanthos* in South Africa; (D) Predicted distribution of *Gleditsia triacanthos* in South Africa.

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2.4 Discussion

Species distribution models are very important in invasive species management, because they can predict areas where the species in question could potentially become problematic and the models are therefore important for conservation efforts (Newbold *et al.,* 2010) in that SDMs allow users to visually identify areas that may require management or pest risk assessments, enabling prioritisation of resources. In this study, we used two commonly used but fundamentally different modelling programmes, MaxEnt and CLIMEX-MCR, to predict areas where *Gleditsia triacanthos* could find favourable growing conditions, with a particular focus on South Africa. We suggest that *G. triacanthos* has not reached its full distribution in South Africa, effectively implying that, if left unmanaged, the species will continue to spread.

Correlative models such as MaxEnt produce climate suitability maps from available distribution data using a set of model parameters determined by the researcher. Accuracy and relevance of these data can be biased and leave room for interpretation (Trethowan *et al.,* 2011). This possible bias suggests all models must be considered with some level of circumspection. Understanding the limitations and strengths of modelling programmes can help in interpreting the final outputs (Merow *et al.,* 2013). One of the core components and common regions of error of these models is in the use of occurrence records, as error can be introduced at any stage. Some basic types of errors include species misidentification, errors within catalogued herbarium records, taxonomic issues, and data capturing errors, all of which result in inaccurate distributions and can significantly influence model accuracy. In the case of *G. triacanthos,* thousands of distribution points are available from a number of countries and sources from around the world. Therefore, as much effort as possible was put into cleaning the distribution points used. In addition to occurrence record cleaning, the occurrence records which were selected were refined to areas where greater confidence could be place in the accuracy of these occurrence points. These were the points from South

America refined by Fernández *et al.* (2017); The SAPIA points were confirmed during the surveys described in Chapter 3; the occurrence points in Australia suggested by Csurhes and Kriticos (1994) were confirmed by *Atlas of Living Australia*; and areas from the plant's native range described by the US Forest Service and Thompson *et al.* (1999). The models produced with these localities were regarded as good models. However, in the case of the Global SDM, when the distribution points from the invaded ranges of the USA, Europe (GBIF, 2021) and Asia (which were much harder to confirm accurately) were added to the models, a much poorer model was predicted, thereby highlighting the importance of ensuring high quality occurrence data and showing that more occurrence data points is sometime less important than accurate occurrence points (Graham *et al.,* 2008).

A number of authors who have used correlative modelling techniques have repeatedly called for caution when extrapolating to novel climates (e.g., Elith *et al.,* 2011; Kriticos *et al.,* 2014), as we have done here. They have suggested these models might be poorly suited to addressing questions concerning novel climates, such as are encountered in biosecurity; climate change application correlative models, including MaxEnt (Kriticos *et al.,* 2014). One of the problems arises from the selection of pseudo-absence points in which MaxEnt requires the modeller to specify pseudo-absence data in the form of a defined background layer that spans the known positives, and to a greater or lesser degree, regions in which there are no presence records (Barbet-Massin *et al.,* 2012). The background of every population was refined, and different backgrounds were tested to see if they dramatically affected the outputs. Calibrating the models using data from both the native range of the species and the invaded range has been shown to provide a better indication of a species' potential to spread (Trethowan *et al.,* 2011). This calibration did not seem to influence the model too significantly in this case; adding the species' native range distribution did not significantly change the predicted potential distribution in South Africa. A second problem with these correlative models is that they rely on biological data, such as the impact of natural enemies and competition from other species (Kriticos *et al.,* 2014). In order to determine if these issues influenced the model, we attempted to circumnavigate them by using true presence-only correlative model- CLIMEX-

MCR. CLIMEX-MCR relies solely upon geographical data to infer climate suitability (Kriticos, 2012). The CLIMEX-MCR produced an area of South Africa very similar to the MaxEnt models, suggesting that our backgrounds in the MaxEnt modelling were appropriate and therefore our models could be used with a certain level of confidence.

The final possibility for confirming the potential distribution of *G. triacanthos* in South Africa would be to develop more mechanistic models using packages such as CLIMEX 'match climates regional' (Sutherst *et al.,* 2007) or NicheMapper (Kearney & Porter 2009). These models have been designed specifically to produce ecologically plausible results when projected onto novel climates (Sutherst & Maywald 1985; Magarey *et al.,* 2007; Sutherst *et al.,* 2007; Kearney & Porter 2009; Sutherst & Bourne 2009; Webber *et al.,* 2011; Sutherst *et al.,* 1999). However, they require the modeller to have extensive knowledge of the environmental parameters which drive the distribution of a species and rely on having physiological data available as well. The wealth of data available for *G. triacanthos* suggests this kind of modelling could be considered for the species, but it was not in the scope of this study.

Not many attempts have been made to predict the distribution of *G. triacanthos,* making comparisons with our study impossible. One example was by Csurhes and Kriticos (1994) who used the CLIMEX-MCR to predict the potential distribution of *G. triacanthos* in Australia. The difference between the study by Csurhes and Kriticos (1994) and this study is that output from the model was interpreted in terms of known distributions of the species from the United States of America only. Interestingly, using a threshold of 0.7 (less than the 0.8 used in this study) and prime locations in the United States produced very few climate matches in Australia, despite the tree being widely established there. Csurhes and Kriticos (1994) were forced to change the climate match index to 0.5 (normally recorded regarded as a poor match) to find a suitable threshold which showed the potential plant distribution in Australia. Their prediction suggests that *G. triacanthos* has the potential to colonise large areas of south-eastern Australia. This result was very similar to that predicted by the MaxEnt models used in this study when all the southern hemisphere data were used to make predictions. However, in this study when far more occurrence data points were used to create a 'HOME' location, a larger area of Australia was predicted as being suitable, even under a CMI of 0.8, than was predicted by Csurhes and Kriticos, (1994). The CLIMEX model suggested large areas were suitable for *G. triacanthos* in Australia. It is noteworthy that since the modelling conducted by Csurhes and Kriticos, (1994), new distributions of *G. triacanthos* have been recorded outside the regions originally predicted (Atlas of Living Australia, 2021; GBIG, 2021) suggesting they should have attempted to use more occurrence records potentially from the Southern hemisphere than just those from the USA.

This extremely broad prediction of the SDMs in South Africa is not particularly surprising. *Gleditsia triacanthos* can tolerate a wide range of types of soils, and can tolerate salinity, drought and frost, and variations in soil temperature (Blair, 1990; Graves *et al.,* 1991; Kebbas *et al.,* 2018; CABI, 2021). In the plant's native range, the species survives under quite diverse climates. In the western portion of its range, *G. triacanthos* grows in a sub-humid climate, while in the middle and eastern portions, the climate is humid. Normal annual precipitation varies from about 510 mm (20 in) in South Dakota and Texas to more than 1520 mm (60 in) in southern Louisiana. The plant tolerates extensive snow up to 102 cm and extremely low temperatures of down to -34° C. In addition to surviving in drought and dry conditions, it also grows in very moist conditions in the Appalachians where annual precipitation varies from 890 mm in the valleys to as much as 2,040 mm on the highest peaks (Blair, 1990).

Studies in Argentina show that *G. triacanthos* has established and is flourishing in four different ecoregions: the dry Chaco, the subtropical mountain forests, the Pampean grasslands and the Espinal (Marco & Páez, 2000; Ghersa *et al.,* 2002; Bortoluzzi *et al.,* 2008; Hoyos *et al.,* 2010; Ferreras *et al.,* 2014; Fernández *et al.,* 2016). Therefore *G. triacanthos* effectively surviving in mean temperatures ranging between 13.8–19 °C, and mean annual precipitation of 650–1390 mm (Fernández *et al.,* 2017). It has been suggested that since seeds of *G. triacanthos* need water to germinate, rainfall may be a limiting factor for the germination and subsequent development of the species (Chaneton *et al.,* 2004; Mazía *et al.,* 2010; Muschetto, 2012). This could potentially limit the spread of the species in the drier regions of the Karoo in North West Province and Western Cape Province of South Africa. However, the tree is already present in the north-west of South Africa because the plant has been so widely planted and it can spread from suckers. The growth of seedlings is so rapid it requires only one season to establish (Fernández *et al.,* 2017), following which, the tree can survive extremely dry conditions. A study in Algeria by Kabbas *et al.,* (2018) showed that *G. triacanthos* seedlings can survive drought as the plants respond with an osmatic adjustment that will improve their resistance to osmotic stress caused by drought (Kabbas *et al.,* 2018). These adaptations make *G. triacanthos* a great invader, and demonstrate why its distribution is likely to spread in South Africa into like the Karoo, the warm tropical weather of KwaZulu-Natal Province, and the cold, high-altitude regions of the Free State and Eastern Cape provinces (SAPIA, 2018).

Gleditsia triacanthos is currently regarded as a Category 1b invasive under the NEMA regulations. By law, an invasive species in Category 1b requires a management plan for its control and should be actively managed. Now that the potential for the species to spread in South Africa is evident, management should be prioritised. Unfortunately, there are very few examples of mechanical or chemical control methods available for *G. triacanthos* in South Africa, but South Africa's experience of invasive tree management (see Chapter 1) suggests management should be possible. Furthermore, as mentioned in Chapter 1, biological control is often an important component, ensuring invasive tree management success. In this case, biological control may be an option for reducing growth of the populations of *G. triacanthos*. In Argentina, Di-Iorio, (2005) documented a case of unplanned biological control of *G. triacanthos*, whose seeds are consumed by the exotic seed beetle species, *B. endotubercularis*. Each larva affects one seed by consuming its cotyledons and embryo, causing the death of the embryo and thus becoming a spontaneous biological control of the species (Di-Iorio, 2005). A similar situation exists in South Africa with the seed beetle *Megabruchidius tonkineus,* but its impact and distribution are not yet well understood.

Chapter Three: Biology of *Megabruchidius tonkineus*, host specificity and impact

3.1 Introduction

South Africa has been working to control invasive plants species for more than a hundred years; work that has been well documented and studied (Moran *et al.,* 2013; Richardson *et al.,* 2020). The greatest success with regard to the management of invasive species, particularly tree species, has been attributed to biological control (Chapter 1; Moran *et al.,* 2013; van Wilgen *et al.,* 2020). Many of the best examples of tree biological control in South Africa have been on species within the Fabaceae; for example, the biological control programme against *S. punicea* which is regarded one of the most successful biological control programmes in South Africa, possibly even globally (Hoffmann & Moran, 1998; van Wilgen *et al.,* 2004; Chapter 1). In addition, South Africa pioneered the biocontrol of invasive Fabaceae species which were previously avoided due to conflicts of interest. For example, the successful use of two species of gall-forming pteromalid wasps, *Trichilogaster acaciaelongifoliae* and *Trichilogaster signiventris,* against long-leaved wattle, *Acacia longifolia*, and golden wattle, *Acacia pycnantha*, (both Fabaceae). These trees have been commercially exploited, but they were also problematic because of their invasive characteristics (Dennill & Donnelly, 1991; Zimmermann *et al.,* 2004). The seed-attacking wasps were able to decrease the population of the trees without harming the parts of the tree that are considered useful. (Dennill & Donnelly, 1991; Zimmermann *et al.,* 2004). Both trees are now regarded as being under substantial biological control (Moran *et al.,* 2021).

In a recent review of biological control programmes in South Africa, Moran *et al.* (2020) used the method developed by Hoffmann *et al.* (2019) to rank biocontrol success against weeds at a population level in South Africa. They considered only those species on which biocontrol agents had been established for more than 10 years. Of the 54 invasive alien plants considered by Moran *et al.,* (2021), 15 (28%) were from the Fabaceae family (Moran et al., 2013, 2021), and of these 15 species, seven were regarded as being under substantial control, while *S. punicea* was considered as being under complete control. These examples provide the incentive that suitable biological control options for *G. triacanthos* could be implemented and may prove successful.

In 2016 a SAPIA (South African Plant Invader Atlas) newsletter highlighted that *G. triacanthos* had doubled its distribution area between 2001 and 2016 and was becoming a major invader species requiring urgent intervention (Klein, 2011; Henderson, 2016). As the seeds of *G. triacanthos* are spread by stream flow or distributed by birds and mammals, the seeds were an obvious target for biological control (Robinson *et al.,* 1992). However, before an official biological control programme was implemented, the seed-feeding bruchid, *Megabruchidius tonkineus* (Pic, 1914) (Coleoptera: Chrysomelidae: Bruchinae) was recorded in the plant's seed pods in South Africa. Even though the insect was not released and was therefore an unintentional introduction, it was still considered a biological control agent (Klein, 2011). This decision was unusual as very little is known about the insect's host range or its impact upon the plant in South Africa (Klein, 2011). It was therefore suggested, at the 45th Annual Workshop on Biological Control of Weeds, Research and Implementation held in Durban that, until the insect is shown to be suitably host specific, it is not considered as a biological control agent.

Usually, a biological control agent is only released in a new country once it has been deemed suitably specific and potentially damaging toward its target. In order to achieve this recognition, a candidate biological control agent undergoes rigorous host-specificity testing. During this testing many potential agents are rejected because they feed on non-target plant species (Klein, 2011). When the agent is deemed safe to release, permission is needed in terms of the legislation that regulates the introduction of exotic biological control agents into the country. In the case of *M. tonkineus,* a number of these steps cannot be followed as the insect is already established in South Africa. However, should the agent be deemed suitably host specific in South Africa, the agent could be considered a biological control agent for *G. triacanthos* with the ability to mass produce and release *M. tonkineus*. The aim of this study, then, was to conduct suitable trials to determine if *M. tonkineus* could be considered a biological control agent in South Africa. This chapter aimed to determine: the biology of *Megabruchidius tonkineus*; the realised and fundamental host range of *M. tonkineus* in South Africa; the distribution of *M. tonkineus* in South Africa; and finally, the impact of *M. tonkineus* on *G. triacanthos* seeds in South Africa.

3.1.1 Study species - Megabruchidius tonkineus

In general, bruchis are oliophagous, meaning they feed on the seeds of only a single or a few closely related species (Pintilioaie *et al.,* 2018). The genus *Megabruchidius* (subfamily Bruchinae: tribe Bruchidini) was established by Borowiec in 1984 and has mating behaviour similar to Bruchinae (Takakura, 1999; Shimada *et al.,* 2001). Species from this genus are reported to feed only on seeds of *Gleditsia* and *Phaseolus*, both of which genera belong to the Fabaceae family. *Megabruchidius* is a genus native to East Asia and has been studied widely in the past few years because *Megabruchidius tonkineus* and *M. dorsalis,* two species that belong to this genus, have been reported as invasive seed beetles in Europe where they feed on the seeds of *G. triacanthos*.

Megabruchidius tonkineus as mentioned previously is native to East Asia and was described by Pic in 1914 from northern region of Vietnam (György, 2007). In its native range the seed beetle feeds on *Gleditsia australis, Dolichos lablab* and *Tagetes sp.* (Southgate, 1979; Borowiec, 1984), The first record of *M. tonkineus* in Europe was in Germany, where Wendt (1980) identified the seed beetle as *Bruchidius tonkineus*. The species was transferred to the new genus at the time, *Megabruchidius,* by Borowiec (1984), since when the name has remained unchanged. In Europe it has completely changed its host range to a novel host: *G. triacanthos* (Yus-Ramos *et al.,* 2011, 2014).

In Europe, *M. tonkineus* is regarded as an invasive species, and there has been much concern owing to its adaptability to new environments and hosts. The main concern is that the seed beetle may start feeding on additional plant species, like native plants, plants for human consumption, or economically important crops (Yus-Ramos *et al.,* 2014; Kurtek *et al.,* 2017), however, this has not yet occurred. The beetle has now been reported in Asia (Vietnam), Europe (Bulgaria, Croatia, France, Germany, Greece, Hungary, Romania, Russia, Serbia and Switzerland), South America (Argentina and Chile) and Africa (South Africa) (Yus-Ramos *et al.,* 2011; György & Germann, 2012; Gavrilović & Savić, 2013; Korotyaev, 2016; Fernández *et al.,* 2017; Kurtek *et al.,* 2017; Pintilioaie *et al.,* 2018).

In Europe the beetle has managed to survive the low winter temperatures (below -20 °C), which was unforeseen for a species that belongs to a warm climate (György, 2007; Izhevsky & Maslyakov, 2008; Kurtek *et al.,* 2017). No studies have yet been conducted on the species in Africa, so very little is known about its distribution, hosts or impact.

3.2 Material and Methods

3.2.1 Surveys for Megabruchidius tonkineus *in South Africa*

Megabruchidius tonkineus larvae live inside the seeds of *G, triacanthos*, therefore, to locate the beetle, seeds of *G. triacanthos* were collected from across the plants distribution in South Africa. Occurrence records of *G. triacanthos* were obtained from the Southern African Plant Invaders Atlas (SAPIA) database and consultations with relevant researchers. Areas identified as suitable for the growth of *G. triacanthos* in Chapter 2 were also considered. Plants were subsequently located during roadside surveys, and provisionally identified as *G. triacanthos* using morphological keys (Henderson, 2001; Glen & van Wyk, 2016). Ultimately, surveys were conducted in 231 sites across South Africa (Fig. 1.4; Chapter 1). These surveys took place along the roadsides in the Eastern Cape, Northern Cape, Free State and KwaZulu-Natal provinces (Table 3.1). The surveys were conducted during winter and spring, when the developing pods were still on the trees.

In addition to collecting pods containing insects, plant parameters were also recorded. When the tree was recorded at locations in high densities, sites were sampled >10 km from the previous sites. Of the 231 sites, only 60 *G. triacanthos* sites could be accessed for sampling (Fig. 3.1) due to be in private property. Data recorded at sampled sites included date, GPS coordinates, plant morphology data, such as the presence of thorns, leaves and flowers, basal offshoots, seedlings, and a site description that included associated vegetation. Genetic samples were taken from the new leaves, placed into small envelopes and dried in silica gel to preserve the genetic material, following the protocol of Chase and Hills, (1991). At the sites old pods were collected from the ground to ensure that the total damage of the beetle could be determined; because pods last longer than a year, all the seasonal fluctuations of insect numbers would therefore be included in the total damage count.

Table 3.1: Date and duration of surveys of *Gleditsia triacanthos* conducted in South Africa.

Date	**Duration**	**Province**	**Report Authors**
08/06/2019-13/06/2019	5 days	Eastern Cape and Free State	G. Martin, A. Wolmarans, S. Salgado
14/06/2019-18/06/2019	4 days	KwaZulu-Natal	T. Westwood, B. Mason, S. Salgado
12/11/2020-17/11/2020	5 days	Eastern Cape, Free State and KwaZulu-Natal	G. Martin, E. Mauda, S. Salgado

In addition to collecting pods, seeds which had escaped the pods and had entered the soil were collected to determine if *M. tonkineus* was damaging seeds already in the seed bank. Seeds in the seed bank were collected from below the canopy of *G. triacanthos* infestations from soil samples randomly taken from 50 sites (Fig. 3.1). Samples were taken using an auger (diameter 7.5 cm × 20 cm deep). Four soil cores were taken per site and the soil samples were passed through a sieve (5 mm), to remove fine particles, leaving only seeds and large particles in the sieve. *Gleditsia triacanthos* seeds could be clearly distinguished as they are quite large (Fig. 3.2). All seeds collected from the field were stored inside paper bags and returned to the laboratory. In the laboratory, the pods which were collected from the ground and trees from the 50 survey sites were transferred into individual plastic cages (20 cm×20 cm×17 cm) to record the presence and abundance of *M. tonkineus*.

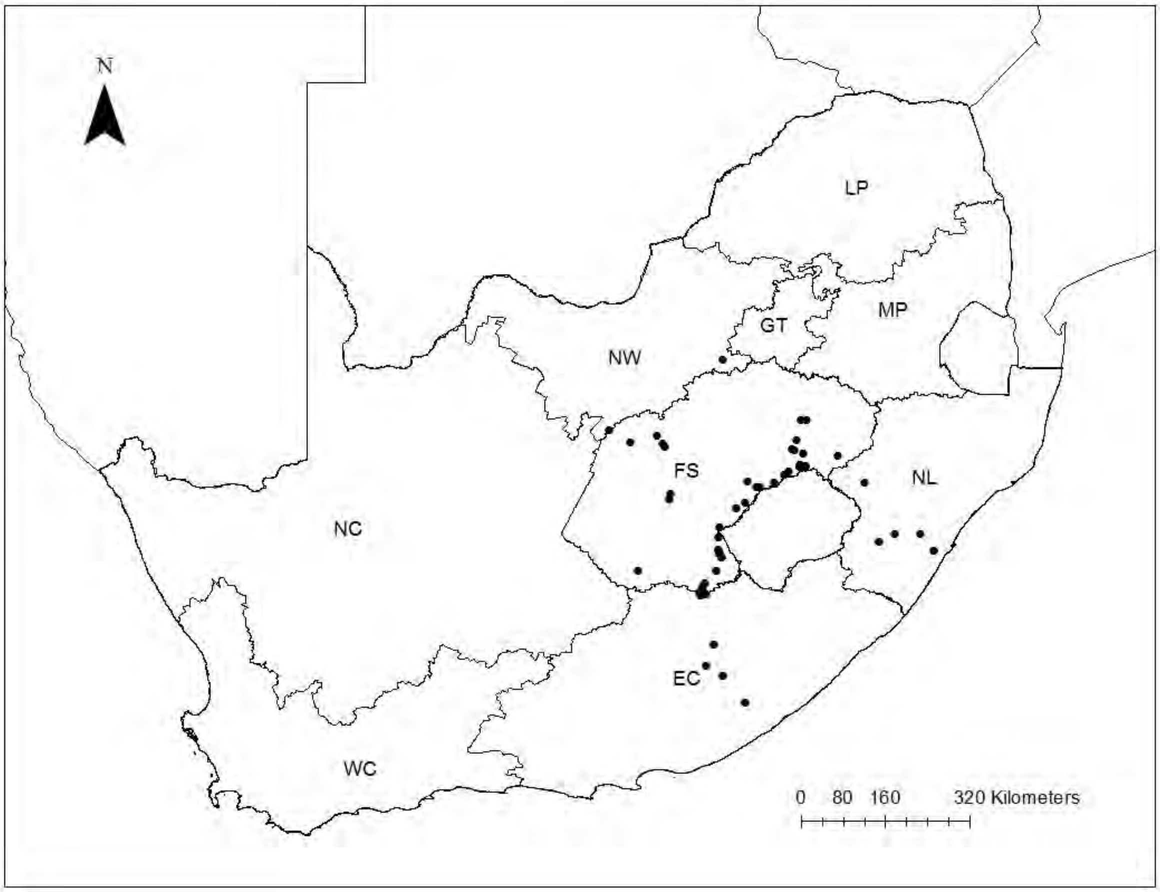

Figure 3.1: Survey locations of *Gleditsia triacanthos* L. (Fabaceae) in South Africa.

Figure 3.2: Seeds of *Gleditsia triacanthos* L (Fabaceae) in the following order (left to right): normal seed, seed with scarification process (cut in the top of the seed to break the seed coat) and seed soaked for 3 days.

In the laboratory, the pods which were collected from the 60 survey sites and kept in paper bags, were transferred into individual plastic cages (20 cm×20 cm×17 cm) to record the presence and abundance of *M. tonkineus* based on the number of seeds with exit holes. Emerging adults were counted and identified using György's (2007) notes on their biology as a reference. A selection of the emerging adults were sent to the National Collections of Insects - Agricultural Research Council (ARC-PPRI) Biosystematics for full species identifications, confirmations and accessioning in the National Insect Collection. Remaining voucher specimens are housed at the Centre for Biological Control (CBC), Rhodes University and referred to by Rhodes University (RH) accession numbers. This gave an indication of the distribution of *M. tonkineus* across the surveyed sites in South Africa

3.2.2 Impact study of Megabruchidius tonkineus

Twenty pods per site were selected from the collected pods to determine how many seeds per site were being damaged by *M. tonkineus*; a total of 60 sites and 1200 pods were examined. The pods were placed into plastic cages (20 cm×20 cm×17 cm), using mesh as a lid to allow ventilation. The cages were kept in laboratory conditions at the Centre for Biological Control, Rhodes University, Makhanda. The pods were in the cages for a period of two months until all the adults had emerged and the seeds were dissected to determine if beetles were remained within the seeds. Emerging adults were removed from the plastic cages and the remaining seeds were processed to determine the number of damaged, deformed, and healthy seeds.

3.2.3 Parasitoids

It is common for biological control agents to recruit native parasitoids in their adventive range. These parasitoids may severely affect the impact of a potential biological control agent (Hill & Hulley, 1995; Paynter *et al.,* 2019). The bruchid has accumulated Hymenoptera parasitoids in Europe (Sajna, 2019) so all cages were monitored for the emergence of parasitoids.

3.2.4 In-field host-specificity testing

At *G. triacanthos* sampling sites, the area was searched for any closely related Fabaceae species. If located, 20 pods were collected from these trees and placed into paper bags. Once back in the laboratory the pods were placed in plastic cages (20 cm×20 cm×17 cm), using mesh as a lid to allow ventilation, and the seeds were inspected weekly for the emergence of any insects, including *M. tonkineus*. It is worth noting that *G. triacanthos* is primarily distributed in the Grassland Biome at this stage and therefore it is not common to find native tree species close to the populations.

3.2.5 Life history, impact and host specificity

All life history, impact and host-specificity trials of *M. tonkineus* were conducted at the Centre for Biological Control, Rhodes University Research facilities, Makhanda, Eastern Cape Province, South Africa.

3.2.5.1 Insect cultures

Laboratory cultures of *M. tonkineus* were created from adults emerging from seeds of *G. triacanthos* collected in the Eastern Cape, Free State, North West, Northern Cape and KwaZulu-Natal provinces of South Africa (Fig. 3.1). The cultures were kept under laboratory conditions in the Centre for Biological Control during 2019–2021. The cultures were kept in op-Up mesh cages (60 cm×60 cm×90 cm) at an average temperature of 21 °C and a 12:12 hour day: night-light ratio. The mesh cages allowed ventilation to the pods. Water and sugar water was provided to the adult beetles: 5 ml of sugar water was placed in 5 cm diameter petri dishes. The culture was used to provide adults for host-specificity tests and to study the biology of *M. tonkineus* under laboratory conditions.

3.2.6 Life history of Megabruchidius tonkineus

3.2.6.1 Oviposition preference

In order to determine where the seed beetle prefers to lay eggs within the pods, an experiment was set up with ten replicates. Each replicate consisted of a closed plastic cage (20 cm×20 cm×17 cm) with mesh as a lid to allow ventilation. Each cage contained ten adult beetles and five different oviposition options, namely: 1) healthy pods, 2) damaged pods (old pods), 3) pods with exit holes, 4) healthy seeds and 5) seeds with exit holes. Water and sugar water were provided as nourishment for adults in each of the cages. After eight days the oviposition options were removed and carefully examined for eggs.

3.2.6.2 Biology

In order to determine how fecund female *M. tonkineus* were, newly eclosed adults (one male and one female) were taken from the cultures and placed into five cm diameter petri dishes lined with moist filter paper, each containing five seeds of G. *triacanthos*; this was repeated ten times. Adults were allowed to mate and to oviposit. The oviposition was monitored on a daily basis. After oviposition had occurred, the eggs were counted.

These eggs and seeds were then used to determine the insect's biology and development time under laboratory conditions. Where seeds contained more than one egg, the extra eggs were removed from the seed using forceps and placed onto undamaged honey locust seeds. Ten replicates were prepared; each replicate included a petri dish with ten seeds containing one egg on each seed (i.e., 100 seeds with one egg each). The length and breadth of ten eggs were measured and they were monitored every day until hatching; larval development was then recorded. Once the eggs had hatched, the head capsule width of ten instar larvae was recorded. The measurements were taken with a stereo microscope with an ocular micrometer. The seeds were then monitored until new adults emerged. The filter paper was replaced every two days to reduce the probability of fungal growth.

3.2.7 Laboratory host-specificity testing

3.2.7.1 Test plant selection

Southern Africa has a very diverse flora and is rich in Fabaceae species with a total of 140 genera and 1839 species. Twenty five genera and 115 species are naturalised and a further 88 genera and 429 species are cultivated in the region (Crisp *et al.,* 2000). Members of this family are ecologically and economically important for timber and valued for traditional and contemporary uses such us food and medicine (Van Wyk & Gericke, 2018; Van Wyk, 2019). The centrifugal phylogenetic method (Wapshere, 1974; Briese, 2003) was used to select the test plants for host specificity of *G. triacanthos.* A list of plants closely related to *G.*

triacanthos was selected, based on the phylogenetic tree for Fabaceae (Wojciechowki *et al.,* 2004; Fig. 3.3). The GenBank taxonomy database was used to identify plant species that are closely related to the target species present in South Africa (Clark *et al.,* 2016). Plants were selected from the Fabaceae family, followed by closely related families (Table 3.2; Fig. 3.3). Three families, Anacardiaceae(1), Brassicae(1), Fabaceace (21) and 21 species were selected to test for host-specficity trials within Fabaceae, species that belong to the genera *Albizia*, *Arachis*, *Cassia*, *Cicer*, *Cyclopia*, *Dipogon*, *Indigofera*, *Libidibia*, *Mimosa, Peltophorum*, *Phaseolus*, *Pisum*, *Podalyria* and *Senegalia*. Four species were selected from the genus *Senegalia*. Of the 23 plants selected, thirteen species were native to southern Africa (Coates & Coates, 2007).

With the plant species selected for host specificity, a phylogenetic tree was generated using the software R version 3.6.3 (Fig. 3.3) (R Core Team, 2021). The packages 'ape' and 'phytools' were used (Paradis & Schliep, 2019; Didier & Heibl, 2020; Revell, 2020). S.PhyloMaker is a tool for generating phylogenies using the R language; the phylogenies were standardised according to the Plant List (Qian & Jin, 2016). The most closely related species to *G. triacanthos* in South Africa is *Umtiza listeriana* Sim followed by *Peltophorum africanum* Sond. *Umtiza listeriana* was important to species to test as the species is endemic to South Africa and is facing extinction as there are less than ten wild surviving trees remaining (Fig. 3.3) (Coates & Coates, 2007; Williams & von Staden, 2008)

Seeds for host range testing were either collected from the field, bought online or requested from herbariums across South Africa.

3.2.7.2 No-choice trials

Host-specificity trials were conducted under the same laboratory conditions as the insect biological studies described above. No-choice tests are an accepted methodology of determining host specificity; in this study, no-choice trials used adult *M. tonkineus* to evaluate oviposition, feeding, survival and reproduction of the insect on non-target species (closely related plants or plant of economic importance) (Harris & Zwolfer, 1968; Marohasy, 1998). Ten adults of *M. tonkineus* were confined in a five centimeter petri dish lined with moist filter paper and were provided sugar water for a week. The food source consisted of a single seed of the test plant in the centre of the petri dish, without the presence of the target plant species seed (Fig. 3.4). Each plant species had five replicates and the sugar water was replaced on day three after the start of the trials. Oviposition, feeding damage and survival of adults were measured. Individual seeds were prefered for host specificty testing as obtaining pods of the test species proved difficult. This was also not considered problematic as suitable controls were achieved on all replications.

Table 3.2: Non-target species selected for host-specificity testing of *Megabruchidius tonkineus* on closely related plant species. Asterisks (*) indicate exotic plant species.

Family	Genus	Species
Anacardiaceae	*Searsia*	*Searsia lancea* (L.f.) F.A.Barkley
Brassicaceae	*Eruca*	*Eruca vesicaria* (L.) Cav.*
Fabaceae	*Senegalia*	*Acacia fleckii* Schinz
	Vachellia	*Vachellia sieberiana* DC.
	Albizia	*Albizia julibrissin* Durazz*
	Arachis	*Arachis hypogaea* L.*
	Cassia	*Cassia abbreviata* Oliv.
	Cicer	*Cicer arietinum* L.*
	Cyclopia	*Cyclopia genistoides* (L.) Vent.
	Dipogon	*Dipogon lignosus* (L.) Verdc.
	Indigofera	*Indigofera cytisoides* Thunb.
	Libidibia	*Libidibia férrea* (Mart. Ex Tul.) L.P Queiroz*
	Mimosa	*Mimosa púdica* L.*
	Peltophorum	*Peltophorum africanum* Sond.
	Phaseolus	*Phaseolus vulgaris* L var. Mex*
	Phaseolus	*Phaseolus vulgaris* L. var. Rola*
	Pisum	*Pisum sativum* L.*
	Podalyria	*Podalyria buxifolia* Willd.
	Senegalia	*Senegalia brevispica* (Harms) Seigler & Ebinger
	Senegalia	*Senegalia burkei* (Bent.) Kyal. & Boatwr
	Senegalia	*Senegalia nigrescens* (Oliv.) P.J.H. Hurter
	Senna	*Senna spectabilis* (DC.) H.S. Irwin & Barneby*
	Umtiza	*Umtiza listeriana* Sim.

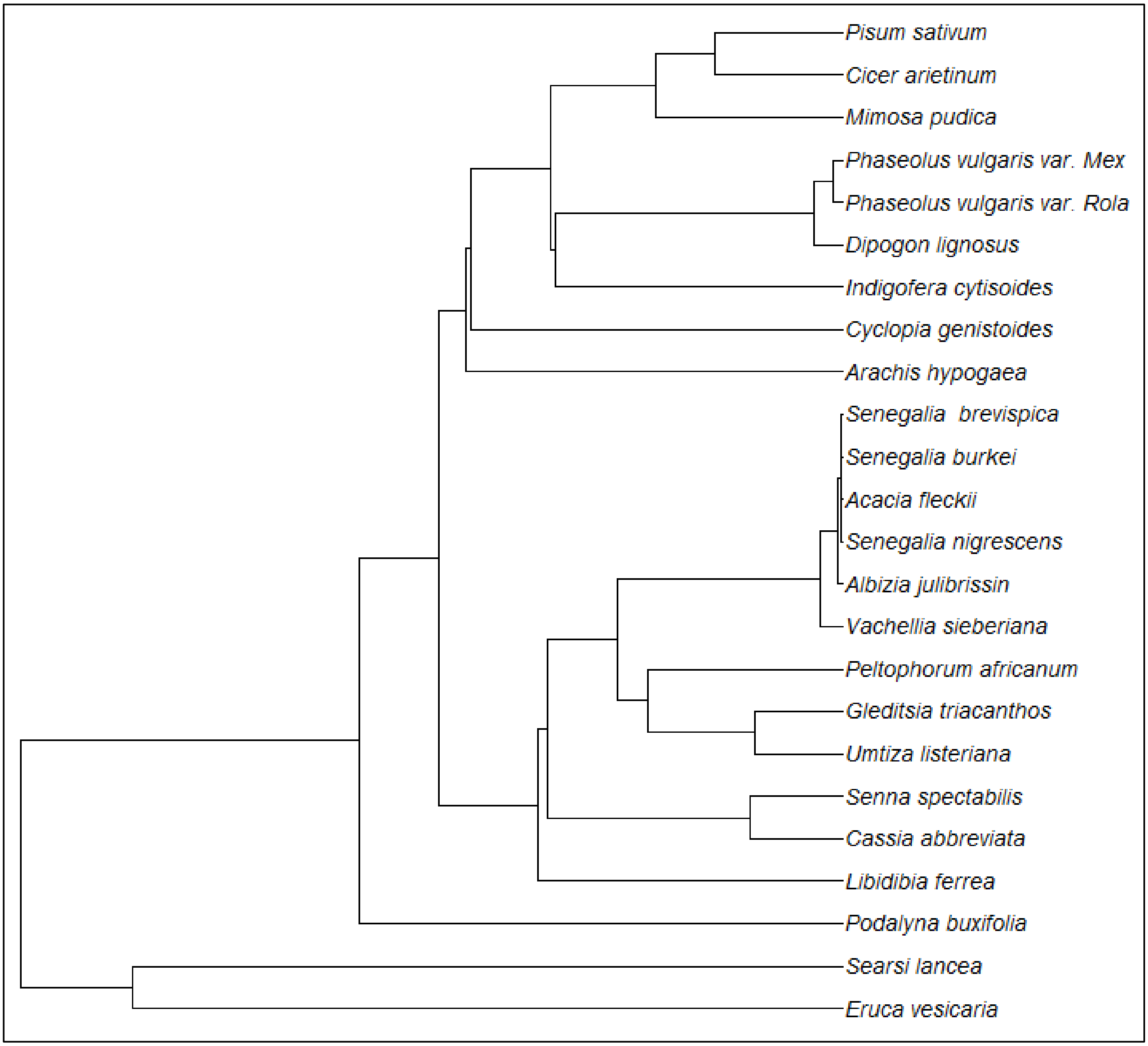

Figure 3.3: Phylogenetic tree of plant species used for host-specificity trials to test *Megabrudichius tonkineus*. Analysis was carried out in R version, using Package ape (Paradis & Schliep, 2019).

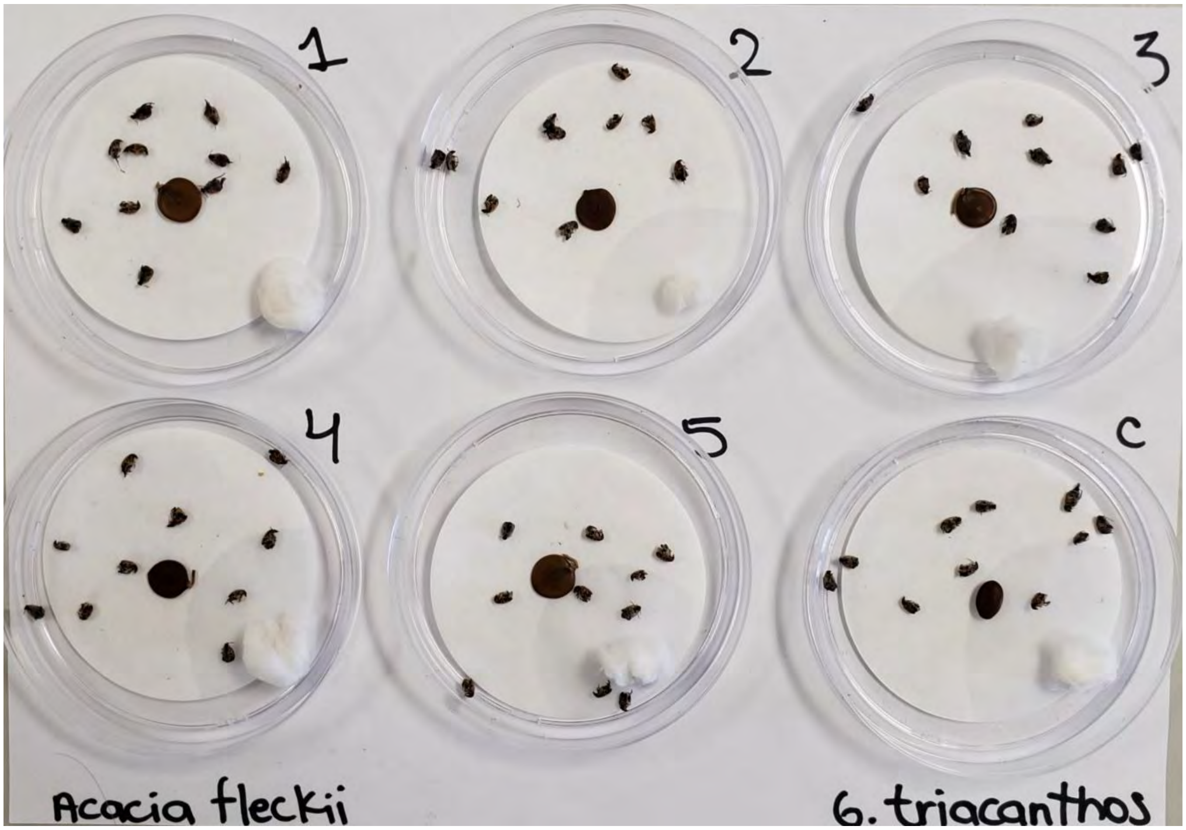

Figure 3.4: No-choice trials with *Megabruchidius tonkineus* and the native plant species *Acacia fleckii* and *Gleditsia triacanthos* as a control.

3.3 Results

3.3.1 Surveys

Gleditsia triacanthos roadside surveys were conducted in 231 sites across the Eastern Cape, Free State, North West, Northern Cape and KwaZulu-Natal provinces, South Africa (Fig. 3.5). *Gleditsia triacanthos* is most abundant in the Free State province where the tree creates dense monocultures (Figs. 3.6; 3.7). At these sites a high number of pods and seedlings of *G. triacanthos* were recorded around the trees (Fig. 3.7).

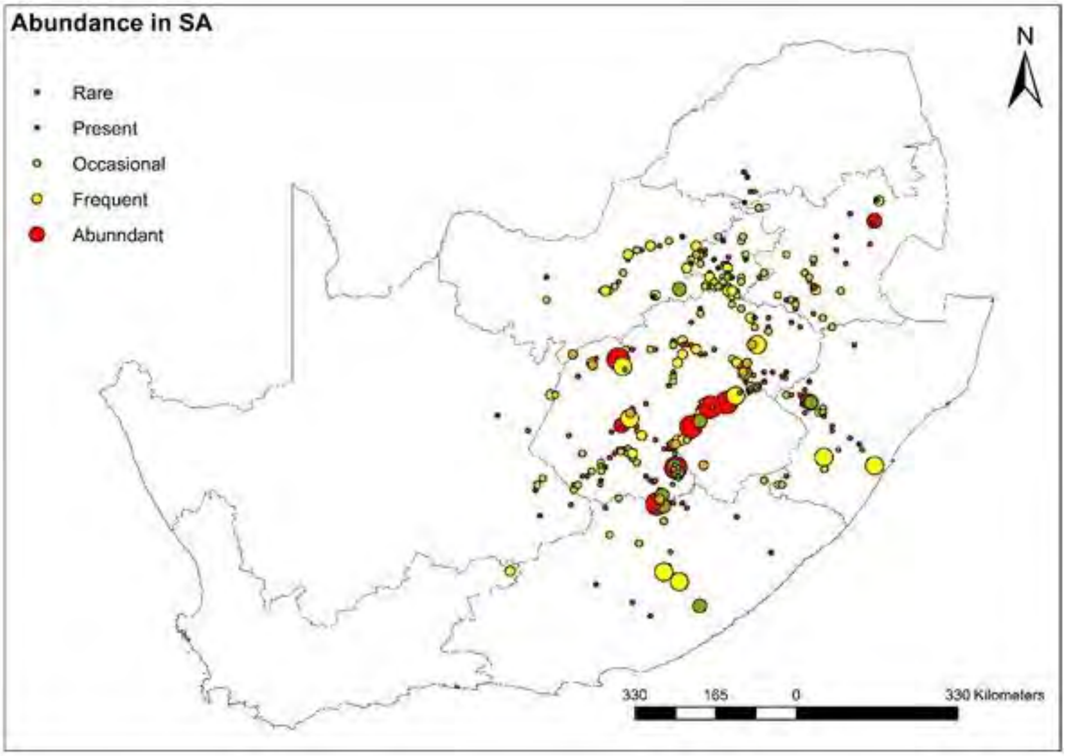

Figure 3.5: Abundance of *Gleditsia triacanthos* in South Africa based on SAPIA (South African Plant Invader Atlas) and survey data.

Figure 3.6: *Gleditsia triacanthos* growing roadside on the Road- R700 in Free State Province.

Figure 3.7: *Gleditsia triacanthos* offspring, basal shoots and seed pods in N6 near the town of Aliwal North, Eastern Cape Province.

The seed beetle*, M. tonkineus,* was present in 47 of the 60 sampled sites (Fig. 3.8). The insect was recorded from the North West Province, Free Sate, KwaZulu-Natal and Eastern Cape provinces. The seed beetle distribution closely followed the distribution of *G. triacanthos.*

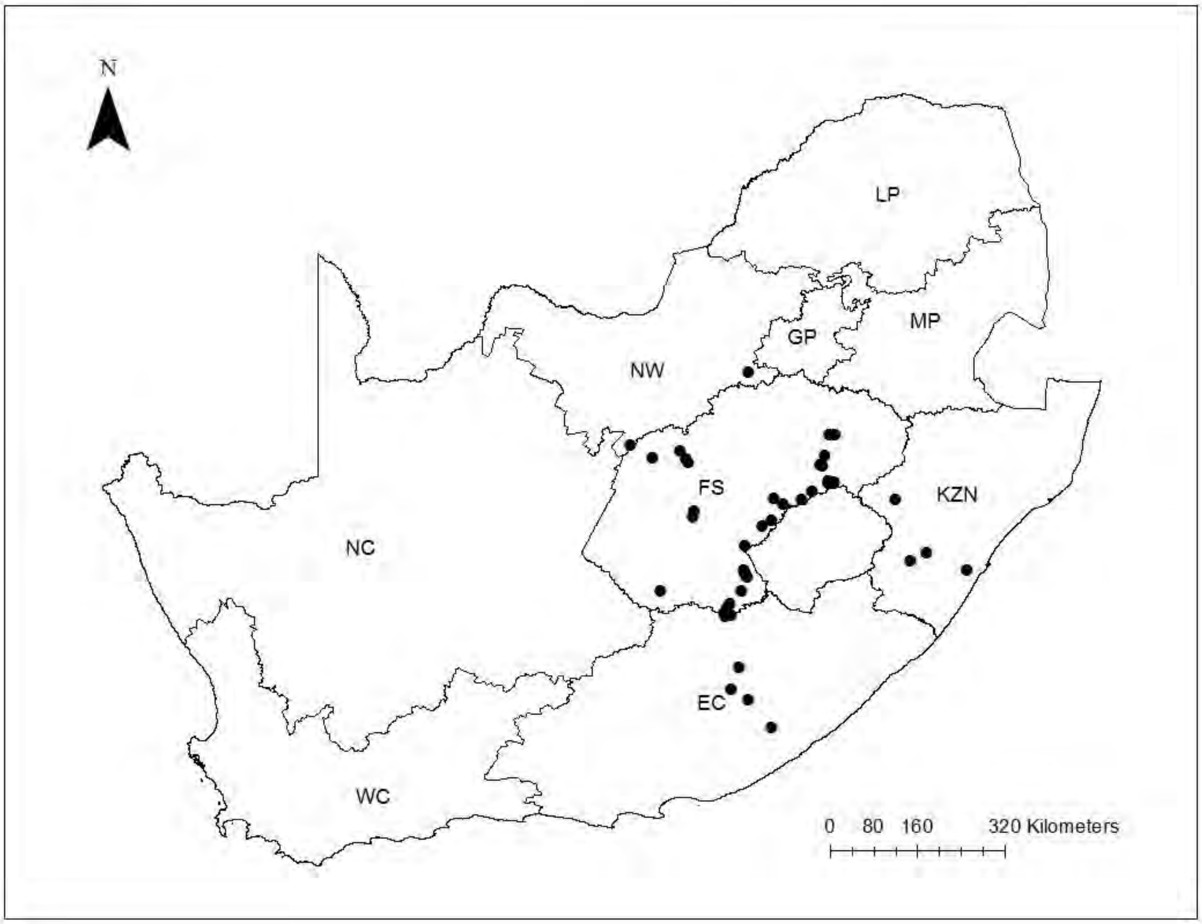

Figure 3.8: Distribution of *Megabruchidius tonkineus* in South Africa. Data collected during 2019 and 2020.

3.3.2 In-field impact data of Megabruchidius tonkineus

To determine the impact of *M. tonkineus* on seeds of *G. triacanthos,* 60 sites across South Africa were sampled, and 20 mature pods per site were collected from each site. The seeds were removed from the pods and checked for damage (Table 3.6; Appendix 1). The mean percentage of seeds damaged by *M. tonkineus* from all the sites in South Africa was 9.17 ± SE 1.28 (Fig. 3.9; 3.10), deformed seeds 10.72 ± SE 1.70 and healthy seeds where the beetle had not caused any damage, 80.11 ± SE 1.33 (Table 3.3). The site with the highest damage of seeds was 37. 87% recorded along the road R 26 to the west of Ficksburg in the Free State province with 124 damaged seeds from the 20 pods. No insect damage was recorded from 13 sites. There was discernible difference in levels of damage across the *G. triacanthos* distribution (Fig. 3.10).

Table 3.3: Mean percentage of damage of *Megabruchidius tonkineus* on *Gleditsia triacanthos* taken from 20 pods from each sampled site, where damaged seeds are the seeds

Seeds	**Mean % with SE**
Damaged	9.17 ± 1.28
Deformed	10.72 ± 1.70
Healthy	80.11 ± 1.84

with presence of exith holes and deformed are seeds with abnormalities.

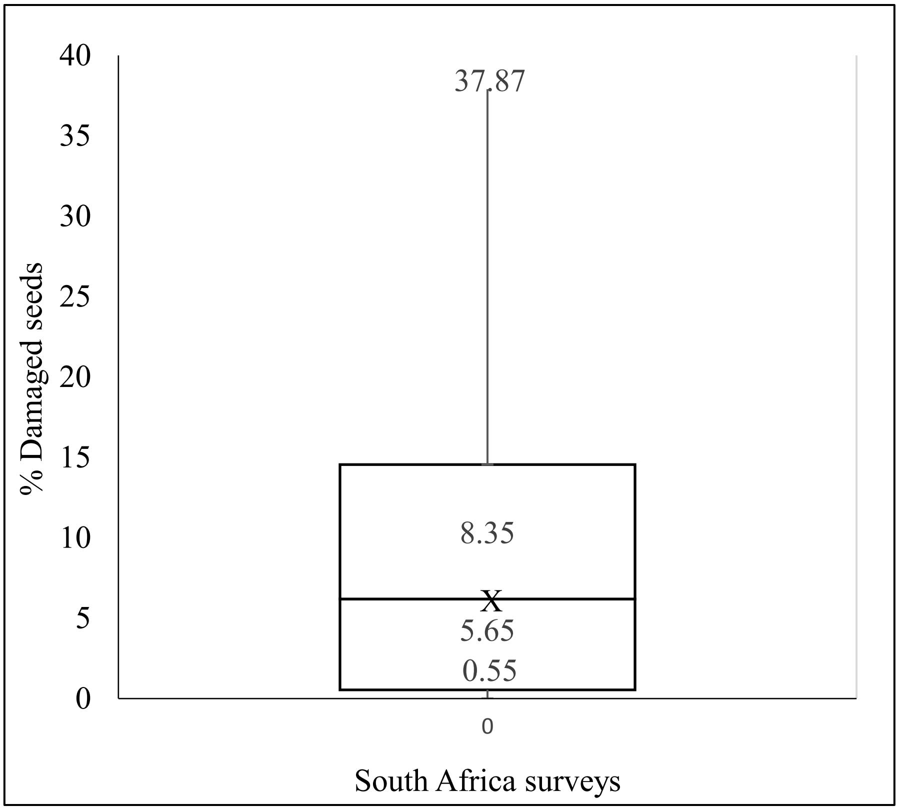

Figure 3.9: Number of damaged *Gleditsia triacanthos* seeds across sampled sites in South Africa (n=60). Maximum and minimum damage levels of the seeds are indicated and the mean is marked with an X.

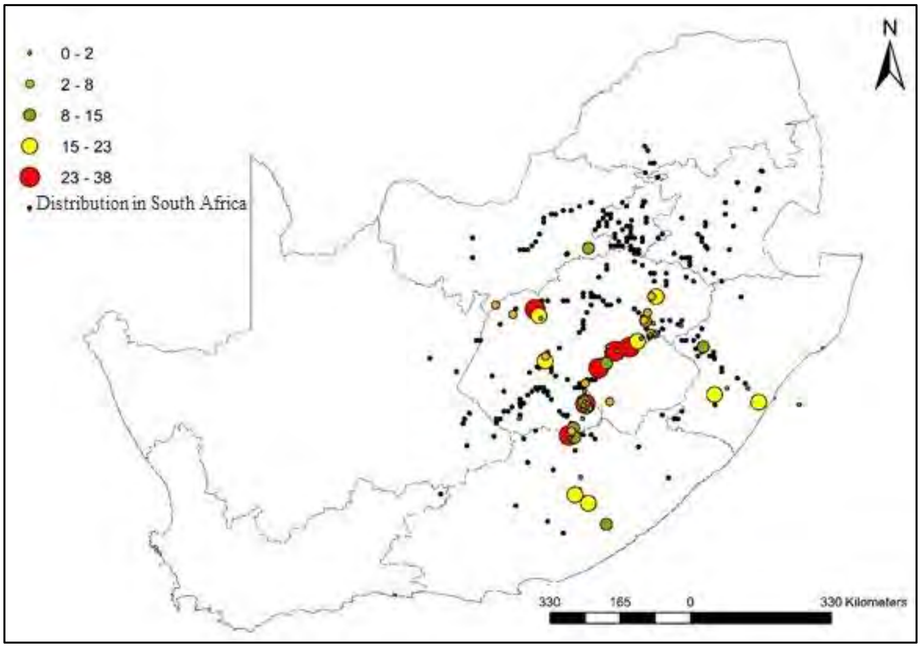

Figure 3.10: Distribution of *Megabruchidius tonkineus* in South Africa with the percentage of damaged seeds.

Two hundred soil cores were conducted between the 50 sites and 1309 seeds were collected, with an average of 26.18 ± SE 13.99 m^2 per site. No seed beetles were recorded emerging from any of the seeds collected from the soil cores neither seeds with exit holes.

3.3.2.1 Description of Megabruchidius tonkineus

Adults are about 3–5 mm with light colouration on the dorsum. Male and females can be differentiated by their ventral side and the pygidium; adult males have an impression in the middle of the first ventrite with a hair in the drop-shaped spot, whereas females have the same impression but lack the hair in the drop-shaped spot. The pygidium in both sexes has two small round impressions; the pygidium in males is immaculate, that is, they have light brown spots, but in females the pygidium has a black, longitudinal, bare apical fovea (György & Germann, 2012). The females lay eggs in the pods, seeds with exit holes, healthy seeds (Fig. 3.12). The seeds described as healthy are new seeds, seed without exit holes (Fig. 3.10 B).

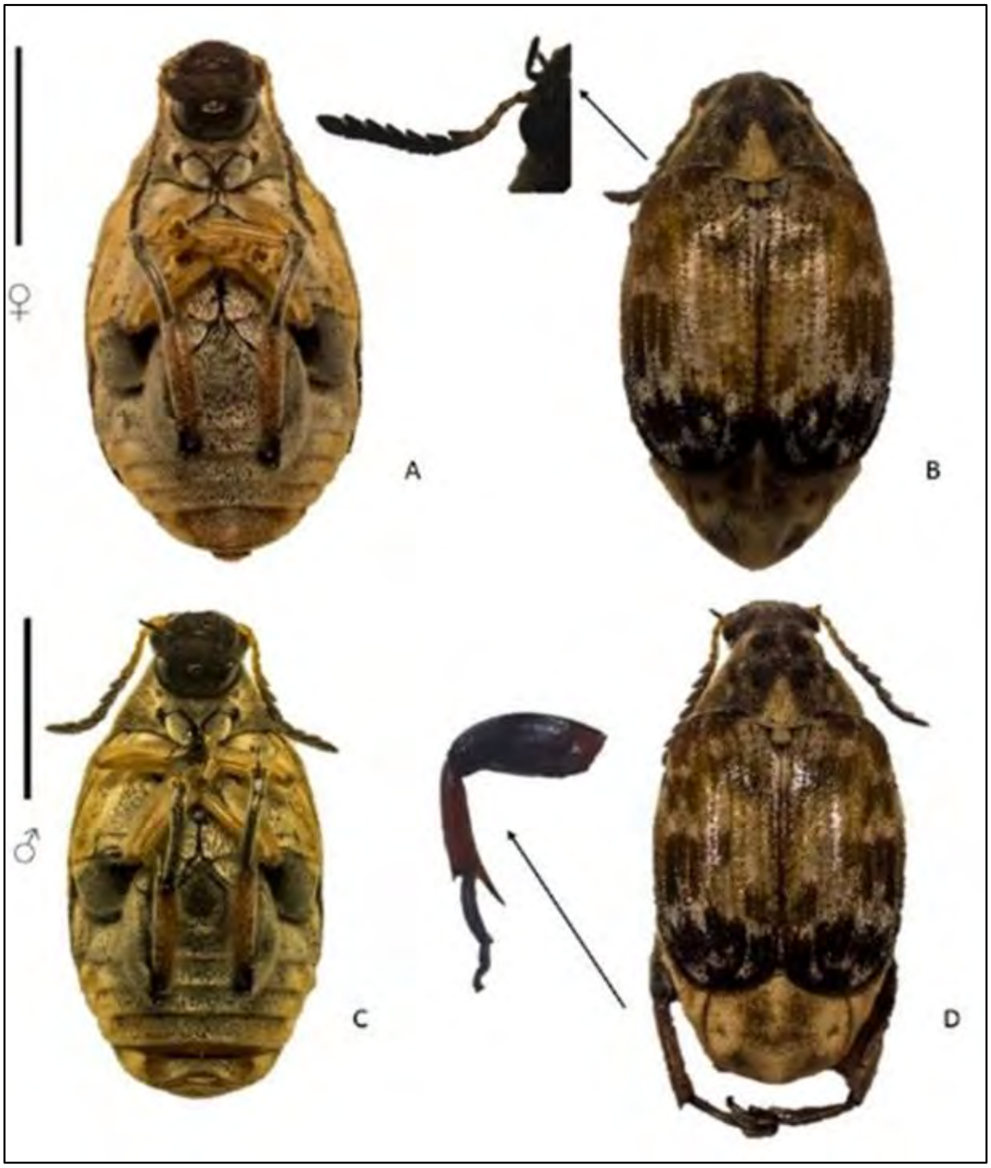

Figure 3.11: *Megabruchidius tonkineus* (Coleoptera: Chrysomelidae: Bruchinae), Female (A and B); a magnification of the diagnostic antennae is shown between the images. Male (C and D) and a magnification of the leg, showing the diagnostic hook in the hind tibiae.

3.3.2.2 Oviposition preference

When five different options are provided, females prefer to lay eggs in the following order: damaged pods (45.89 ±SE 5.44), healthy pods (24.46 ±SE 5.04), pods with existing exit holes (17.85 ±SE 6.54), healthy seeds (9.75 ±SE 2.80) and seeds with exit holes (2.05 ±SE 1.11) (Fig. 3.12).

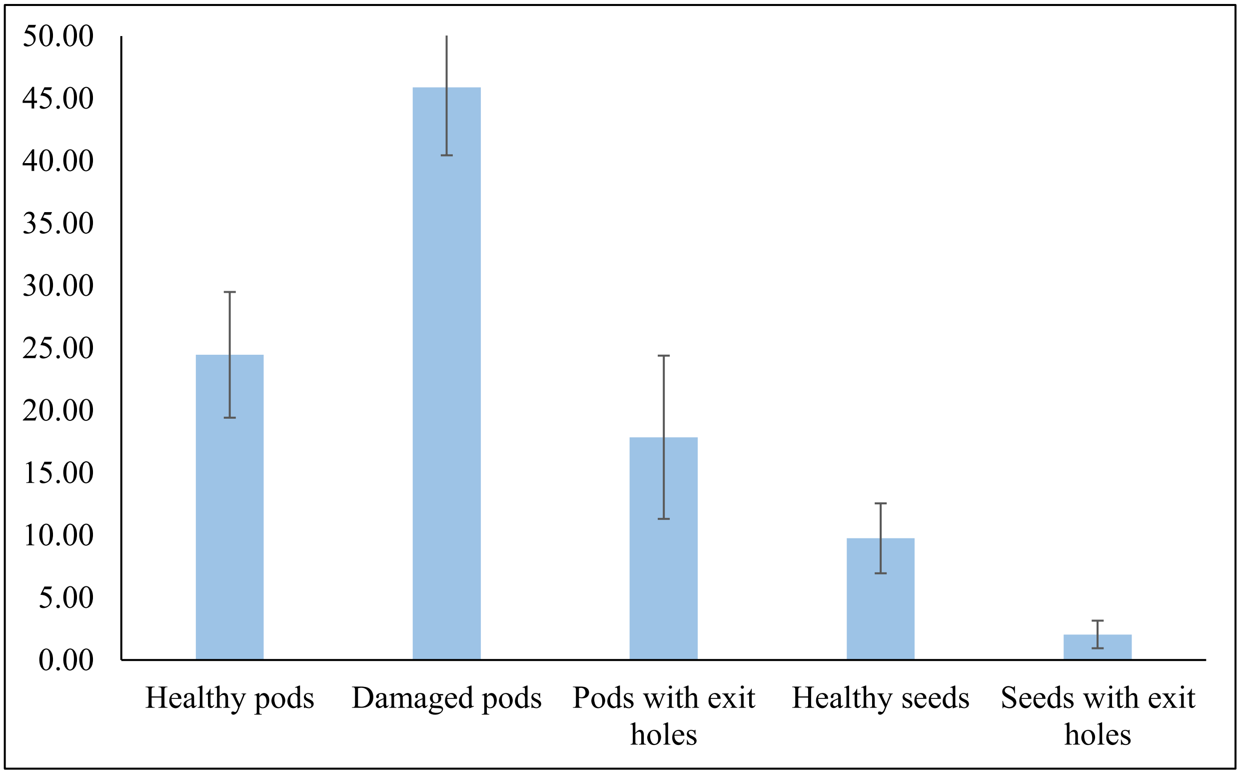

Figure 3.12: Percentage of eggs laid by female *Megabruchidius tonkineus* on different parts of the *Gleditsia triacanthos* pods and seeds. Error bars show standard error.

3.3.3.3 Biology

Females laid 2–13 eggs (n=25, Mean7.32 ±SE 0.708) on the seeds or pods (Fig. 3.10 B), laying one, two or more eggs in the same place. The eggs are yellow, oval in shape, elongated, with a pointed end. The colour changes to creamy-yellow with age (Fig. 3.13 B). The average length and width of the eggs was (n=10) 0.835 mm ± SE 0.0182 and 0.324 mm ± SE 0.00763, respectively. Eggs hatched around 12 days (n=25) 11.64 days ± SE 0.1137 (Table 3.3).

The neonate larvae are pale yellow to white with a few erect hairs; the width of the head capsule (n=10) is 0.22 mm ±0.001. The first larval instar has legs that allow it to move to find the seeds; when the larva finds the seed, it makes an entry hole (Fig. 3.10 C), opening the seed with its mouth parts and closing the entry hole (Fig. 3.10 D) with the last pair of legs. The larvae complete their development inside the seed (Fig. 3.10 E), but the number of instars is unknown. The larvae pupate inside the seed (Fig. 3.10 F). From the hatching of the egg to emergence as an adult takes approximately 50 to 60 days (n=25) 55.16 days ± SE 0.6992 (Table 3.3).

When the adult is ready to emerge, it starts making an exit hole to leave the seed (Fig. 3.10 G). Adults are about 3–5 mm (n=25, 4.44 mm ± SE 0.699) and can be recognised by their colouration, ventral side and pygidium (Fig. 3.10 H). The observed sex ratio of the 25 adults that emerged was 18 females and seven males. The lifespan of the adults is 24.64 ± SE 0.5846 days n= 25 (Table 3.3).

Table 3.3: Mean duration, in days, of each life stage of *Megabruchidius tonkineus*. The sample size and the percent of the lifecycle are provided (±SE).

Adult Longevity (days)	24.64 ± 0.5846
Mean development time (days)	
Egg stage	11.64 ± 0.1137
Larval-pupal stage	55.16 ± 0.6992
Total development time	**66.80± 0.6880**

3.3.3.4 Parasitoids

During the study 1410 *M. tonkineus* adults emerged from pods collected from the 60 sites across South Africa (Fig. 3.6). No parasitoids were recorded.

Figure 3.13: Life cycle of *Megabruchidius tonkineus* (Coleoptera: Chrysomelidae: Bruchinae). Mating pairs (A); female lays eggs on the inner side of the pods (B); first larval instar (C); seed with an entrance hole made by *M. tonkineus* (D); larval development inside the seed (E); pupa stage (F); exit hole made by adult ready to emerge (G); adult (H).

3.3.4 Host-specificity testing

3.3.4.1 No-choice larval development trial

Oviposition and larval development trials were conducted on *G. triacanthos* seeds and the test plant seeds between 2019 and 2021. Damage was easily recorded because the larvae make an entrance hole in the seed then eat the inside of the seed in which it completes its larval instars and pupae. In laboratory conditions, oviposition was recorded on ten different seed plant species: *Arachis hypogaea, Albizia, julibrissin, Cicer arietinum, Pisum sativum, Dipogon lignosus, Peltophorum africanum, Podalyria buxifolia Senegalia burkei, Umtiza listerina* and *Vachellia sieberiana* but larval feeding damage was recorded only on *Arachis hypogaea* (peanut), *Cicer arieatnum* (chickpea) and *Pium sativum* (pea); however, the beetle did not complete its life cycle in these seeds (Table 3.4).

Table 3.4: Host-specificity trials of *Megabruchidius tonkineus* (Coleoptera: Chrysomelidae: Bruchinae) in laboratory conditions with related plant species of economic value and native trees of Fabaceae, Anacardiaceae and Papilionaceae families in South Africa. Each seed species has five replicates (n=115 seeds). Ovipositions are shown with (X) absent and (✓) present.

Laboratory test					
Family	**Closely related plants (Botanical and common name)**	**Oviposition**	**#eggs**	**Larval feeding damage**	**Survival/ Reproduction**
Fabaceae	*C. abbreviate* (Sjambok pod)	X	0	0	X
Fabaceae	*C. genistoides* (Coastal tea)	X	0	0	X
Fabaceae	*P. vulgaris var. Mex* (Beans)	X	0	0	X
Fabaceae	*P. vulgaris var. Rola* (Beans)	X	0	0	X
Fabaceae	*A. hypogaea* (Peanut)	✓	1	1	X
Fabaceae	*I. cytisoides* (Fire pea)	X	0	0	X
Fabaceae	*V. sieberiana* (Paperbark)	✓	11	0	X
Fabaceae	*A. burkei* (Black monkey thorn)	✓	53	0	X
Fabaceae	*A. nigrescens* (Knob thorn)	X	0	0	X
Fabaceae	*A. fleckii* (Blade thorn)	X	0	0	X
Fabaceae	*A. brevispica* (Prickly thorn)	X	0	0	X
Fabaceae	*L. férrea* (Brazilian ironwood)	X	0	0	X
Fabaceae	*A. julibrissin* (Silk tree)	✓	23	0	X
Fabaceae	*C. arietinum* (Chickpea)	✓	1	1	X
Fabaceae	*P. sativum* (Pea)	✓	1	1	X
Fabaceae	*S. spectabilis* (Golden wonde tree)	X	0	0	X
Fabaceae	*D. lignosus* (Cape sweet pea)	✓	20	0	X
Fabaceae	*P. africanum* (Rhodesian blackwood)	✓	6	0	X
Anacardiaceae	*S. lancea* (Karee)	X	0	0	X
Fabaceae	*M. pudica* (Sensitive plant)	X	0	0	X
Fabaceae	*P. buxifolia* (Box-leaf bush-sweetpea)	✓	9	0	X
Brassicaceae	*E. vesicaria* (Arugula)	X	0	0	X
Fabaceae	*U. listerina* (Omtisa)	✓	3	0	X
Fabaceae	*G. triacanthos* (Honeylocust tree)	✓	61	24	19

3.3.4.2 In field host-specificity testing

None of the pods of the ten closely related Fabaceae species collected in the fiels were damaged by *M. tonkineus*, moreover, no weevils emerged from any of the pods collected from field (Table 3.5).

Table 3.5: In field host specificity, 20 pods of the trees were collected in the field, kept in the laboratory and inspected for *Megabruchidius tonkineus*.

Species name	***Megabruchidius tonkineus***
Albizia adianthifolia	X
Peltophonum africanum	X
Senegalia brevispica	X
Senegalia burkei	X
Senegalia mellifera	X
Vachellia erioloba	X
Vachellia karoo	X
Vachellia robusta	X
Vachellia sieberiana	X
Vachellia tortilis	X
Gleditsia triacanthos (1200 pods)	1410

3.4 Discussion

Under laboratory conditions *M. tonkineus* could complete its life cycle only in the seeds of *G. triacanthos*. However, the adult beetles did lay eggs on a few non-target species: *Arachis hypogaea, Albizia julibrissin, Cicer arietinum, Pisum sativum, Dipogon lignosus, Peltophorum africanum, Podalyria buxifolia Senegalia burkei, Umtiza listerina* and *Vachellia sieberiana*. After the eggs hatched, the larvae made an entrance hole, penetrating the seed coat on seeds of *A. hypogaea*, *C. arietinum* and *P. sativum*. However, the larvae died after they entered the seed, so did not complete development in any of the non-target plants.

A study by Kergoat *et al.,* (2007) showed that species from bruchine can feed on Caesalpinioideae and Mimosidaeae, both of which are subfamilies of Fabaceae. Other studies have shown that *M. tonkineus* laid eggs and developed into adults on the following Caesalpinoid seeds: *Gymnocladus dioica* (L.) K. Kosh (Fabaceae: Caesalpinoideae), *Gleditsia caspia* Desf., *Gleditsia delavayi* Franch., *Gleditsia ferox* Desf., *Gleditsia japonica* Miq. And *Gleditsia macracantha* Desf. (T. Jermy, pers. comm., 2006; György, 2007a). Although it seems that *M. tonkineus* has a restricted diet in South Africa, we cannot exclude the possibility that *M. tonkineus* might be able to complete its life cycle on seeds not tested within the Fabaceae. *Megabruchidius dorsalis,* a beetle closely related to *M. tonkineus,* has been recorded feeding on *Gleditsia* spp., *Gleditsia fera* (Lour.) Merr., *Gleditsia japonica* Miq., *Gleditsia sinensis* Lam., *Gleditsia triacanthos* L as expected. However in contradiction to other publications a new study in Hungary (Gyorgy & Tuda, 2020), reports *M. dorsalis* completing its life cycle in the seeds of an American tree, *Gymnocladus dioica* (Fabaceae: Caesalpinioideae), showing that species from the genus *Megabruchidius* can feed on different genera within the same subfamily. At this stage, the results from this study indicate that, currently, *M. tonkineus* is suitably safe in South Africa and only damaged the seeds of *G. triacanthos*. However, the results of this study are based in a small percentage of species that were tested, and on seeds only. Using pods should be considered for further studies as well as choice testing and open-field trials where possible.

The results from this study indicate that *M. tonkineus* is already widely distributed in South Africa, closely matching the distribution of *G. triacanthos*, but has a limited impact on seed numbers. From seed pods collected from below adult trees, only 9% of seeds (n=60 sites) were damaged by the beetle. Additionally, viable seeds were recorded in the seed bank which were not damaged by *M. tonkineus*.

As mentioned earlier *M. tonkineus* originates from Asia while *G. triacanthos* is from North America, which is potentially concerning as this does not follow the principles of classical biological control and makes the relationship a new association. Literature in biological control of weeds suggests that a new association can be formed between plants and insects that have not co-evolved together. It is believed that a new association could result in an increased impact on the target weed (Dennill, 1993). However it is more common that insect that are not associated with the host usually perform poorly. As, *G. triacanthos* is not its principal host plant this might be a reason why the beetle has a low damage impact on the seeds of *G. triacanthos*. However this is unlikely as the beetle has been shown to do damage up to 90 % of *G. triacanthos* seeds in Argentina (Fernandez *et al.,* 2017). However, the low levels of damage may also be because the insect numbers have not yet built up sufficiently as it is estimated *M. tonkineus* has only been around in South Africa for approximately 20 years and its rate of spread is currently unknown (Neser pers comm. 2020).

The average percentage of seeds damaged by *M. tonkineus* was only around 9%, meaning that the seed beetle causes damage to a small portion of the seeds of *G. triacanthos*. These numbers are potentially too low for *M. tonkineus* to impact the population of *G. triacanthos* significantly. Moran (1991), for example, showed that the 98% seed reduction in *Sesbania puniceniaa* caused by the weevil *Trichapion lativentre* (Beguin-Billecocq) did not lead to a decline in the density of mature plants. Another example is *Melaleuca quinquenervia*, an invasive plant species in the Everglades in the south of Florida and the

Caribbean wetlands which has displaced native species in the Everglades region and reduced the biodiversity of freshwater marshes by 60–80% (Austin, 1978). To reduce the impact of these invasive trees, a combination of manual (cutting), chemical and biological control was implemented. The biological controllers introduced were the weevil, *Oxyops vitiosa,* and the psyllid, *Boreioglycaspis maleucae*, both of which have decreased the spread of *M. quinquenervia* owing to the weevil reducing the seed viability, killing 85% of seedlings (Pratt *et al.,* 2007; Rayamajhi *et al.,* 2009). These examples suggest that a far higher percentage of seed would need to be damaged before control is achieved. Henderson (2016) reviewed the status and geographical extent of the tree in southern Africa and showed that *G. triacanthos* has doubled its areas of distribution measured in QDS (Quarter degree squares) in 15 years, despite the presence of *M. tonkineus.* It is not yet certain if the agent numbers of *M. tonkienus* are increasing over time, but at the current level of damage, effective control of *G. triacanthos* cannot be achieved by *M. tonkineus* alone.

Chapter Four: General discussion

4.1 Thesis synthesis

Gleditsia triacanthos has been present in South Africa for over a decade, and as an invasive alien species under the existing legislation, it requires a formal management plan (NEMBA 2014; Henderson & Wilson, 2017) which has not yet been developed. The first objective described in Chapter 2, was to use different species distribution modelling techniques (MaxEnt and CLIMEX-MCR) to determine if *G. triacanthos* has reached its full distribution in South Africa, or if the weed will continue to spread in South Africa. One of the most important findings described in Chapter 2 was that the predictive models from both programmes showed that a significant proportion (1 220 000 km^2) of South Africa, including all its nine biomes, are climatically suitable for *G. triacanthos*, meaning that *G. triacanthos* has not reached the full ecological niche in South Africa, and that, if left unmanaged, the species will continue to spread. This is concerning as it is already recognised as one of the fastest spreading invasive species in South Africa (Henderson & Wilson, 2017).

The objectives in Chapter 3 were to determine the distribution and impact on *G. triacanthos* seeds of the unintentionally introduced seed predator, *M. tonkineus.* The chapter describes a series of laboratory experiments conducted to determine the biology and the fundamental host range of the insect. *Megabruchidius tonkineus* is widely established in South Africa, and the seed beetle was found across the country; however, *M. tonkineus* only damages a mean of nine percent of seeds with a maximum damage of 37. 87% of *G. triacanthos* seeds. The investigation also showed that *M. tonkineus* does not complete its life cycle on any of the non-target plant species tested, only in the seeds *G. triacanthos*. Since only 23 non-target species were considered, we cannot ignore the possibility that *M. tonkineus* might be able to complete its life cycle on other plant species that were not tested, although we suggest that this is unlikely as we tested a number of close relatives to *G. triacanthos*, including its closest South African relative, *Umtiza listeriana*.

4.2 The role of mapping and predictive modelling in invasion biology

Biological invasion is a major threat to biodiversity worldwide, with severe economic and social impacts (Levine *et al.,* 2003; Newbold *et al.*, 2010; Pimentel *et al.,* 2001). Propagules of potential new invasive species are continuously being introduced, making the management of invasions more challenging and the need for prioritisation more important (Fournier *et al.*, 2019; Richardson & Kluge, 2008). It is important to know how far an invasive species can spread so management efforts and resources can be appropriately used and not wasted on regions unsuitable to the species. Several modelling programmes are available to predict areas where a species could find favourable growing conditions; the programmes used in this study were MaxEnt and CLIMEX-MCR. The models developed by species distribution modelling programmes are not perfect, but if used correctly, their results can be extremely valuable in assisting with invasive species management (Trethowan *et al.*, 2011). For example, a recent study by Martin *et al,* (2020) using MaxEnt showed that *S. terbinthifolia* has not reached its full distribution in South Africa and that a change in the national legislation is required in order to protect regions previously not protected by legislation from the impacts of this invasive tree. Unlike *S. terbinthifolia,* which was a registered invasive only in some South African provinces, *G. triacanthos*, is currently listed as Category 1b under the NEMBA for the entire country, and no higher categorisation for the species can be implemented. Therefore, management needs to be prioritised according to regions where the tree is already established, as well as regions it has not yet reached.

For regions where the species has not yet arrived or only a very few scattered populations exist, early detection and a rapid-response type of management should be implemented. The successful programme control in Queensland, Australia, is a good example of how this intensive management method can work. The programme was a joint effort of the government with landholders. Together they adopted a ‘search and destroy’ campaign, and recognising that the seed would need follow-up control measures, they continued to eradicate seedlings growing from the seedbank years after the initial clearing of mature trees. Managing the seeds is important as *G. triacanthos* produces high numbers of seeds (Marco & Paez, 2000), which are durable and persistent for many years in the soil (Ferreras *et al.,* 2015; Fernandez *et al.,* 2017). This kind of management should be possible in South Africa through the Working for Water programme which has extensive experience with this kind of management (van Wilgen et al. 2020). In regions where the tree is already extensively established like most of the grassland biome it is unlikely the tree will be eradicated even with significant financial investments. Therefore containment efforts should be made to stop the tree from spreading further from these regions as well as protecting conservation areas within these regions.

In South Africa fire is normally considered a management approach in the grassland biome for managing invasive trees and shrubs because it is the most effective method for killing seeds near the soil surface (Richardson & Kluge, 2008; van Wilgen, 2009). In addition it can stimulate germination of the deeper seeds buried in the seed bank which can then be managed, effectively reducing the soil seed bank (Richardson & Kluge, 2008) This is particularly important where biological control has effectively sterilized adult trees such as with a number of *Acacia* species (Impson *et al.,* 2021). However, fire should not be promoted for the management of *G. triacanthos* because it can have other undesirable effects. Fire does not trigger the germination of the seeds of *G. triacanthos*, but helps their invasion by making the plant resprout from the roots, and thereby promoting spread while not reducing the seed bank (Ferreas *et al.,* 2015; Herrero *et al.,* 2016). This has been shown in the Pampean Grasslands of Argentina, where fire regimes have promoted the spread of *G. triacanthos* seedlings by reducing competition from grasses (Chaneton *et al.,* 2004; Faggi *et al.,* 2006; Mazía *et al.,* 2010; Fernandez *et al.,* 2017). This however is contrary Fitch and Kettle, (1983) who suggests that in the prairie of Kansas honey locust appears to be excluded from prairies by frequent fire, and expands where fire is excluded.

It is hoped that biological control can offer an effective, long-term, cost-effective management option, either working in combination with other management options or on its own. However, developing the appropriate biocontrol agent takes time and therefore more conventional methods such as those suggested above should be implemented immediately.

4.3 Prospects for the biological control of Gleditsia triacanthos *in South Africa*

Klein (2011) is the last edition of an annual catalogue of organisms (insect, mite and pathogen species, or biotypes thereof) that have featured in biological control of invasive alien plants (weeds) in South Africa lists *M. tonkineus* as a 'not released biological control agent' for *G. triacanthos,* with an undetermined degree of control. In 2017 it was decided to remove *M. tonkineus* from the 2021 edition of the catalogue as its host range was undetermined. This study has shown *M. tonkineus* has a narrow host range in South Africa, limited to *G. triacanthos* and is therefore not a threat to native plant species (Chapter 3). This finding was important as having insects considered a biological control agent without knowing their host range goes against standard biological control best practice and could potentially have direct implications for the science of weed biocontrol who relies heavily on its safety record. The insect could now potentially be re-considered a biological control agent in South Africa.

This study has also contributed to the 'damage inflicted' by the species in South Africa. Klein (2011) originally listed the damage as 'undetermined'. This study, which found that *M. tonkineus* is distributed across the invaded range of *G. triacanthos* in South Africa and showed that the mean damage caused by *M tonkineus* is only 9 % (n=60 sites). By the standards of the catalogue this would be regarded as negligible damage, and that the seed beetle is not causing enough damage to reduce the rapidly spreading population of *G. triacanthos* in the country. As this study was conducted over a single year only, there is a chance that the level of damage may increase as the seed beetle may not have yet reached its potential, or might have simply have had a year of low damage for some or other biotic or abiotic reasons. It is well known that the interaction of these abiotic factors play an important role in the abundance of insect populations (Hoffmann *et al.,* 2010; Savopoulou-Soultani *et al.,* 2012; Naithani *et al.,* 2014).

The climate experienced by both weeds and their natural enemies affect the rates of growth and development, uptake or ingestion of nutrients, rate of reproduction, and, therefore, the time taken for agent populations to build up and therefore reduce the population of the weed (Julien *et al.,* 1996; Coetzee *et al.,* 2007). In Europe where the insect has been for at least two decades *M. tonkineus* damages between 0.0% and 9.15% (Ferus *et al.*, 2013; György & Germann, 2012). The low impact of *M. tonkineus* may be because of the more temperate climate of Europe compared to the insect's native range. In the warmer dryer climates of Argentina, the seed beetle has been shown to damage up to 94.00% of seeds (Di Iorio, 2005, 2015). South Africa climate is more similar to the Argentina suggesting that if climate is currently limiting the damage in Europe high levels of damage similar to Argentina should be attainable in South Africa.

An option for increasing the number of seeds being damaged in South Africa could be to mass rear the agent in insect mass-rearing facilities. Weed biological control mass-rearing facilities in South Africa are non-commercial facilities, established to mass produce high numbers of healthy biological control agents for release against IAPs (Martin *et al.,* 2018). These facilities could be used to mass produce *M. tonkineus* in order to make augmentative releases to increase the abundance of the beetle in the country. Rearing the seed beetle in the laboratory for use in host specificity testing was straightforward, suggesting that mass rearing could be used to facilitate insect numbers in South Africa.

Additional seed-feeding agents could be considered for release in South Africa. As mentioned previously there is *M. dorsalis* another Asian seed-feeding insect that damages the seeds of *G. triacanthos* (Herford, 1935). This agent has also been recorded in Europe damaging the seeds of *G. triacanthos.* This agent could provide another biological control option depending in host-specificity trials. In addition the CBC at Rhodes University is also currently considering the seed-feeding weevil, *Amblycerus robiniae* (Fabricius, 1781)

(Chrysomelidae: Bruchinae), as a candidate agent for *G. triacanthos*. *Amblycerus robiniae* is a very common bruchid in the plant's native range in the USA (Mathwig, 1971) where it has been recorded as damaging seeds of *Gleditsia* spp. (Rădac *et al.,* 2021) only, including *Gleditsia aquatic* (Marshall) Rasf. (Kingsolver, 2004). The bruchid is also established in Europe, where the first record of *A. robiniae* was 1986 in Hungary; it has been suggested that it emerged from *Gleditsia triacanthos* var. *inermis* seeds (Merkl, 2001; Rădac *et al.,* 2021). Host-specificity trials for *A. robiniae* will be conducted in quarantine conditions in South Africa to determine the specificity of the weevil; if proved safe, the weevil could be released in the country. *Amblycerus robiniae* might be a good agent in the colder regions of South Africa because it survives cold temperatures in the USA (Mathwig, 1971).

The introduction of multiple agents in programmes to control weeds biologically has often been debated (Jackson & Myers, 2008), the charge being that agents might compete for resources, resulting in less effective control (Myers & Bazely, 2003; Myers, 2008). Crower and Boucheir (2006) showed that when the gall fly, *Urophora affinis* Frauenfeld (Diptera: Tephritidae), and the weevil, *Larinus minutus* Gyllenhal (Coleoptera: Curculionidae), were released onto spotted knapweed, *Centaurea stoebe micranthos* L. (Asteraceae), in various combinations in southern British Columbia, Canada, the agents competed with each other and there was less impact on plant growth than when the agents acted alone. Whether releasing three seed feeding agents will increase or decrease the level of damges in undetermined. However investigations in Europe where all thee agent have been unintentionally released might provide some additional information.

In a recent study in Romania, Rădac *et al.,* (2021) focused on *A. robiniae* and sampled seeds of *G. triacanthos* to determine if the bruchid was established in the country. The researchers sampled 60 sites during 2018, and obtained individuals of *A. robiniae*. The results of the study suggested that the presence of *Megabruchidius* sp. reduced the abundance of adults of *A. robiniae* because the localities with more abundance of *M. tonkineus* and *M. dorsalis* had lower numbers of *A. robiniae*. The authors suggested that the lower abundance of *A. robiniae* may be because the larval food was reduced by the occurrence of *Megabruchidius* species (Rădac *et al.,* 2021). Whether this would occur in South Africa remains to be seen and whether that will result in less damage is also uncertain. Such competition might not occur as, currently, it seems that *M. tonkineus* is less abundant in South Africa than in Europe, and *M. dorsalis* had not yet been reported in the country. However, if *M. dorsalis* is shown to be suitably specific to *G. triacanthos* in South Africa, it could prove to be a better biological control agent than the associated seed predator *A. robiniae.* It has also been shown that the two Asian beetles might work well together (Rădac *et al.,* 2021).

The recent success of acacia control in South Africa (Impson *et al.,* 2021) once again shows that agents which, directly or indirectly, suppress seed-production should be fundamental in any long-term management strategy for invasive plants (Impson *et al.,* 2021). The exact combination of which biocontrol agents will destroy the highest number of available seeds is yet to be determined, as discussed above, but there are additional options to reduce the number of seeds being produced. *Acacia mearnsii* De Wild (Fabaceae) is an invasive alien plant in South Africa whose production of high numbers of long-lasting seeds drives its spread. Owing to a conflict of interest with the timber industry, only biological control agents which reduced the reproductive output of the species could be considered. Two agents have been released: the seed-feeding weevil, *Melanterius maculatus* (Curculionidae), and a flower-galling midge, *Dasineura rubiformis* (Cecidomyiidae). On their own, the weevils could not cause a significant decline in the density or distribution of mature populations of the target plants (Impson *et al.,* 2019); however, the release of the flower-galling midge, *Dasineura rubiform* (Cecidomyiidae), has seen seed production decline to extremely low levels (Impson *et al.,* 2021). A similar example could be used for *G. triacanthos*. Like *A. mernsii*, *G. triacanthos* has a pod-gall midge associated with it, *Dasineura gleditchiae* (Osten Sacken, 1866) (Diptera: Cecidomyiidae). The combination of the species might provide higher levels of damage than just the seed feeding beetles. The midge has been described as monophagous and cause damage to the plant in its native range (Cranshaw, 2003). Like the seed-feeding weevil, the pod-galling midge was inadvertently introduced into a number of

European countries, where it is regarded as a pest of *G. triacanthos* since severe infestations can cause growth reduction, twig death, and partial defoliation.

Unlike the examples of *Acacia* spp. in South Africa, where biological control was limited to seed-reducing biological control agents because of conflicts of interest there is no conflict associated with *G. triacanthos*. Therefore other insects such as the apple wood strainer, *Monarthrum mali* Wood & Bright (Coleoptera: Curculionidae) could also be considered as biological control.

4.4. Genetic populations in South Africa

Even though *M. tonkineus* was found throughout the invaded range in South Africa, it is not known if its performance differed on certain populations for any specific biotic or abiotic reason. For example it is not known why 14 sites had no insect damage. Currently, in South Africa it is unknown whether different varieties of *G. triacanthos* are present, nor we do know the origin or origins of introduction of the tree into South Africa. During field work, it was evident that there was a difference in size and colour of the pods, and the number of seeds per pod varied from site to site which might suggest some differences within the population. In addition in her handbook, Henderson, (2001) mentions that sterile cultivars of *G. triacanthos* are excluded from the legislation of Category 1 NEMBA – Category 1b, implying that there is more than one cultivar of *G. triacanthos* in South Africa. The genetic differences of plant species can affect the performance of the biological control agent; a well-known example of the performance of biological control agents depending of the different genetic of the plant species is *S. terbinthifolia*. This plant is regarded as an invasive alien plant species, found in the USA, South Africa and other countries. The Brazilian peppertree has two different haplotypes (A and B) introduced in Florida but several haplotypes in the native range, and several studies have showed that the natural enemies perform differently on the different haplotypes of the tree (Hight *et al.,* 2002; Wheeler *et al.,* 2016; Cuda *et al.,* 2019). As mentioned in Chapter 3 genetic samples were taken from all sampled locations however they are yet to be assessed. Determining the population structure and whether different biotypes or hybrids are present in South Africa would greatly assist in developing effective management structures should differences be found

In conclusion, *Gleditsia triacanthos* cannot be left unmanaged. This study shows that large parts of South Africa are climatically suitable for *G. triacanthos*, and therefore the weed will continue to spread. Integrated chemical and mechanical management should be implemented until suitable biological control options are available. It is hope biological control will assist in the management of the species.

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Appendix

Table S1.1. Percentage of damage of *Megabruchidius tonkineus* on *Gleditsia triacanthos* taken from 20 pods from each sampled site. Damaged seeds are the seeds with presence of exit holes, deformed seeds are abnormal seeds and healthy seeds are viable seeds.

Latitude	Longitude	Species	Damaged	Deformed	Healthy
-32.1155	27.1115	*G. triacanthos*	17.26	2.61	80.13
-31.5681	26.9455	*G. triacanthos*	0.85	4.70	94.44
-30.7259	26.8238	*G. triacanthos*	11.22	6.12	82.65
-30.6814	26.7034	*G. triacanthos*	33.33	1.34	65.32
-30.3238	25.6562	*G. triacanthos*	0.55	2.20	97.25
-30.029	31.55	*G. triacanthos*	0.41	36.18	63.41
-29.7528	27.0325	*G. triacanthos*	0.00	9.73	90.27
-29.5797	27.0422	*G. triacanthos*	6.67	2.96	90.37
-29.1512	27.4941	*G. triacanthos*	11.16	2.89	85.95
-28.7973	27.5156	*G. triacanthos*	2.07	36.51	61.41
-28.5365	28.5211	*G. triacanthos*	0.00	11.23	88.77
-28.5366	28.5211	*G. triacanthos*	3.23	9.03	87.74
-28.5354	28.5193	*G. triacanthos*	7.17	5.28	87.55
-28.5504	28.4229	*G. triacanthos*	0.00	10.95	89.05
-28.5472	28.4201	*G. triacanthos*	6.11	9.92	83.97
-29.8166	29.7692	*G. triacanthos*	16.55	9.80	73.65
-29.6837	30.0342	*G. triacanthos*	0.00	86.42	13.58
-29.9759	30.7018	*G. triacanthos*	21.63	11.02	67.35
-28.2588	28.3167	*G. triacanthos*	10.66	4.10	85.25
-28.2426	28.2815	*G. triacanthos*	6.43	1.79	91.79
-29.6831	30.4761	*G. triacanthos*	0.00	9.09	90.91
-26.7185	27.1111	*G. triacanthos*	12.25	14.81	72.93
-32.5637	27.4881	*G. triacanthos*	13.39	6.28	80.33
-29.2567	27.3243	*G. triacanthos*	37.87	1.78	60.36
-28.3571	29.0628	*G. triacanthos*	0.00	19.92	80.08
-29.9791	27.029	*G. triacanthos*	9.35	11.94	78.71

-27.7428	28.54	*G. triacanthos*	20.25	1.23	78.53
-30.521	26.7968	*G. triacanthos*	11.99	3.51	84.50
-31.9379	26.8266	*G. triacanthos*	17.97	2.29	79.74
-29.965	27.031	*G. triacanthos*	0.00	4.35	95.65
-28.8161	27.9763	*G. triacanthos*	31.83	6.23	61.94
-29.105	26.1986	*G. triacanthos*	22.10	3.75	74.16
-28.0807	28.3574	*G. triacanthos*	7.69	5.13	87.18
-28.8912	27.7245	*G. triacanthos*	0.00	2.54	97.46
-28.5163	28.4167	*G. triacanthos*	3.11	8.89	88.00
-29.9679	27.5594	*G. triacanthos*	4.87	9.42	85.71
-30.6406	26.7414	*G. triacanthos*	7.09	0.79	92.13
-30.3222	26.987	*G. triacanthos*	1.18	5.33	93.49
-27.9212	25.1558	*G. triacanthos*	3.27	8.33	88.39
-27.7428	28.4426	*G. triacanthos*	3.76	4.51	91.73
-28.3089	28.4822	*G. triacanthos*	0.00	7.04	92.96
-28.5518	28.4227	*G. triacanthos*	0.00	15.43	84.57
-30.0261	27.0498	*G. triacanthos*	4.79	0.53	94.68
-30.0817	27.0799	*G. triacanthos*	15.26	6.02	78.71
-30.7378	26.7151	*G. triacanthos*	0.83	9.96	89.21
-28.6866	28.1456	*G. triacanthos*	18.10	1.29	80.60
-30.5934	26.7551	*G. triacanthos*	4.85	2.27	92.88
-28.2039	26.1115	*G. triacanthos*	1.15	34.23	64.62
-28.1485	26.0677	*G. triacanthos*	22.80	18.00	59.20
-30.0811	27.0794	*G. triacanthos*	0.00	36.18	63.82
-29.0073	26.2079	*G. triacanthos*	6.30	16.54	77.17
-28.8952	27.6792	*G. triacanthos*	26.77	13.85	59.38
-28.012	25.9873	*G. triacanthos*	29.00	6.49	64.50
-28.6272	28.2249	*G. triacanthos*	0.00	7.80	92.20
-30.0155	27.0476	*G. triacanthos*	26.56	12.03	61.41
-28.5363	28.5206	*G. triacanthos*	0.00	11.59	88.41
-28.1186	25.5172	*G. triacanthos*	6.96	16.52	76.52
-28.8071	29.5312	*G. triacanthos*	14.34	17.36	68.30
-30.738	26.715	*G. triacanthos*	9.05	10.48	80.48
-30.1786	27.0845	*G. triacanthos*	0.00	4.90	95.10