Rural R&D for Profit Program

New biocontrol solution for sustainable management of weed impacts to agricultural profitability

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AgriFutures®
Biocontrol of Weeds

New biocontrol solution for sustainable management of weed **impacts to agricultural profitability.**

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Rural R&D for Profit program final report: New biocontrol solution for sustainable management of weed impacts to agricultural profitability

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AgriFutures Australia is the new trading name for Rural Industries Research & Development Corporation (RIRDC), a statutory authority of the Federal Government established by the Primary Industries Research and Development Act 1989.

Summary

This project aimed to develop biocontrol agents for the control of ten weeds of importance in Australia. Five of these weeds are Weeds of National Significance (WoNS): cabomba, Sagittaria, prickly acacia, silverleaf nightshade and African boxthorn. Fleabane and sowthistle have become major weeds of cropping land while mother-of-millions and giant rat's tail grass impact on grazing land. The final weed, ox-eye daisy is becoming a serious environmental weed in crown land.

Biocontrol agents, when released and established in the Australian environment, will benefit primary producers through the general landscape level reduction in weed pressures on rangelands, croplands and water assets, thereby enabling better integrated weed management outcomes. Farmers directly affected by the weeds targeted with these new biocontrol agents will see a gradual reduction in their control costs, as the released agents build-up their populations and cause increasing damage on the weeds.

The methods used combined

- Stakeholder engagement to identify management goals for the targeted weeds and opportunities for integrated weed management,
- Literature reviews to identify prospective biocontrol agents,
- Molecular characterisation of the weeds and bioclimatic models to select most appropriate region(s) to survey in the native range,
- Native range field surveys to characterise the diversity of pathogens and insects that may be potential agents and prioritised their further study,
- Investigations on the biology and host range of prioritised agents in the native range and in quarantine facilities in Australia to determine if they are safe for release into the Australian environment.

The project identified the following potential biocontrol agents

- **African boxthorn** (*Lycium ferocissimum*): the rust, (*Puccinia rapipes*) two leaf-chewing beetles, (*Cassida distinguenda* and *Cleta eckloni*) and a leaf-mining weevil (*Neoplatygaster serietuberculata*).
- **Cabomba** (*Cabomba caroliniana*): the cabomba weevil (*Hydrotimetes natans.*).
- **Fleabane** (*Conyza bonariensis*):- the rust (*Puccinia cnici-oleraceil)* and a stem gall forming tephritid fly.
- **Ox-eye daisy** (*Leucanthemum vulgare*):-a rhizome-feeding moth (*Dichrorampha aeratana*) and a root-feeding beetle (*Cyphocleonus trisulcatus*).
- **Silverleaf nightshade** (*Solanum elaeagnifolium*):- a tingid (*Gargaphia arizonica*) and a, mite (*Aceria* sp.).
- **Sagittaria** (*Sagittaria platyphylla* and *S. calycina*):- a fruit-feeding weevil (*Listronotus. appendiculatus*), a crown-boring weevil (*L. sordidus*) and the tuber-feeding weevil (*L. frontalis*)
- **Giant rat's tail grass** (*Sporobolus pyramidalis* and *S. natalens*): - the fungus *Ustilago sporoboli-indici* and a wasp (*Tetramesa* sp.).
- **Prickly acacia** (- Gall thrips (*Acaciothrips ebneri*), a gall mite *Aceria* sp.) and a gall fly (*Notomma mutilum*).

Despite extensive surveys and testing, no suitable agents for sowthistle or mother-of-millions were identified during this project, due to lack of host specificity.

Applications have been submitted or are in preparation to release for agents for Sagittaria, cabomba, ox-eye daisy, African boxthorn, fleabane and prickly acacia

These agents will be imported into Australian quarantine in 2020.

Further host specificity testing both in Australia and at overseas facilities will continue as part of Round 4 Rural R&D for Profit program of the Australian Government. Specifically, this will involve agents for African boxthorn, giant rat's tail grass, prickly acacia, fleabane and silverleaf nightshade.

This project brought together a network of international collaborators spanning Australia, Europe, Africa, South America, Asia and North America

As well there were significant resources committed from industry project partners both financially and in-kind. The contributors to each weed project are as follows

- African boxthorn: Primary Industries Research South Australia (PIRSA), and rangelands and pastoral stakeholders and land managers, Ravensthorpe Shire,
- Cabomba: SEQwater, Sun Water and other rural water asset managers,
- Fleabane: Grains Research and Development Corporation(GRDC)
- Sowthistle Grains Research and Development Corporation
- Mother-of-millions: Northwest LLS, QDAF, NSW DPI
- Ox-eye daisy: NSW Biocontrol Taskforce, NSW DPI
- Giant rat's tail grasses: (QDAF, NSWDPI NSW Weed Biocontrol Taskforce (via Rous County Council), Bundaberg Regional Council, Gladstone Regional Council and HQPlantations Pty Ltd.)
- Sagittaria: Goulburn Murray Water, Murrumbidgee Irrigation, Coleambally Irrigation, Goulburn Broken CMA, NQ dry tropics, Central Coast Council, Central Murray Council NSW Office of Environment and Heritage, Wyong Shire, Murray LLS
- Silverleaf nightshade: PIRSA, GRDC, Bland Shire Council, NSW DPI, Murrumbidgee Landcare
- Prickly acacia. (*Vachellia nilotica*).

These established partnerships will facilitate widespread adoption of the findings of this project both within and between industry sectors and between agricultural and environmental stakeholders.

Abbreviations and Glossary

ARC, PHC	Agricultural Research Council, Plant Health and Protection (Pretoria, South Africa)
BRI	Queensland Herbarium
BRIP	Queensland Plant Pathology Herbarium
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DAWE	Department of Agriculture, Water and the Environment (Australian Government)
DEDJTR	Department of Economic Development Jobs Training and Resources (Victoria)
DJPR	Department of Jobs Precincts and Region, (Victoria)
ESP	Eco Sciences Precinct
GPG	Giant Parramatta Grass refers to *Sporobolus fertilis*
GRT	Giant Rat's Tail Grass refers to *Sporobolus natalensis* and *Sporobolus pyramidalis*
NCAR	National Centre for Agronomic Research (Senegal)
NSW DPI	New South Wales Department of Primary Industries
OAI	Orange Agricultural Research Institute
PCR	Polymerase chain reaction
QDAF	Queensland Department of Agriculture and Fisheries
RIRDC	Rural Industries Research and Development Corporation
UoA	University of Anatananarivo
WoNS	Weed of National Significance
WSG	Weedy *Sporobolus* Grasses

Contents

Section 1 Project rationale and objectives

The organisations involved in the delivery of this project were CSIRO, Department of Jobs Precincts and Regions (DJPR), Victoria (formerly Department of Economic Development of Jobs Training and Resources – DEDJTR), NSW Department of Primary Industries (NSW DPI) and Queensland Department of Agriculture and Fisheries (QDAF).

Managing threats from weeds to soil, water and natural resources is a key challenge to Australia's agricultural sector. Weeds impact vast areas of agricultural and pastoral lands and their significant impacts conservatively cost Australia in excess of $6 billion/year. Aquatic and riparian weeds significantly affect the flow and quality of water that is an important component of irrigated agriculture. Biocontrol is the most cost-effective solution for landscape scale management of these weeds, with historical benefits outweighing R&D costs by over 23:1. It is a sustainable approach that requires little further investment once biocontrol agents are established, thus enhancing Australia's agricultural competitiveness.
The project was undertaken to improve the long-term profitability of primary producers by developing novel biocontrol solutions that will reduce recurrent costs of control for farmers affected by the targeted weeds. These focal weed species were identified through consultations with agricultural and livestock stakeholders and water asset managers.

The weeds targeted in this project are of importance to many different agricultural sectors, including small industries, in Australia. The significance and rationale for the selection of these weeds are detailed below.

1.1 African Boxthorn *(Lycium ferocissimum)*

African boxthorn is a Weed of National Significance (WoNS; Figure 1). It is regarded as one of the worst weeds in Australia because of its invasiveness, potential for spread, and economic and environmental impacts.

African boxthorn can spread quickly if left unchecked. Having established, it can rapidly form impenetrable, spiny thickets reducing stock movement and land available for pasture. Since birds are often the dispersing agent, infestations are commonly found around the base of taller trees. Dense infestations may provide a haven for feral animals such as rabbits and sparrows.

Figure 1. *Lycium ferocissimum* (African boxthorn)

The fruit of African boxthorn is a breeding ground for insect pests such as fruit flies major horticultural pests that impact yields and market access for Australian growers.

In Australia, *L. ferocissimum* is widespread in coastal to semi-arid inland habitats and islands of southern Australia, with records from every jurisdiction (GBIF.org, 24th July 2018; Figure 2). It is found predominantly in the southern part of the Australian continent in coastal and island situations (except Queensland). Inland, *L. ferocissimum* is abundant in areas of New South Wales, Victoria and South Australia, where it is a common weed of semi-arid pastures and rangelands and is often found growing along dry stream beds. It has a lesser, but significant presence in south-east Queensland, southern Western Australia, and Tasmania.

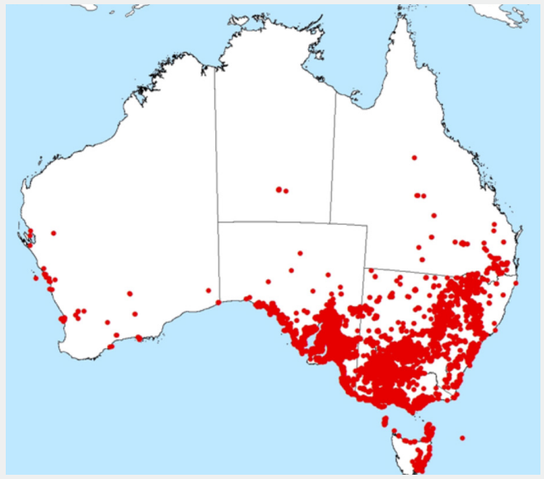

Figure 2 . Current distribution *Lycium ferocissimum* (African boxthorn) in Australia (GBIF.org 24th July 2018c).

1.2 Cabomba *(Cabomba caroliniana)*

Cabomba (Figure 3) is a Weed of National Significance. It is regarded as one of the worst aquatic weeds in Australia because of its invasiveness, potential for spread, and economic and environmental impacts. It is choking waterways along Australia's east coast. Cabomba grows quickly and produces a large amount of plant material. It can significantly reduce water storage capacity and taint drinking water supplies. Water treatment costs can be increased by up to $50 a megalitre. Heavy infestations can also raise water levels to a point where overflows and heavy seepage losses occur. It is extremely persistent and can take over a water body, excluding native plant species. Cabomba's dense mass of underwater stems and leaves provide a hazard for recreational water users. When this vegetation dies off, decomposition causes dramatic oxygen reductions and foul-smelling water.

In Australia, most *C. caroliniana* infestations occur in southern Queensland and the northern New South Wales hinterland. In Queensland it occurs in shallow, permanently flowing creeks and deep, slow-flowing pools of coastal river systems. There are smaller infestations found in Victoria and the Northern Territory. *Cabomba caroliniana* infestations have not yet been found in WA, SA, TAS or the ACT (Figure 4).

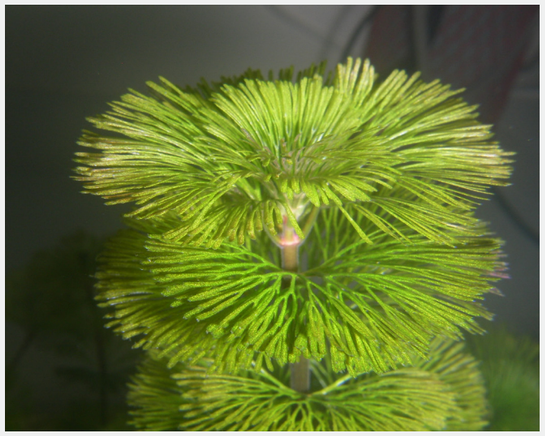

Figure 3. Cabomba caroliniana

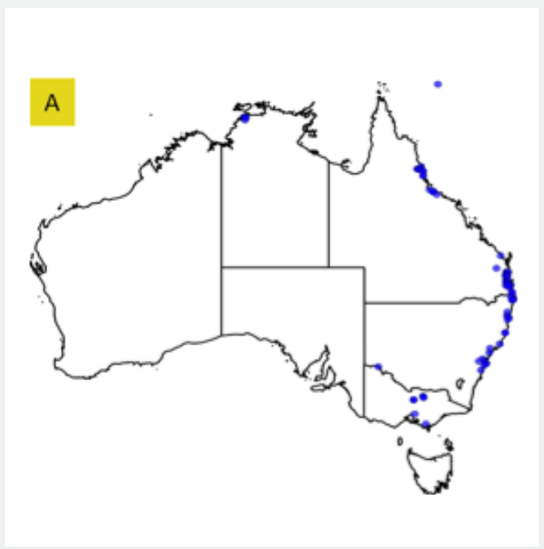

Figure 4. Current distribution of *Cabomba caroliniana* in Australia (source: Atlas of Living Australia)

1.3 Fleabane *(Conyza bonariensis)*

Fleabane (Figure 5) has been present for a long time in Australia but it has only become a widespread weed of cropping systems in recent years due to the development of herbicide resistance. Fleabane is a small seeded weed that requires several days of moist soil on the surface to germinate. Therefore, it is favoured by no-till, stubble-retention farming systems. It germinates primarily in spring, but if water is available, it can germinate through summer into early autumn provided temperatures are not too hot.

Figure 5. *Conyza bonariensis* (flaxleaf fleabane)

Many fleabane populations in Australia are resistant to glyphosate, which makes them extremely difficult to control in the summer fallow period.

Fleabane is present in all states of Australia, occurring predominantly in temperate and Mediterranean coastal regions, and with restricted distributions in semi-arid to arid central regions (GBIF.org 2nd November 2018) (Figure 6).

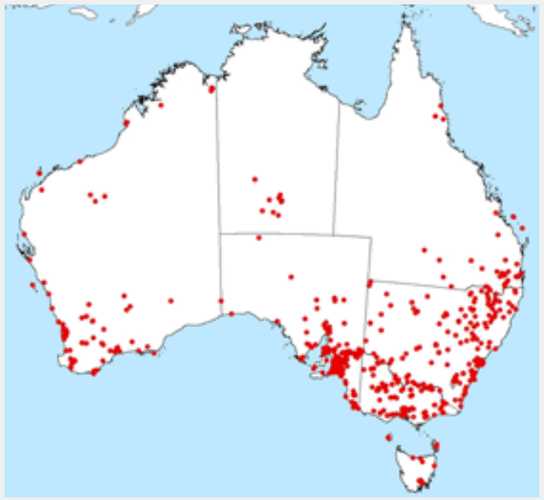

Figure 6. Current distribution *Conyza bonariensis* (flaxleaf fleabane) in Australia (GBIF.org 24th July 2018c).

1.4 Sow thistle *(Sonchus oleraceus)*

Sowthistle (Figure 7) is a widespread weed of grain crops and cotton that has developed extensive herbicide resistance in recent years making it extremely difficult to manage with currently available methods. It has flourished in no till situations and is able to germinate and set seed year round. Sowthistle can produce up to 25,000 seeds per plant with the seed being readily dispersed by wind.

In Australia, *S. oleraceus* is widespread and found in all States and Territories but appears to be most prevalent in the southern half of the continent. There are more than 33,000 records of *S. oleraceus* in the Atlas of Living Australia (ALA 2017; Figure 8).

Figure 7. *Sonchus oleraceus* (sow thistle)

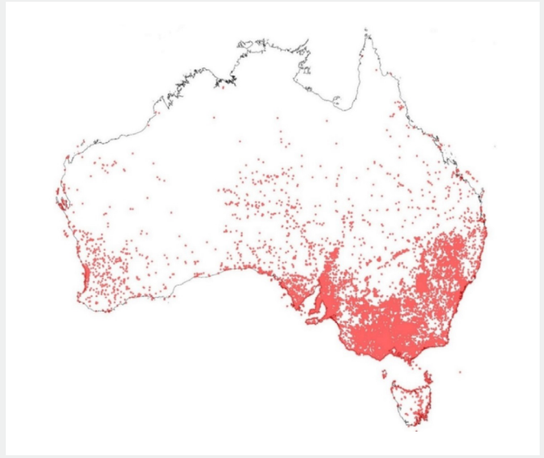

Figure 8. Occurrence records of *Sonchus oleraceus* (common sowthistle) in Australia (ALA 2020).

1.5 Mother-of-millions *(Kalanchoe delagoensis)*

Mother-of-millions (Figure 9), a Madagascan endemic, is a major weed in Queensland, New South Wales, Victoria, South Australia, Western Australia and Norfolk Island, Australia. Three species of *Bryophyllum* are invasive in Australia: Mother-of-millions (*K. delagoensis*), hybrid mother-of-millions (*K. daigremontianum* x *K. delagoensis)* and resurrection plant (*K. pinnatum*).

As its common name suggests, mother-of-millions (MoM) produces hundreds of tiny plantlets which quickly form new colonies. It was introduced into Australia in the 1940s and has now invaded thousands of hectares of grazing land. Figure 10 shows the distribution of mother-of-millions in Australia.

Figure 9. *Kalanchoe delagoensis* (mother-of-millions)

Figure 10. Current distribution of *Kalanchoe delagoensis* in Australia (Atlas of Living Australia)

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Mother-of-millions is adapted to dry conditions and can survive long periods of drought. This increases the plant's potential to persist and spread. Seed production is prolific, with each inflorescence able to produce about 20,000 seeds. However, the species is best known for its vegetative reproduction. Six to eight daughter plantlets are produced at the terminal ends of each phyllode.

Mother-of-millions is toxic when ingested by livestock. It is also poisonous to humans and household pets. The toxic effects of these plants are due mainly to bufadienolides (a type of cardiac glycoside) which cause heart failure. The toxins are present in all parts of the plant.

Established infestations are difficult and expensive to eradicate mechanically or chemically. Infestations can increase by up to 20-30% per year if left uncontrolled. For control of large infestations, the integrated use of herbicides, grazing management and fire can be effective. However, continued use of herbicides and fire can bring about deleterious changes to the composition of native vegetation.

This project aimed to improve available control options for farmers by exploring option of biocontrol. The project built on from an earlier biocontrol program which identified three Madagascan insect species as holding potential in this regard: the stem-boring weevil *Ospihilia tenuipes* (Coleoptera: Curculionidae), the phytophagous wasp *Eurytoma bryophylli* (Hymenoptera: Vespidae) and the phyllode- and bulbil-feeding beetle *Rhembastus* sp. (Coleoptera: Chrysomelidae).

1.6 Ox-eye daisy (*Leucanthemum vulgare*)

Ox-eye daisy (Figure 11) is a serious environmental weed in Australia, with the potential to become a problem for primary producers (as observed in America and Canada). Biological control in environmentally sensitive areas (containing threatened species) where herbicides cannot be readily sprayed will be an important future management strategy.

Ox-eye daisy is a rhizomatous perennial herb, native to Europe that has become an invader in over 40 countries (including Australia and New Zealand). Seed longevity is high and up to 80% of propagules are viable for six years. The weed is not palatable to cattle and affects pastoral lands by reducing carrying capacity. Dense infestations exclude other plant species, leading to soil erosion and depletion of soil organic matter.

Figure 11. *Leucanthemum vulgare* (ox-eye daisy)

Ox-eye daisy is invasive in Victoria (where it is a declared noxious weed), New South Wales (where one of the more alarming infestations is in Kosciuszko National Park), South Australia, ACT and Tasmania. This weed species thrives in disturbed areas, however, of greatest concern is its ability to aggressively invade areas of conservation importance.

While mechanical and chemical control can be implemented to manage localised infestations of ox-eye daisy, there is an urgent need for the sustainable management of this invasive plant at the landscape level, especially in conservation areas.

In 2008, a programme was initiated to investigate the prospects for the biological control of ox-eye daisy in North America. Over the last twelve years CABI Switzerland have identified and studied a suite of promising biological control agents including a root-feeding moth, *Dichrorampha aeratana* Pierce & Metcalfe (Lepidoptera: Tortricidae), a root-feeding weevil *Cyphocleonus trisulcatus* Herbst (Coleoptera: Curculionidae) and a flower head-mining fly, *Tephritis neesii* Meigen (Tephritidae), among others. Of these, *D. aeratana* seems to hold the most immediate promise in terms of specificity and is being developed further as the first biological control agent for North America. In 2016, a programme to investigate prospects for the classical biological control of ox-eye daisy was initiated for Australia. Since then, extensive testing (in Australia and Switzerland) on key Australian native Asteraceae has demonstrated that *D. aeratana* looks very promising as a potential biocontrol agent for ox-eye daisy. The distribution of Ox-eye Daisy in Australia is shown in Figure 12.

Figure 12. Distribution of *Leucanthemum vulgare* in Australia.

1.7 Giant Rat's Tail Grass (*Sporobolus spp.*)

Sporobolus R.Br. (Poaceae) is a genus of almost 200 grass species from tropical and subtropical parts of the world, including Africa, temperate and tropical Asia, Australasia, North and South America (Clayton 1965, Simon and Jacobs 1999), http://www.theplantlist.org/tpl1.1/search?q=sporobolus). The distribution of sporobolus species in Australia is shown in Figure 13.

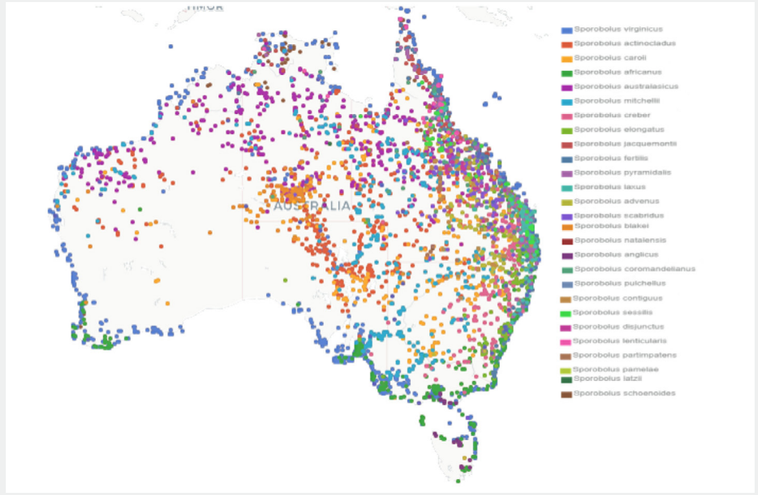


Figure 13. *Sporobolus* spp. distribution within Australia. Source Australia Virtual Herbarium 2020.

Giant rat's tail grass is the common name of two species, namely *Sporobolus pyramidalis* and *S. natalensis*. They were introduced into Australia through contaminated seed. *Sporobolus pyramidalis* is now found widespread from Cooktown in north Queensland south to the NSW Central Coast. *Sporobolus natalensis* is found widespread from Rockhampton in central Queensland to Port Macquarie on the mid north coast of NSW. Populations of both species are present in the Northern Territory (Figure 14). The importance of these species is reflected in both being Weeds of National Significance.

Current control efforts for giant rat's tail grasses centre on the use of chemical and mechanical control, plant competition and pasture management. Despite the production of a best practice manual for giant rat's tail grasses and the widespread use of the recommended control strategies, control has not been achieved and giant rat's tail grasses continue to spread into new In 2000, a biological control program was implemented by the Queensland Government to survey *S. pyramidalis*, *S. natalensis* and *S. africanus* in southern Africa for insects, mites and pathogens as potential biocontrol agents. The study identified only two agents showing promise; a leaf smut (*Ustilago sporoboli-indici*) and a wasp (*Tetramesa* sp.) (Palmer 2008). Unfortunately, the smut infects four native Australian species of *Sporobolus* and was rejected as a biological control agent. The wasp larvae feed in the stem of

Project rationale and objectives

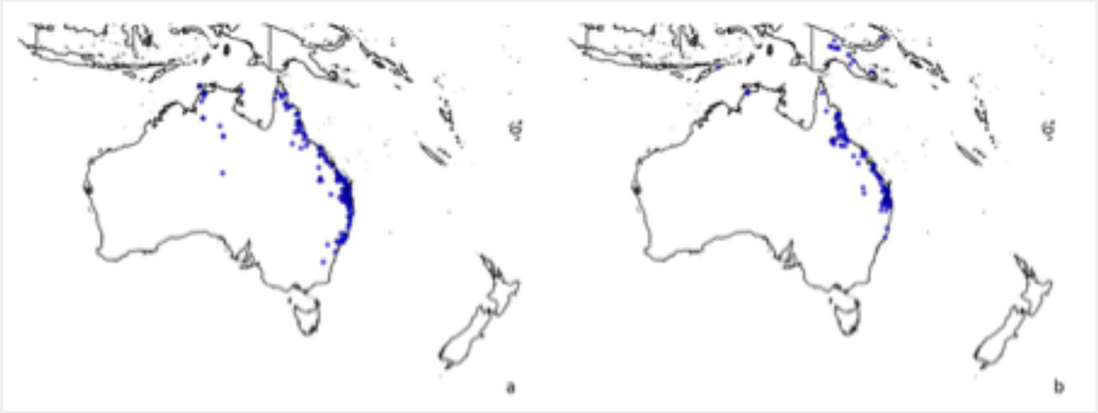


Figure 14. Map showing the distribution of *Sporobolus pyramidalis* (a) and *S. natalensis* (b) in Australia. Source: Atlas of Living Australia.

GRT resulting in malformation of the seed head flower. However all efforts to rear this species in the laboratory failed (an essential prerequisite for further study), hence work on this agent was discontinued in 2007. In more recent years an Australian strain of the crown rot (*Nigrospora oryzae*) has been considered as a biological control agent for GPG (Ramasamy *et al.* 2008). However, when tested on GRT it proved to be ineffective (Fletcher and Leemon 2015).

Exploring environments within Australia where both native and naturalised species within the *S. indicus* complex coexist could provide a nursery of pathogens capable of controlling GRT.

1.8 Silverleaf Nightshade (*Solanum elaeagnifolium* Cav.)

Silverleaf nightshade (Figure 15) is one of the world's most invasive alien plants and a Weed of National Significance in Australia (Australian Weeds Committee 2012; Knapp *et al.* 2017). It reduces productivity and profitability across the wheat-sheep agricultural zone of Australia Figure 16), infests over one million hectares in Australia, and costs farmers $70 million every year. Silverleaf nightshade is a summer growing perennial weed with a large root system. The root system may grow more than 3m deep and 10m or more across. Silverleaf nightshade has the ability to grow new stems from small root pieces. Controlling the shoots of silverleaf nightshade does not necessarily control the root system and control of the root system is necessary to achieve long-term control.

It grows in a wide variety of environments and presents wide phenotypic variations. The weed is native to the Americas and may have originated in North America (Boyd *et al.* 1984). In Argentina it is widely distributed throughout most of the country, from the Patagonian province of Río Negro (cold arid steppe) to the northern province of Salta (subtropical climate with seasonal rains).

The beetles *Leptinotarsa defecta* (Stal) and *L. texana* Schaeffer (Coleoptera: Chrysomelidae), and the moth *Frumenta* spp. were introduced as biocontrol agents in South Africa, with different levels of establishment (Winston *et al.* 2014). In Australia, the nematode *Ditylenchus phyllobius* (Thorne) was imported into quarantine and tested but was not considered specific enough for release (Field, Kwong & Sagliocco 2009).

Figure 15. *Solanum elaeagnifolium* (silverleaf nightshade)

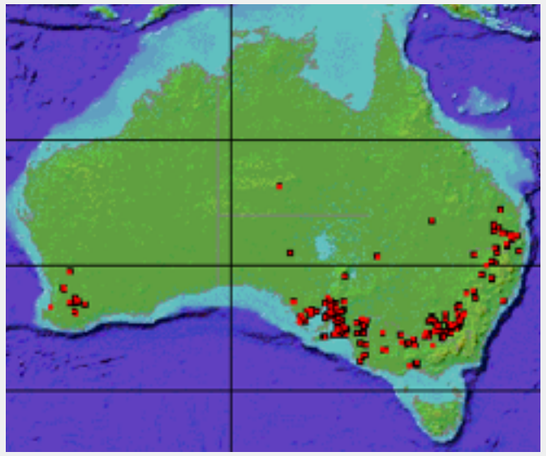


Figure 16. Distribution of *Solanum elaeagnifolium* (silverleaf nightshade) in Australia

Leptinotarsa texana also underwent extensive host-specificity testing in Australian quarantine but was rejected when it utilised Australian native *Solanum* and certain potato *S. tuberosum* cultivars in quarantine cage experiments (Lefoe *et al.* 2020). Effective and host-specific biocontrol agents are still sought for Australian conditions, prompting renewed survey efforts in parts of Argentina and USA through this Rural RnD4P Round 2 sub-project (See 3.2.1).

The key objective of the Silverleaf Nightshade sub-project was to undertake native range surveys in areas where climatic, genetic and other factors maximised the likelihood of collecting and testing agents suitable for introduction to Australia.

1.9 Sagittaria (*Sagittaria platyphylla and Sagittaria calycina*)

Sagittaria platyphylla (*Sagittaria platyphylla* (Engelmann) J.G.Smith) and *Sagittaria calycina* (Engelmann) (Alismataceae) (Figure 17) are emergent aquatic herbs native to north America that have become serious weeds of shallow ephemeral or permanent water bodies, in natural and ruderal habitats. In Australia, *S. platyphylla* extends from the tropical (Townsville) to the temperate regions of New South Wales, Australian Capital Territory, Victoria, South Australia and Western Australia. It is a serious invader of irrigation channels and drains in south-eastern Australia, forming dense monocultures that impede water flow, increasing risk of flooding and damaging irrigation infrastructure. In natural waterways, extensive infestations threaten native biodiversity and potentially impede the movement of native fish *Sagittaria calycina* is much less widespread than *S. platyphylla* and is currently only present in NSW where it is a major crop competitor in rice crops of the Murrumbidgee and Coleambally irrigation areas, causing yield reductions of up to 75%, increased production costs and reductions in rice quality.

Sagittaria platyphylla and *S. calycina* were declared targets for biological control in Australia in November 2015 after an in-depth biogeographical study on the genetic, demographic and herbivory differences between native USA and invasive Australian populations concluded that the prospects for successful biological control were high (Kwong, 2016).

Figure 17. *Sagittaria calycina*

Few effective options are available for the management of *S. platyphylla* and *S. calycina*, particularly in sensitive aquatic habitats or where off-target damage to horticultural and rice crops is a concern.

Surveys for natural enemies in the southern USA conducted between 2010 and recorded 32 arthropod and 29 fungal taxa. The most common and abundant insect species encountered was the fruit, flower and petiole-feeding weevil, *Listronotus appendiculatus* (Boheman) (Coleoptera: Curculionidae) which was collected at 74% of sites. Two further weevils, *Listronotus sordidus* (Gyllenhal) and *Listronotus frontalis* LeConte were identified as promising candidates due to the damaged caused to plant crowns, roots and tubers.

The key objective of the AgriFutures-led RnD4P Round 2 Sagittaria sub-project was to undertake host specificity testing of three candidate agents, the fruit-feeding weevil (*L. appendiculatus*), the crown-boring weevil (*L. sordidus*) and the tuber-feeding weevil (*L. frontalis)* to assess their safety for release into Australia.

1.10 1.10 Prickly acacia (*Vachellia nilotica ssp. indica*)

Prickly Acacia (Figure 18) is a Weed of National Significance. It infests over six million hectares of natural grasslands and over 2,000 km of bore drains in Queensland, with potential to spread throughout northern Australia (Figure 19). Prickly acacia trees form dense impenetrable thickets, restricting stock access to watercourses, compete with native pasture species, and prevent growth of plants beneath the canopy. Prickly acacia costs primary producers about AUD $9 million per year in lost pasture production.

Biological control efforts so far have focused on agents from Pakistan, Kenya, South Africa and India, with limited success to date. Hence, the search for new agents, focussing on gall-inducers, was redirected to Ethiopia and Senegal, based on plant genotype and climate matching.

Figure 18. Vachellia nilotica (Prickly acacia)

Figure 19. Australian distribution of prickly acacia

Section 2 Method and project locations

2.1 Methods

The methodology followed in this project was tailored to the situation of each target weed. In general, the following methodological steps were followed.

Step 1
Stakeholder engagement

The weeds in this project have been identified through consultation of primary industry and affiliated stakeholders, spanning sectors and states/territories. Early in the project, clear goals for the management of each target weed were developed in consultation with key stakeholders, to delineate a role for biocontrol in achieving these goals. The research carried out by the project teams was done within the context of expectations for biocontrol as part of integrated weed management strategies. This guided the search for biocontrol agents to deliver the required level of management, and the locations where releases of agents should ideally be made.

Step 2
Literature searches

Prior to field surveys for potential agents, the literature was searched extensively to gather information on the taxonomy of the target weed, its distribution and its known natural enemies. Knowledge of the evolutionary centre of origin and diversity of the weed helped plan subsequent field surveys as this is the area where natural enemies that have evolved to attack the target weed are more likely to be host-specific and most abundant. The centre of origin of the target weed species was sometimes inferred from botanical records obtained from various herbaria when the species distribution is limited to a single country or region. Insect collections, mycological herbaria and web-based databases, as well as the commonly available literature, were consulted to develop the list of natural enemies recorded on the target weed in both the putative centre of origin and invaded range. However, as many more natural enemies have been described worldwide from plants of economic importance than from those of no commercial interest, it was common to find some previously undescribed species on target weeds during field surveys in the native range.

Step 3
Surveys for candidate biocontrol agents

Molecular characterisation of the target weed using efficient cutting-edge technologies (e.g. genome-wide polymorphic molecular marker systems such as genotyping-by-sequencing) was used to determine the weed genetic structure which help identify the area(s) of the native range where the weed originates from. To further refine the area(s) to be surveyed, the species distribution modelling tools (e.g. CLIMEX Climatchand MAXENT) were used to characterise and compare the climate of the target weed's native and invaded ranges. By comparing meteorological data from the different regions, specific area(s) of the native range were identified where potentially best climatically suited candidate biocontrol agents may be found. The results from molecular characterisation of the weeds and bioclimatic models informed field surveys for candidate agents. These surveys involved spatially extensive and temporally intensive surveys between 2017 and 2020, resulting in a catalogue of candidate agents.

Step 4
Host-specificity testing of promising agents

Host-specificity testing is necessary to determine the potential range of plants (hosts) which will be attacked by the candidate agent in Australia. Research to develop rearing/propagation methods was undertaken prior to conducting host specificity testing. Experimental investigations were generally undertaken in quarantine facilities in Australia (including obtaining export and import permits from the relevant regulatory authorities in the native range and Australia), and in some cases were also performed in the field and collaborators' laboratories overseas. Testing methods were always tailored to the particular agent. Testing followed well established evolutionary and ecological understanding of host-range in plant pathogens and insect herbivores. It concentrated on closely related plant species to the target weed and extended out to plants of increasing phylogenetic distance to the focal weed, particularly on plants which occur in the same climatic and ecological zone as the target weed.

Method and project locations

Step 5

Prepare and submit application for release

Where required, the individual weed will be formally nominated through the Invasive Plant and Animal Committee (IPAC); replaced by the Environment and Invasives Committee (EIC), which assesses possible conflicts of interest before approving it as a biocontrol target. This step is a key regulatory requirement before the release of any agents. The regulatory timelines for Activity 5 are currently up to 2 years, and therefore it was not possible to undertake releases as part of the current project; this will be a core part of the new Rural R&D for Profit project (18-04-014; 2019-2022), which builds on the achievements of this project.
Progress towards these outcomes are indicated in this report.

Step 6

Release of agent(s)

Following risk assessment approved agents will be released at suitable locations.

2.2 Method modifications

African Boxthorn
Nil

Cabomba
Nil

Fleabane
Nil

Sowthistle
Nil

Mother-of-millions

Two potential biocontrol agents from Madagascar were to be imported into Australian quarantine for all the host range testing. However, Madagascan permitting requirements stipulated that there had to be an official relationship with the University of Antananarivo (UoA) and that a post-graduate student needed to be included in order to export any species the stem-boring weevil, *Osphilia tenuipes*, was exported as planned in 2016. However, the root-feeding beetle, *Rhembastus* sp. and the phyllode-feeding wasp, *Eurytoma bryophylli*, were not found during the life of this project in southern Madagascar despite repeated field visits over the life of the project at different times of the year.) A new species of root-feeding beetle, *Bikasha* sp., was however discovered and cultures were established at UOA and the containment facility in Brisbane.

Ox-eye daisy

The initial plan for this project was to import and test *D. aeratana* entirely in Australia. However, due to delays in securing funding to upgrade the quarantine facility at NSW DPI Orange, it was deemed necessary early on in the project to outsource some of the work to CAB Switzerland. A subcontract was therefore negotiated with CABI, in addition to a Material Transfer Agreement (MTA) which gave NSW DPI access to the moth, *D. aeratana*. This decision was beneficial to NSW DPI as the Swiss were able to conduct open field choice tests with Australian native plants, something which could not be conducted in Australia. Seeds of native Australian Asteraceae required for testing were sourced and mailed to CABI to be propagated at their laboratory in Delemont. Species, for which seed was not available, were sourced in Australia as vegetative material and propagated at NSW DPI Orange.

Three Australian ox-eye daisy long-term field sites were established Mount Hotham (Victoria), Mongarlowe (NSW) and Tantangara Reservoir (NSW). The sites were initially planned to be in New South Wales, ACT and Victoria, however, due a decision by the ACT conservation authorities to declare ox-eye daisy an eradication target, the proposed ACT site was therefore relocated further west to Mongarlowe (NSW).

Giant rat's tail grass
Nil

Silverleaf nightshade

In the Meat & Livestock Australia-led RRnD4P Round 1 project (B.WBC.0080 SLN Biocontrol), laboratory host-specificity tests of the silverleaf nightshade leaf beetle (*Leptinotarsa texana*) revealed that several Australian native *Solanum* species and certain cultivars of potato could be at risk of attack. Hence *L. texana* was considered an unacceptable risk to the Australian environment and economy and as such, an application for release was not pursued.

The key objective of the AgriFutures-led RRnD4P Round 2 Silverleaf Nightshade sub-project was to undertake native range surveys in areas where climatic, genetic and other factors maximised the likelihood of collecting and testing agents suitable for introduction to Australia.

Sagittaria

A variation of the project milestones was made on the 19th November 2019. The original milestone provided for the as it was anticipated that none of the Sagittaria biological control agents were likely to be approved for release by the original milestone due dates.

Prickly acacia
Nil

2.3 Project Locations

Table 1 shows the location of activities associated with the project for each of the weeds investigated

Release of approved agents will be a core part of the new Rural R&D for Profit project (18-04-014; 2019-2022), which builds on the achievements of this project.

Table 1

Location of project activities

Name & type of site (field site, laboratory, project partner sites, RDC headquarters)	Address	State
African boxthorn (CSIRO)		
CSIRO, quarantine facility,	Clunies Ross St, Black Mountain	ACT
Field surveys - agents	Eastern Cape, Western Cape	South Africa
Cabomba (CSIRO)		
CSIRO, quarantine facility, laboratory	Clunies Ross St, Black Mountain	ACT
S. America, native range field sites, project partner sites	Various	Paraguay, Argentina, Uruguay, Brazil
N. America, native range field sites, project partner sites	Various	USA
Fleabane (CSIRO)		
CSIRO, quarantine facility, laboratory	41 Boggo Rd, Dutton Park	QLD
S. America, native range field sites, project partner sites		Colombia

Method and project locations

Name and type of site (field site, laboratory, project partner sites, RDC headquarters)	Address	State
Sowthistle (CSIRO)		
CSIRO, quarantine facility, laboratory	41 Boggo Rd, Dutton Park	QLD
Europe, native range field sites, project partner sites	Various	France, Spain, Italy
Laboratory	Campus International de Baillarguet, Montferrier-sur-lez NA	France
Field surveys - agents		Southern Spain
Field surveys - agents		Southern Portugal
Field surveys - agents		Morocco
African native range field sites, project partner sites	Various	South Africa, Morocco
Mother-of-millions (NSW DPI)		
Laboratory (quarantine; OED & MoM) –DPI Orange	Orange Agricultural Institute 1447 Forest Road, Orange	NSW
Laboratory (quarantine; MoM) – QDAF Brisbane	Biosecurity Queensland Department of Agriculture and Fisheries. Level 3C West. Ecosciences Precinct	QLD
Laboratory - University of Antananarivo	Faculte Des Sciences Entomologie c/- Universite de Madagascar, Antananarivo	n/a
Maryvale (field site - MoM)	Janewindi Creek Road, Wee Waa	NSW
Turrawan (field site - MoM)	Turrawan Road, Turrawan	NSW
Inglewood (field site - MoM)	Cunningham Highway between Inglewood and Glenarbon	QLD
Dalby (field site - MoM)	Property: Jindabyne, 146 Humphrys Road, Moola	QLD
Ox-eye daisy		
Laboratory (quarantine; OED) –DPI Orange	Orange Agricultural Institute 1447 Forest Road, Orange	NSW
Laboratory – CABI, Switzerland	Rue des Grillions 1, CH-2800, Delemont, Switzerland	n/a
Kosciuszko National Park (field site – OED)	Tatangara Reservoir, KNP	NSW
Mount Hotham (field site – OED)	Brandy Creek, Mt Hotham	VIC
Mongarlowe (field site – OED)	28 Warragun Lane, Mongarloe	NSW
Giant rat's tail grass (QDAF)		
DAF (office & lab)	41 Boggo Road	QLD
Rhodes University (office and lab)	Grahamstown	Eastern province, South Africa
Various sites	Eastern Cape, Kwa-Zulu Natal, Mpumalanga and Limpopo Provinces	South Africa
Field Site	Taunton	QLD
Field Site	Miriam Vale	QLD
Field Site	Connondale	QLD
Silverleaf nightshade (DEDJTR)		
Laboratory	AgriBio, Bundoora	Victoria
Field Survey	FuEDEI, Gral. Simón Bolívar 1559, Buenos Aires, Argentina	Argentina, Paraguay
Laboratory	University of Texas 1201 W University Dr, Edinburg,	Texas
Sagittaria (DEDJTR)		
Laboratory	AgriBio, Bundoora	Victoria
Field Survey	FuEDEI, Gral. Simón Bolívar 1559, Buenos Aires, Argentina	Argentina, Paraguay
Field Survey	Texas	USA
Prickly acacia(QDAF)		
Laboratory	Ecosciences Precinct, Boggo Road	Brisbane
*Project partner: Institute of Forest Genetics and Tree Breeding, Coimbatore, India	Coimbatore	Tamil Nadu, India

Name and type of site (field site, laboratory, project partner sites, RDC headquarters)	Address	State
Field Surveys	Adama, Arba Minch, Awasa, Awash, Debre Birhan, Dessie, Harar, Mille, Shewa Robit regions	Ethiopia
Laboratory	Ethiopian Forest Research Centre Addis Ababa	Ethiopia
Field Surveys	Borno, Adamawa, Kano and Kaduna	Nigeria
Field Surveys	Bambey, Kaolack, Ndioum Wadi, Podol and Senegal River Valley	Senegal
Laboratory	Dr Sebahat Ozman Sullivan Ondokuz Mayis University,	Turkey
Laboratory	Agricultural Research Council - Plant Protection Research Institute Pretoria	South Africa
Project partner: National Herbarium of Tanzania	Arusha	Tanzania
Project partner: National Museum of Kenya, East African Herbarium.	Nairobi	Kenya
Project partner: Ondokuz Mayis University, Samsun, Turkey	Samsun	Turkey
*Project partner: Kogi State University, Nigeria	Anyigba	Nigeria
Prof Merv Mansel	University of Pretoria,	South Africa

2.4 Regional Applicability

African Boxthorn

Research into biocontrol for African boxthorn will be relevant SA, WA, QLD, VIC, NSW, ACT, those areas as shown in Figure 2.

Cabomba

Figure 4 shows the distribution in QLD, NSW which will be relevant to a biocontrol agent for cabomba.

Fleabane

A biological agent for fleabane would have applicability as shown in Figure 6.

Sowthistle

Sowthistle is widespread in QLD, NSW, VIC, SA, WA as shown in Figure 8.

Mother-of-millions

The research will be applicable to all areas where mother-of-millions are a problem. This includes coastal and subcoastal areas from north Queensland south to the NSW-Victorian border. There are significant infestations in inland southern Queensland and northern NSW. There are also a few infestations in Victoria, South Australia and Western Australia (See Figure 20).

Ox-eye daisy

The research findings from the ox-eye daisy biocontrol project will be applicable to all areas where the weed is invasive in Australia. The moth, *D. aeratana* has evolved under alpine conditions, with the ability to diapause during the winter period (to endure periods of snow and cold temperature). It is not anticipated that there are any parts of the invasive range where the moth should not be able to establish due to climatic constraints.

Giant rat's tail grass

The regions where the research findings are applicable are all regions where *Sporobolus* spp. are considered a problem. *Sporobolus pyramidalis* is found widespread from Cooktown to the

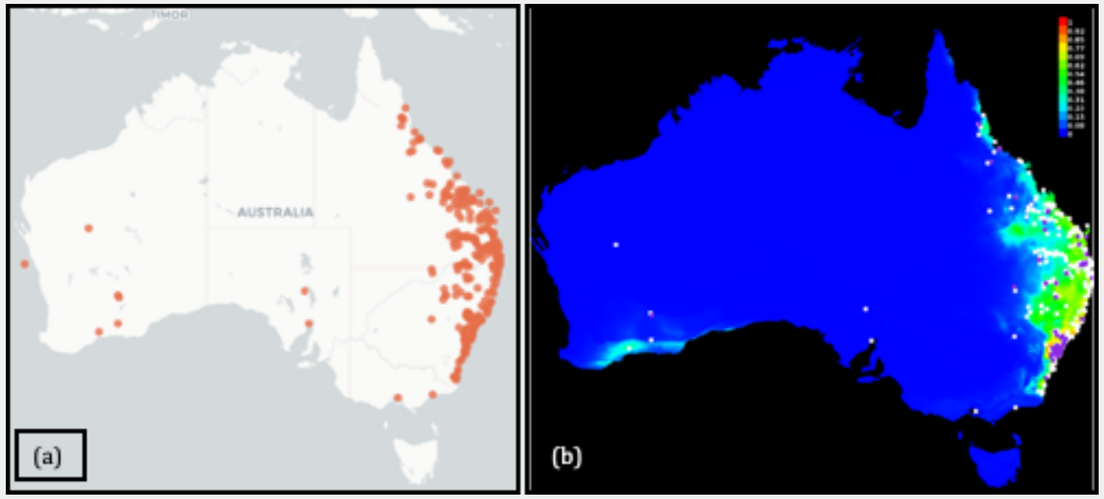


Figure 20. (a) Current distribution of *Kalanchoe delagoensis* in Australia (Atlas of Living Australia). (b) Maxent model of the potential distribution of *K .delagoensis* in Australia.

Project rationale and objectives

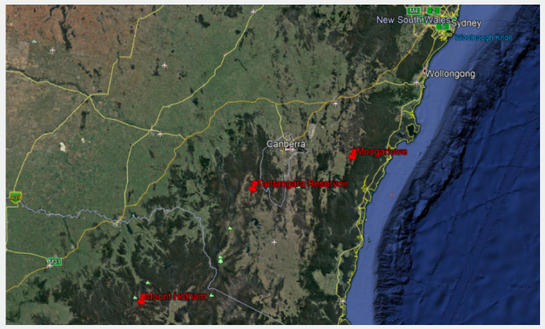


Figure 21. Current distribution of Leucanthemum vulgare in Australia.

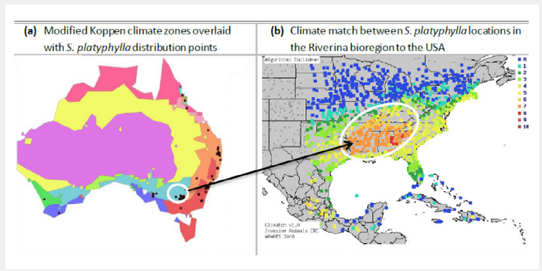


Figure 23 . Climate match between (a) *Sagittaria platyphylla* in the Riverina bioregion (circled) to, (b) southern USA using Climatch. The highest climate matches are represented by red and orange squares and the lowest by green and blue ones.

NSW Central Coast. *Sporobolus natalensis* is found widespread from Rockhampton to Port Macquarie on the mid NSW coast. *Sporobolus pyramidalis* is common in the Northern Territory while S. natalensis is present there at only a few sites (Figure 21).

Silverleaf nightshade

Northern Territory, Queensland, New South Wales, Victoria.

Sagittaria

Climate matching software (Climatch; http://data.daff.gov.au:8080/Climatch/climatch.jsp) was used to identify regions in the southern USA that most closely matched the invaded climatic zones of Australia. Based on the Koppen-Geiger climate classification (Kottek *et al.* 2006) *S. platyphylla* and *S. calycina* infestations from the Riverina bioregion (cold-arid steppe climate represented in light blue in Figure 23a) matched similar climates in the United States across Georgia, Alabama, Mississippi, Tennessee, Arkansas, Louisiana and eastern Texas (circled area in Figure 23b). Field trips to collect agents were focussed on these states. Note: the distribution of *S. platyphylla* in Australia (Figure 23a) is much greater than the Riverina with populations occurring along the east coast. However, the Riverina was chosen for climate matching purposes because this is where both *S. platyphylla* and *S. calycina* have reached greatest abundance and cause the greatest impact.

Prickly acacia

Prickly acacia is weed mainly in Queensland as shown in Figure 19.

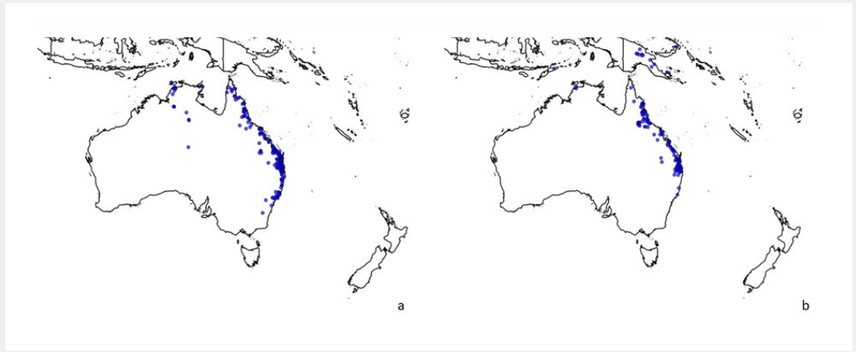


Figure 22. Map showing the distribution of *Sporobolus pyramidalis* (a) and *S. natalensis* (b) in Australia. Source: Atlas of Living Australia.

Section 3 Project outcomes

3.1 Project level achievements

All KPIs related to the project outputs detailed below have been met. A summary of achievements related to each output is indicated below. Additional details are captured in Appendices (available on request from authors).

3.1.1 African Boxthorn (*Lycium ferocissimum*)

Output 4(a) Undertake a literature review on taxonomy and distribution of African boxthorn and known natural enemies of the weed in the introduced and native ranges

Taxonomy

Species within the genus *Lycium* are highly plastic, so delimiting and identifying species within the genus with morphological characters can be difficult (Levin et al. 2006; Venter 2000). This is especially relevant to regions like southern Africa that has a high diversity of species in this genus. Morphological variation within *Lycium ferocissimum* is substantial (Venter, 2000), perhaps influenced by the broad range of climatic and environmental conditions across its distribution in South Africa. Identification of this species in the field is therefore challenging.

Morphometric analyses across *L. ferocissimum* and other *Lycium* species in South Africa were undertaken during this project and this research is now published in McCulloch et al. 2020 (Appendix 1). No leaf or floral characteristics unique to *L. ferocissimum* were identified, making morphological identification of the species problematic. This is not an issue in Australia, because there are only four other Lycium species present, outside of cultivation, all with restricted distributions: the native *L. australe* and the naturalised *L. barbarum*, *L. chinense,* and *L. afrum* of Eurasian origin. To ensure the correct *Lycium* species was surveyed for candidate biocontrol agents, *L. ferocissimum* individuals for which the identity was confirmed with genetic analyses were permanently tagged.

Distribution

In Australia, *L. ferocissimum* is widespread in coastal to semi-arid inland habitats and islands of southern Australia, with records from every jurisdiction (GBIF.org, 24th July 2018, Figure 2). It is found predominantly in the southern part of the Australian continent in coastal and island situations (except Queensland). Inland, *L. ferocissimum* is abundant in areas of New South Wales, Victoria and South Australia, where it is a common weed of semi-arid pastures and rangelands and is often found growing along dry stream beds. It has a lesser, but significant presence in south-east Queensland, southern Western Australia, and Tasmania.

Lycium ferocissimum has a relatively widespread distribution in South Africa (Venter 2000); however, most herbarium records are from the Eastern and Western Cape provinces (Figure 23).

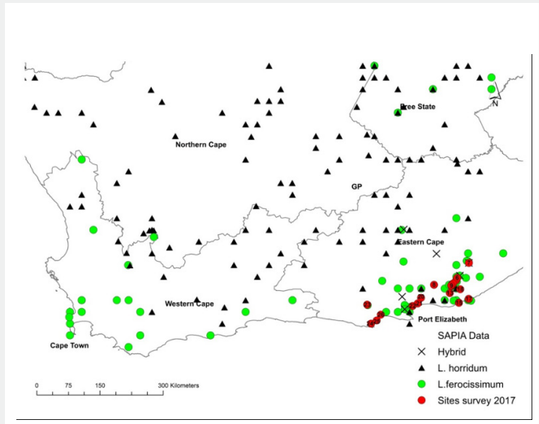


Figure 24. Distributions of *Lycium ferocissimum* (African boxthorn; green circle) and *L. ferocissimum x Lycium horridum* hybrid (black cross) and *Lycium horridum* (black triangle) in South Africa. Locality data was collected from herbarium specimens and the South Africa Biodiversity Institute (SANBI) database. Red circles show the *L. ferocissimum* sites surveyed by Rhodes University between January and April 2017.

Project outcomes

Its distribution overlaps with several morphologically similar and closely related species with which *L. ferocissimum* hybridizes (Figure 23).

A comprehensive review of the literature was undertaken in late 2016 to identify potential agents already recorded on *L. ferocissimum* in the native range, South Africa, and determine if any of these are already present in Australia. At the time of the review, 4 fungi and 13 insects had been recorded on *L. ferocissimum* in Australia. None of these have been recorded in South Africa. We found records of only one pathogen, the rust fungus *Puccinia rapipes*, and eight insect species on *L. ferocissimum* in South Africa. Among these, the rust fungus, tortoise beetles, *Cassida distinguenda*, *Cassida lycii* and *Cassida* sp., and the mirid bug, *Schuhistes lekkersingia* appeared to be specific enough to warrant further study as potential biocontrol agents. (Appendix 2 and Appendix 3).

Output 4(b) - Define goals for management of African boxthorn

In order to better understand the impacts of *L. ferocissimum* on Australian natural ecosystems and agriculture, and to guide the selection of candidate biocontrol agents, a national online stakeholder survey was conducted. Agricultural, community and environmental stakeholders were invited to complete a survey entitled 'African boxthorn management objectives', using the online survey platform SurveyGizmoTM between May and July 2017. Results from this survey are published in Ireland et al. 2019b (Appendix 4).

Of the 239 responses received, respondents primarily identified as natural resource managers, specialists or extension officers (61%). Community, conservation or other interest group members (22%), agricultural landholder or graziers (19%), and agricultural land managers, agronomists or extension officers (7%) made up the remaining respondents, with some respondents identifying with more than one group. A range of reasons were provided by respondents as to why L. ferocissimum was problematic, aside from it being difficult (79% agricultural and 86% environmental) and costly (78% agricultural and 84% environmental) to control in both the agricultural and environmental sectors (see details in Ireland et al. 2019b Appendix 4).

Many respondents (79%) agreed that biocontrol solutions for L. ferocissimum would be useful, particularly in difficult to access and environmentally sensitive areas, and when adopted as part of an integrated weed management framework. Respondents' top six management objectives to which biocontrol needs to make a significant contribution were a reduction in:

- incidence of new infestations,
- management costs,
- negative impacts on native biodiversity,
- weed seed bank over time,
- the need for follow up manual control,
- herbicide use

Output 4(c) - Nominate African boxthorn as a biocontrol target

The Invasive Plants and Animals Committee (IPAC; now the Environment and Invasives Committee; EIC), a cross-jurisdictional sectoral sub-committee of the National Biosecurity Committee, endorsed *L. ferocissimum* as a biocontrol target in August 2016. Unbeknown to us, the nomination had been prepared and submitted to IPAC by DPIPWE, Tasmania prior to the commencement of the project. One of the members of the project team, however, approached the authors of the nomination document to assist in updating and reformatting the information into a review paper on *L. ferocissimum* for publication. (For further information, contact CSIRO).

Output 4(d) - Conduct genetic analysis on samples of African boxthorn from different regions in Australia and the native range

Putative *L. ferocissimum* (i.e. tentatively identified morphologically in the field) samples were collected across its native range in South Africa and introduced range in Australia. A first set of genetic analyses (three chloroplast markers, one nuclear marker) were conducted in order to: (a) confirm plant identity, (b) assess genetic structuring across the native and invaded ranges (to explore the provenance of the invasive lineage), and (c) assess evidence for hybridisation between *L. ferocissimum* and other *Lycium* species that occur in Australia. Results from this study are published in McCulloch et al. 2020. (Appendix 1).

All samples collected in Australia were confirmed as *L. ferocissimum*, with no evidence of hybridisation with any other *Lycium* species. Ten samples from South Africa putatively identified in the field as *L. ferocissimum* were genetically characterised as different (unidentified) *Lycium* species. Nuclear and chloroplast genetic diversity within *L. ferocissimum* across both South Africa and Australia was low, with no evidence of genetic structure.

The lack of any detected genetic diversity and structure across *L. ferocissimum* in South Africa made inferring the introduction history of the invasive lineage challenging. However, one of the two chloroplast haplotypes found in Australia was identified in South African material only from plants collected in the Western Cape Province, near Cape Town. The other common chloroplast haplotype identified in Australia was also found in plants from this area, as well as other regions in South Africa. This suggested that the region around Cape Town may be the provenance of the invasive Australian lineage, though the possibility that *L. ferocissimum* was introduced to Australia from multiple localities could not be excluded.

A second set of genetic analyses using the more powerful next generation sequencing approach, genotyping-by-sequencing (GBS), was performed to better characterise the likely origins of Australian genotypes of *L. ferocissimum* (Paper in preparation. For further information, contact CSIRO). 3409 SNPs across 442 *L. ferocissimum* samples from 64 localities across the entire distribution of the species in South Africa and Australia were compared. Clear geographic genetic structuring was detected across South Africa, with distinct populations across the Eastern and Western Cape provinces. Our analyses indicated that the invasive *L. ferocissimum* plants in Australia originated from the Western Cape Province, with plants from near Malmesbury (in the northern part of the species' distribution) the closest genetic match to the Australian samples (Figure 25). Samples from Australia had similar levels of genetic diversity as those from South Africa, but there was no evidence of genetic structure across Australia. Our results suggested that the search for candidate biocontrol agents for *L. ferocissimum* should be focused in the Western Cape Province.

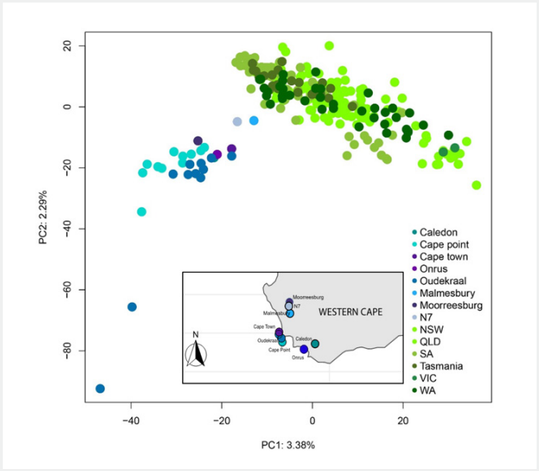


Figure 25 . Principal component analyses showing the genetic relationship between plants of *Lycium ferocissimum* (African boxthorn) sampled in the Western Cape (WC) province of South Africa and Australia.

Output 4(e) - Undertake bioclimatic models to identify optimal locations and conduct native range surveys and host-specificity tests for potential biocontrol agent(s) and import at least one potential agent in quarantine

Bioclimatic modelling

Details of the methodology used for developing the bioclimatic models using the CLIMEX package are in preparation. (For further information, contact CSIRO). The simpler Match climates model was used to identify where to focus the search for candidate biocontrol agents in South Africa (Figure 26A, B). It showed that *L. ferocissimum* is climatically well-matched to the Mediterranean and dry arid and semi-arid climate zones in South Africa, which accords with the rangelands in which it is an invasive problem in Australia.

Surprisingly there were little data on the climatic requirements and physiology of *L. ferocissimum* in the literature to inform the more sophisticated Compare Locations model. An experiment was thus conducted to measure the growth rate of *L. ferocissimum* at different temperatures (Figure 26D) to refine temperature growth-related parameters for the model (Figure 26E, F). From this model, we extracted the Monthly Growth Index values in South Africa for guiding when and where to survey for natural enemies on *L. ferocissimum* (Figure 27). Similar values were extracted for Australia to guide when and where biocontrol agents should be released, so that *L. ferocissimum* is actively growing at the selected sites at the time of release, in order to increase chances of establishment (Figure 28).

Project outcomes

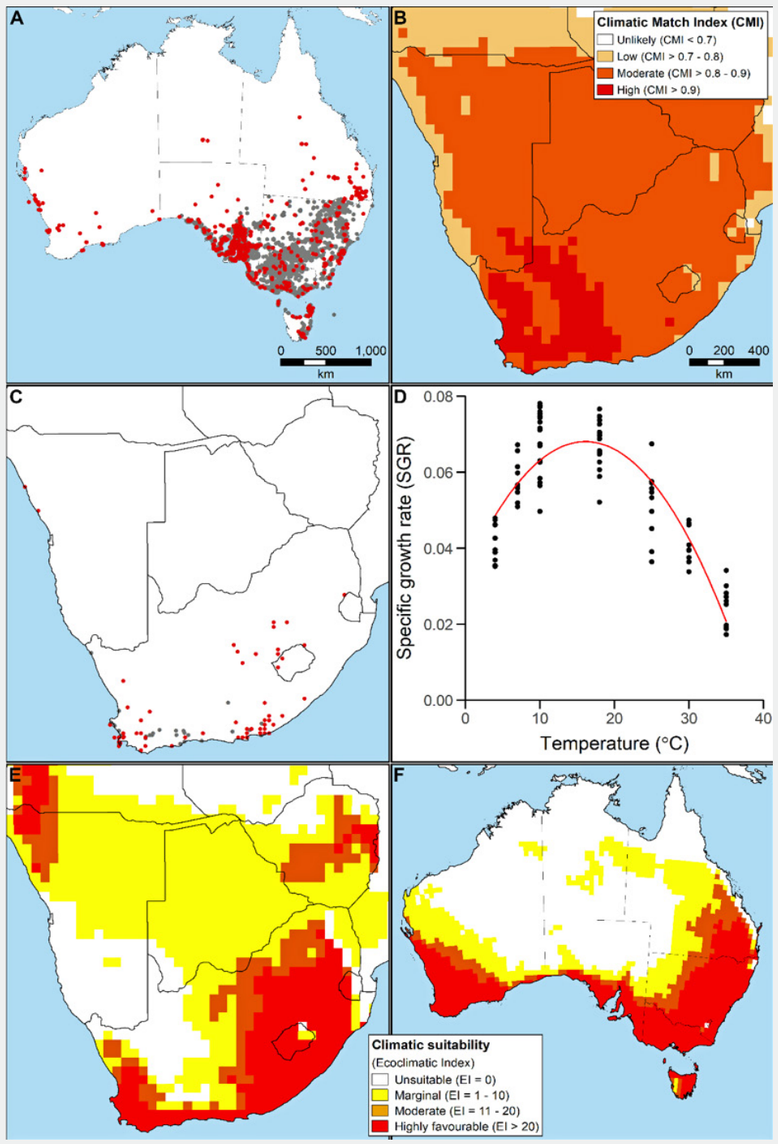


Figure 26. *Lycium ferocissimum*'s current distribution in (A) Australia and (C) the native range of southern Africa. Preserved and living specimen records (red points) projected atop observational records (grey points) (GBIF.org 24th July 2018c). (B) CLIMEX Composite Match Index climate matching model, as projected for South Africa. (D) Temperature response curve from growth experiment. Final fitted polynomial model incorporating both datasets shown as a red line. Projected climatic suitability from the CLIMEX Compare Locations model projected for South Africa (E) and Australia (F). Increased intensity of red colour, starting from yellow, indicates higher climatic suitability.

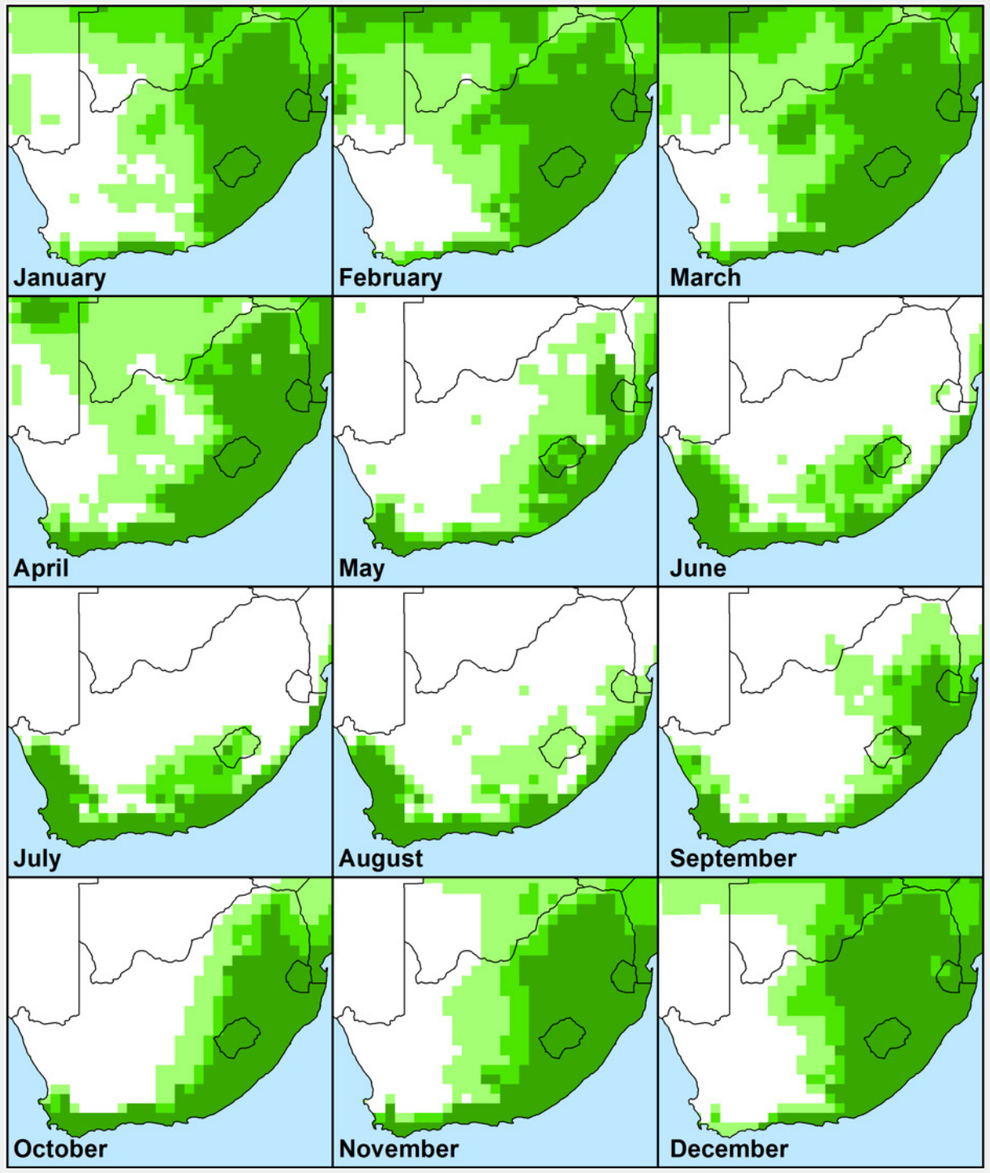


Figure 27. Monthly Growth Index values in South Africa for guiding when and where to survey for natural enemies on *Lycium ferocissimum*. Values are averaged across five years from 2012 to 2017. Surveying is recommended within areas in which the Ecoclimatic Index is most suitable, indicating potential for year-round survival. Increased intensity of green colour indicates higher climatic suitability.

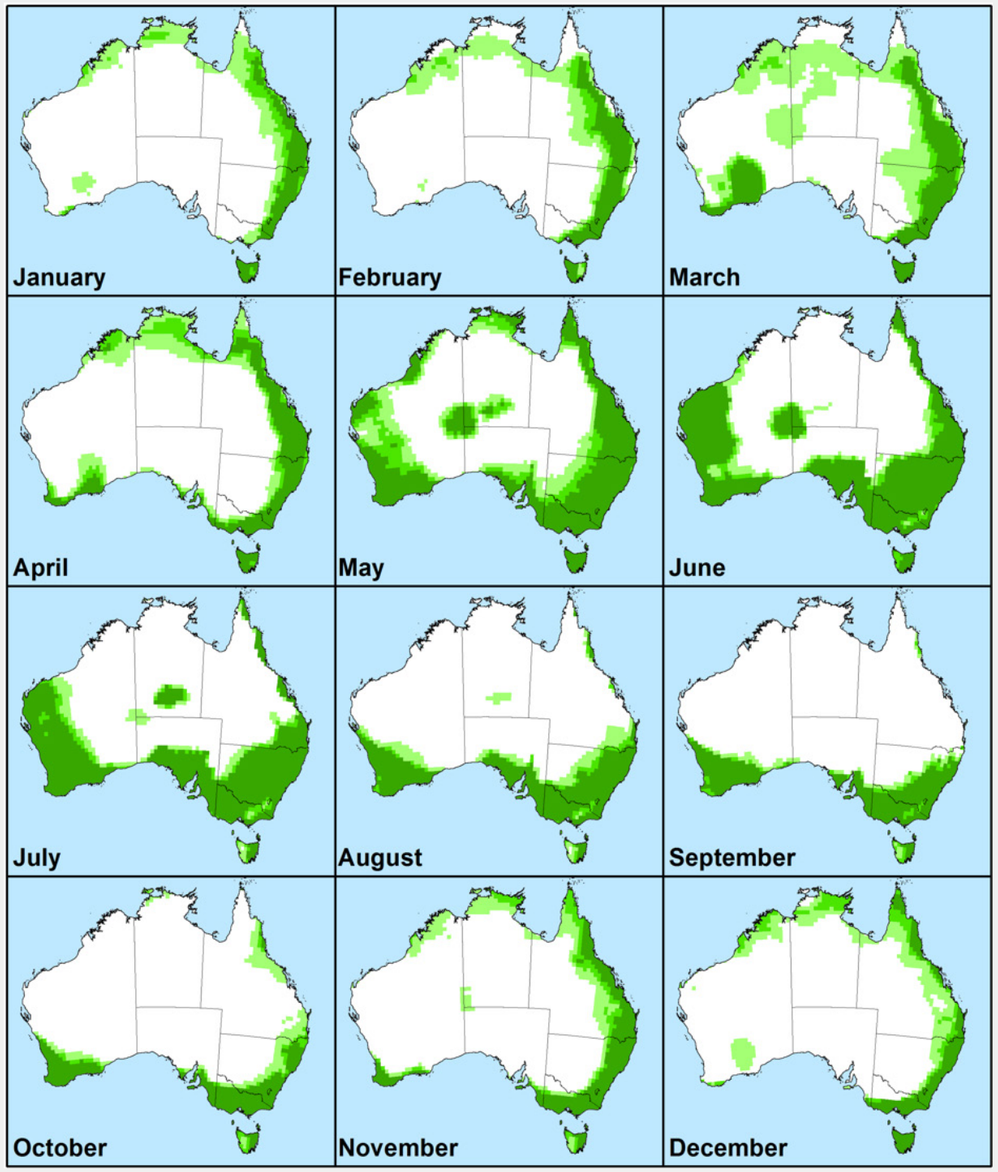


Figure 28. Monthly Growth Index values in Australia for guiding when and where to release biocontrol agents on *Lycium ferocissimum*. Values are averaged across five years from 2012 to 2017. Agents would only be deployed in areas in which the Ecoclimatic Index was most positive, indicating potential for year-round survival. Increased intensity of green colour indicates higher climatic suitability.

Native range surveys

Pathogens

A comprehensive survey for diseases on *L. ferocissimum* was performed in October 2017 at 28 sites across the Eastern (13 sites) and Western (15 sites) Cape provinces of South Africa. Results from this survey are published in Ireland et al. 2019a (Appendix 5). Disease symptoms caused by the rust fungus *Puccinia rapipes* were observed on *L. ferocissimum* at 4 of the 13 sites in the Eastern Cape and 10 of the 15 sites in the Western Cape. The rust fungus was not observed on any other *Lycium* species. The most severe rust symptoms were observed on *L. ferocissimum* at coastal sites in the Western Cape. Disease symptoms of any other primary pathogens were not observed at any of the sites surveyed.

Two sites in each of the Western and Eastern Cape provinces were also visited multiple times between November 2016 and October 2017 to source material of *P. rapipes* to establish cultures in the Australian containment facility and track life cycle development. On each field visit, rust life cycle stages and evidence of any other disease symptoms were recorded. Uredinia, telia, spermogonia and aecia were observed on *L. ferocissimum* at both sites where the Eastern and Western Cape during the course of these visits, confirming that the rust fungus is a macrocyclic and autoicous (no alternate host) rust fungus Ireland et al. 2019a (Appendix 5).

Insects

Native range surveys for potential insect biocontrol agents on *L. ferocissimum* were performed over a two-year period in the Eastern Cape and Western Cape provinces of South Africa. In total, 96 insect species comprising 1315 individuals were collected from both provinces (Chari et al. 2020; see Appendix 6). Of the 96 species, three species, the leaf-chewing beetles *Cassida distinguenda* Spaeth (Chrysomelidae) and *Cleta eckloni Mulsant* (Coccinellidae) and the leaf-mining weevil *Neoplatygaster serietuberculata* Gyllenhal (Curculionidae) were prioritised as potential biocontrol agents based on their distribution, abundance, preliminary biology studies, pilot in-field host-specificity studies (in South Africa) and feeding preference.

Host-specificity tests for potential biocontrol agents

The proposed list of non-target species for host-specificity testing of candidate biocontrol agents for *L. ferocissimum* was submitted to DAWE in December 2018 for posting on their website for feedback (Ireland et al. 2018, Appendix 7).

Pathogens

A preliminary host-specificity study using two purified isolates of *P. rapipes*, from the Eastern and Western Cape provinces of South Africa, was performed in quarantine in Australia (results published in Ireland et al. 2019a; Appendix 5). The experiments comprised two different chloroplast haplotypes of *L. ferocissimum* identified in Australia (McCulloch et al. 2020; Appendix 1) and seven species closely related to the weed that occur in Australia. The *L. ferocissimum* haplotypes and the three *Lycium* species of Eurasian origin tested – *L. barbarum*, *L. chinense* and *L. ruthenicum* – were found to be susceptible to both isolates of *P. rapipes* used, while the Australian native *L. australe* was resistant. The three more distantly related species to *L. ferocissimum* tested were immune to the fungus: *Hyoscyamus albus*, *Hyoscyamus aureus* and *Solanum aviculare*. The susceptibility of Goji berry (*L. barbarum*) to *P. rapipes* was further confirmed by our collaborators in South Africa in a study performed under natural conditions (Paper in preparation. For further information, contact CSIRO).

To better appreciate the implications of such results, further testing was suspended, and we undertook extensive stakeholder consultation (Ireland et al. 2019c; Appendix 8) We first reviewed the known and potential economic and social importance of non-native *Lycium* species propagated and sold within Australia, ascertained by review of the literature, online searches and discussions with stakeholders. We then further consulted growers and retailers of Goji berry to identify possible concerns they would have if a weed biocontrol agent was to be released that could infect their Goji berry plants. With this background information provided, we asked the national Plant Health Committee (PHC) for advice on whether it would be acceptable for an exotic weed biocontrol agent targeting African boxthorn in Australia to also infect Goji berry. This committee is responsible for reviewing applications for release of weed biocontrol agents that are submitted via DAWE and thus is in the best position to provide initial advice on this issue. This consultation process indicated that Goji berry is an extremely low value and volume plant in Australia, and overall growers, wholesalers and retailers were not particularly concerned about the possible release of a biocontrol agent for the *L. ferocissimum* that would also affect Goji berry. Based on the responses received, including those from PHC, it was decided to resume work with *P. rapipes*.

Comprehensive host-specificity testing in quarantine began in September 2019 using one of the purified isolates (ex. Western Cape). The test list comprises a total of 31 closely related, non-target species in the family *Solanaceae* that occur in Australia (ornamental, weed and native). Most species are tested in at least two separate experiments using different accessions of each plant

Project outcomes

species, with *L. ferocissimum* plants used as positive controls in all experiments. Testing is on-going and thus far, 11 experiments have been conducted comprising 17 different species (Ireland et al. in preparation). Similarly, to results of the preliminary study, all the *Lycium* species non-native to Australia included in these experiments to date, including the target weed *L. ferocissimum* and the Eurasian Goji berries *L. barbarum* and *L. ruthenicum* were found to be susceptible to *P. rapipes*. In contrast, all accessions of the Australian native *Lycium* australe tested were found to be immune to the fungus, while all other species tested were either immune or displayed various levels of resistance to *P. rapipes*.

Insects

Pilot in-field host specificity tests with the three promising candidate agents (*C. eckloni, N. serietuberculata*, *C. distinguenda*) were conducted on selected test plants at three sites in the Eastern Cape Province in South Africa: *L. oxycarpum*, *L. barbarum*, *Solanum melongena* (eggplant). There was generally very minimal or no spill over of the insects on the non-target plants. At only one of the sites, three *C. eckloni* and one *C. distinguenda* individuals were observed resting on *L. oxycarpum* and *S. melongena*, but without any signs of feeding. Preliminary host-specificity testing with *C. eckloni* and *C. distinguenda* was also conducted in a glasshouse at Rhodes University in South Africa. These tests showed that the insects have a host range restricted to the genus *Lycium*. Adult feeding and oviposition were recorded on *L. barbarum*, and both insects were able to complete their life cycle on this species.

Among the three prioritized agents, *C. eckloni* from a population in the Eastern Cape province was first imported into an Australian quarantine facility in February 2019 (and again in April and Mary 2019, to bolster colonies). Comprehensive no-choice host-specificity tests with this leaf-feeding beetle revealed that it feeds and reproduces on the three *Lycium* goji berry species (*L. chinense*, *L. barbarum* and *L. ruthenicum*) as well as the native *L. australe* to the same extent observed on *L. ferocissimum* (Figure 29).

As part of the investigations on *C. eckloni* we noticed morphological and molecular variation in this taxon in key diagnostic features between populations from the Eastern Cape and Western Cape provinces. This suggested that this taxon was perhaps a complex of cryptic species. In order to explicitly test this, we have commenced molecular characterisation of this species and, in Feb 2020, we imported a population of this species from the Western Cape to repeat the host-specificity testing undertaken with the Eastern Cape population. This testing will be part of the new Rural R&D for Profit project (Agrifutures Australia Project number: PRJ-12377). If the test results are similar to those with the Eastern Cape population, further testing with this insect will be discontinued and we will shift our focus to other candidate insect agents identified during native range surveys.

Importation of potential agents in quarantine

Pathogens

An accession of *P. rapipes* from the Western Cape Province of South Africa was imported in quarantine in Australia early 2017, but a culture could not be established because teliospores were dormant. Later in 2017 two additional shipments of rust-infected material were imported; an accession from the Eastern Cape Province in August and another accession from the Western Cape Province in October. Material from both accessions contained urediniospores that readily germinated, and a culture of each accession was established in quarantine. These two accessions were purified to generate single-uredinum isolates, bulked-up and used in a series of experiments (see above).

Insects

Accessions of *C. eckloni* from the Eastern Cape and Western Cape provinces were imported into Australian quarantine in May 2019 and February 2020, respectively.

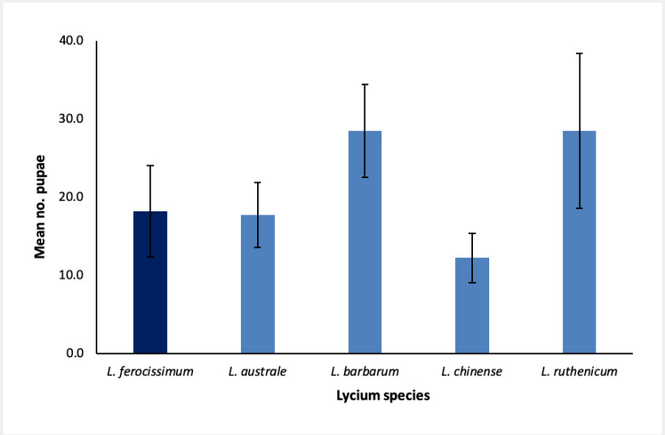


Figure 29. The mean (± SE) number of *Cleta eckloni* (Eastern Cape accession) pupae reared through from the focal weed *Lycium ferocissimum* (dark blue bar) and the four other *Lycium* species tested (light blue bars).

Output 4(f) - Pending risks to non-target plants are acceptable, submit application to the Commonwealth regulators seeking approval to release at least one potential agent. Upon receiving approval, release biocontrol agent(s)

Host-specificity testing with the rust fungus *P. rapipes* is expected to be completed in the following months. A draft of the release application has been prepared. Provided results of the remaining experiments do not identify unacceptable risks with this candidate agent, the application for release will be submitted to DAWE by the end of the project (15-Jun-2020).

Output 4(g) - Explore options for integration of biocontrol with other management techniques

Long term effective control of *L. ferocissimum* requires a combination of treatments over many years due to the capacity of the species to regenerate from rootstock, stems and seed. *Lycium ferocissimum* seed is dispersed predominantly by birds and other fauna, and potential for re-infestation of sites from outside sources should be considered in management planning.

Physical control techniques

Physical control of *L. ferocissimum* includes winching, pulling, bulldozing, stick raking, blade ploughing and cultivation (Noble and Rose, 2013). These techniques are best used when *L. ferocissimum* plants are not carrying seed (or are carrying minimal seed). Otherwise, fresh seed is likely to be deposited into freshly disturbed soil. Winching and pulling are the lowest impact physical control techniques for situations where disturbance is a concern, such as where *L. ferocissimum* is growing within native vegetation. Bulldozing, stick raking and blade ploughing are suitable in less sensitive landscapes (e.g. pasture), and provide a rapid control method for moderate to heavy infestations.

Successful management of *L. ferocissimum* using the above techniques is dependent on follow-up application of herbicide. This includes cut-stump technique with immediate application of herbicide for any remaining base/roots after winching and pulling. For all physical control techniques, there is a need to return periodically and carry out foliar spray application and/or machine-based cut stump treatments until there is no regrowth or seedling presence (Noble and Rose, 2013).

Chemical control techniques

Chemical control of *L. ferocissimum* uses techniques including foliar spraying, cut-stump application (including mechanical cut-stump), stem injection, stem scrap or frilling (e.g. using chisel cuts), basal bark application, and soil-root zone application. Glyphosate, triclopyr, picloram, aminopyralid, hexazinone and tebuthiuron have all been trialed in the chemical control of *L. ferocissimum*. Appropriate formulations, mixes and applications of these chemicals are detailed in Noble and Rose (2013) (For further information, contact CSIRO).

Biocontrol

From the current project, the pathogen *Puccinia rapipes* is showing promise as a candidate biocontrol and, pending final testing, which was initiated in this project and approval of its release by regulators, is likely to be an important component of landscape scale management of this species. This pathogen is likely to establish and perform optimally in wetter or more humid parts of the *L. ferocissimum* distribution in Australia, and in wetter years. Sites that fit these criteria might make good nursery sites, where the agent could initially establish and from where natural or assisted dispersal of the agent could occur.

Integration of management tactics

At the landscape scale, minimising the disruptions of biocontrol agents by other control tactics (e.g. avoiding spraying plants in nursery sites with herbicides) will require coordination among land managers recommending/deploying management tactics for *L. ferocissimum*. In addition, there are likely to be many circumstances where the pathogen (and other biocontrol agents) may act in concert with physical and chemical management. For example, it could be of value to trial the use of the pathogen as a follow-up treatment to control recruiting seedlings in place of herbicide applications. Similarly, the timing of releases of the pathogen with periods of regrowth of the weed following physical treatment, may also aid the integration of biocontrol to be another chronic stressor for this weed.

3.1.2 Cabomba (*Cabomba caroliniana*)

Output 5(a) - Define goals for management of cabomba

Through interactions with water asset managers (SEQwater) who are responsible for managing 90% of the Australian infestation *Cabomba caroliniana*, the following framework for integrated weed management of the weed was developed, with each broad category (presented below) comprising a series of specific objectives:

1. Containment is the principal goal given the current nature of infestations (i.e. four lake/dam systems principally impacted at present).
2. Eradication is the goal for new outbreaks in high priority locations (e.g. any incursions that could result in spread into larger lake/dam systems, i.e. transfer risk).
3. Anticipation of water quality implications of removing large amounts of weed biomass through management.
4. Understanding impacts of local catchment land-use context on *C. caroliniana* invasions.

Containment objectives in freshwater systems may need to be met almost entirely by biocontrol given the impermissibility of use of chemical control methods. Eradication objectives in non-potable water systems may be possible using the one chemical (Shark™; Active ingredient: 240 g/L Carfentrazone-Ethyl) registered for control.

Output 5(b) -Undertake bioclimatic models to identify optimal locations and conduct native range surveys and host-specificity tests for potential biocontrol agent(s) and import at least one potential agent in quarantine.

Bioclimatic modelling

Bioclimatic models were developed in the CLIMEX package to better understand current and prospective distributions of *C. caroliniana* in Australia, to identify optimal areas to survey for candidate biocontrol agents and to identify suitability of release sites in Australia for candidate agents. These initial models are being refined for inclusion in a scientific paper.

In Australia, most *C. caroliniana* infestations occur in southern Queensland and the northern New South Wales hinterland.

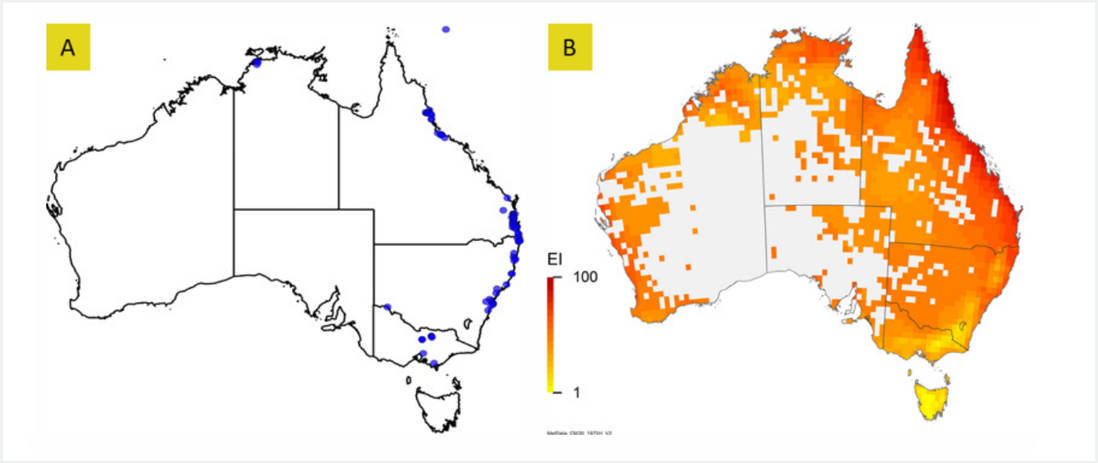
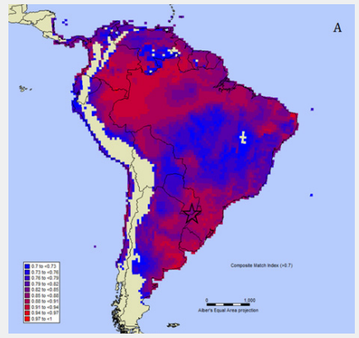


Figure 30 (A) Current distribution of *Cabomba caroliniana* in Australia (source: Atlas of Living Australia), (B) Potential distribution represented as Ecoclimatic index (EI) with temperature parameters derived from the growth experiment and stress parameters extrapolated from the global distribution of *C. caroliniana*. Redder colours indicating areas of greater climatic suitability for *C. caroliniana*. The projections were restricted to areas where perennial water bodies were found (based on National Surface Water Information; https://www.ga.gov.au/scientific-topics/national-location-information/national-surface-water-information).



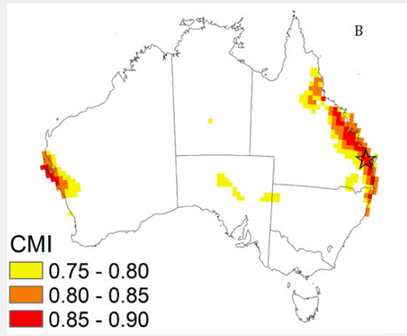


Figure 31. (A) CLIMEX Composite Match Index (CMI) for South America trained on those areas of Australia infested with *Cabomba caroliniana* considering only temperature variables. The star is centred on the largest known population of *C. caroliniana* in South America (Iberá wetlands in northeastern Argentina, and the wet grasslands of southern Paraguay). The highest climate matches are represented by red squares, and the lowest matches are represented by blue.

(B) Regions of Australia that match the climate of the area in the native range (Iberá wetlands, Argentina) where *Hydrotimetes natans* was sourced. The highest climate matches are represented by red squares, and the moderate and lowest matches are represented by orange and yellow respectively. The star is centred on Lake MacDonald, the largest population of *C. caroliniana* in Australia.

In Queensland it occurs in shallow, permanently flowing creeks and deep, slow-flowing pools of coastal river systems. There are smaller infestations found in Victoria and the Northern Territory. *Cabomba caroliniana* infestations have not yet been found in WA, SA, TAS or the ACT (Figure 30A). Bioclimatic modelling, based on the known climatic tolerances of *C. caroliniana* and availability of suitable water bodies, indicated it could potentially spread beyond its current distribution, especially across southern and eastern Australia (Figure 30B).

Matching the climate of Australian infestations of *C. caroliniana*, to its native range revealed that the known populations of the species in north-eastern Argentina, southern Paraguay and north-eastern Brazil were optimal places to survey to source candidate agents that would be the best bioclimatic fit for Australia. Surveys were thus undertaken in these locations over the course of this project. The focal candidate agent for this project, the cabomba weevil, *Hydrotimetes natans* (see 'Native range surveys', below), has only been recorded to date in the region (north-eastern Argentina, southern Paraguay) that is best matched with the climate where *C. caroliniana* infestations exist in Australia (Figure 31A). Projecting the climatic envelope of the native range distribution of *H. natans* onto Australia revealed that the major *C. caroliniana* infestations in eastern Australia were likely to be suitable for establishment of weevil populations (Figure 31B).

Native range surveys

Over the course of the project, surveys of natural enemies of *C. caroliniana* were undertaken in the native range (guided by the bioclimatic modelling): Argentina (Nov-2017; Apr-2018; Jan-2019), Paraguay (Nov-2017; Apr-2018; Jan-2019) and Brazil (Sep-2019). These surveys validated earlier surveys that there was only a small number of insect herbivores present on this submerged aquatic weed: four Coleoptera, three Lepidoptera, three Diptera and one Hemiptera species (Schooler et al. 2009, 2012). Based on the published literature, most of these were likely to be too general in their host range for consideration as potential biocontrol agents for *C. caroliniana* in Australia (Cabrera-Walsh et al. 2011, Schooler et al. 2012). The exception was the cabomba weevil (*H. natans*), which was prioritised for host-specificity testing and imported into quarantine in Australia.

Host-specificity testing

The cabomba weevil, *H. natans* was imported from Paraguay and Argentina into a quarantine facility in Australia for comprehensive host-specificity testing. Testing was concentrated on a broad range of plant species, including native Australian species, selected based on their phylogenetic relationships to the target weed.

Project outcomes

A total of 16 plant species from the families Cabombaceae, Nymphaeaceae and Hydatellaceae were tested – 13 in the laboratory in Australia and 3 in the laboratory in Argentina. In addition, field host-specificity assessments were performed at four sites in Argentina and Paraguay where the weevil was recorded on *C. caroliniana*. Co-occurring non-target aquatic species with *C. caroliniana* (e.g. *Egeria najas*, *Nymphoides indica*, *Nymphaea prolifera*, *Salvinia minima* and *Ludwigia grandiflora*) were examined for the presence of *H. natans* and any sign of feeding by larvae and adults.

Results from field observations and laboratory trials are briefly outlined below:

Field host-specificity: In the field in Argentina and Paraguay, observations revealed the presence of *H. natans* almost exclusively on *C. caroliniana* except for a single *H. natans* adult observed on *N. prolifera* adjacent to *C. caroliniana*. However, no feeding on *N. prolifera* was noticed which suggested that it was likely a casual occurrence.

Adult and larval feeding on leaf discs/sprigs: Feeding lesions caused by adult *H. natans* were observed on *C. caroliniana*, *Brasenia schreberi*, *Nymphaea caerulea*, *N. gigantea*, *N. nouchali*, *N. prolifera* and *Victoria cruziana* but not on *N. mexicana* and *C. caroliniana* var flavida. The feeding lesions on non-target plants were superficial and exploratory. Larval feeding trials on *C. caroliniana* var. *flavida*, *N. prolifera*, *N. caerulea* and *V. cruziana* demonstrated larvae are highly specific and unable to feed on non-target species. All larvae on non-target species died within four to five days of exposure to these species.

No-choice trials: Larval feeding, oviposition and larval development to adult occurred consistently on *C. caroliniana*. No oviposition occurred on any of the *Nymphaea* or *Trithuria* test plant species and hence no progeny development was observed. In *B. schreberi*, oviposition occurred on four of the six replicates tested. Among the four replicates that showed evidence of oviposition, larval feeding was noticed on three replicates, and pupation and adult emergence was observed in only one replicate.

Choice and continuation trials: Choice trials with *C. caroliniana* and *B. schreberi* suggested partial lifecycle development of *H. natans* on *B. schreberi*. Oviposition and larval development were observed on two of the five replicates tested. However, larval development to pupation was observed on only one replicate of *B. schreberi* with two pupae recorded. Only one of the two pupae metamorphosed into an adult, which however died soon after emergence, and the other pupa did not emerge as adult.

In continuation trials, offspring from parental *H. natans* did not complete lifecycle on any of the replicates of *B. schreberi*. No pupation was observed on *B. schreberi* despite egg laying in three replicates and larval development in one replicate. In contrast, a healthy and reproducing colony of *H. natans* was maintained on *C. caroliniana*, which has yielded five generations in the eight-month period between April 2019 to December 2019, in the same period over which the laboratory testing was undertaken.

In summary, as evident from the decision tree below (Figure 32), results from a suite of laboratory/glasshouse-based host-specificity testing in the native range and in a quarantine facility in Australia, as well as field observations in the native range demonstrated that *H. natans* has a high degree of specificity towards the target weed *C. caroliniana*. Based on these results, we concluded that the level of risk *H. natans* poses to non-target native and introduced species in Australia is negligible and that *H. natans* will potentially be an effective biocontrol agent for *C. caroliniana* (Schooler et al. 2006).

Output 5(c) - Pending risks to non-target plants are acceptable, submit application to the Commonwealth regulators seeking approval to release at least one potential agent. Upon receiving approval, release biocontrol agent(s)

A release application for the cabomba weevil, *H. natans* was submitted to DAWE on 10 March 2020.

Output 5(d) - Identify optimal rearing methods and nursery sites for field release of potential biocontrol agent(s)

Mass-rearing methods

We undertook multiple importations of the cabomba weevil because of difficulties with establishing colonies in the quarantine facility due to its unknown biology. Detailed investigations of the biology of the weevil have enabled us to fully characterise the life cycle of this species (Figure 33. For further information, contact CSIRO). This detailed study of the weevil's lifecycle was crucial to the completion of the host-specificity studies (see 'Host-specificity testing', above), and formed the basis for developing a mass-rearing protocol with the following steps (summarised in Figure 34).

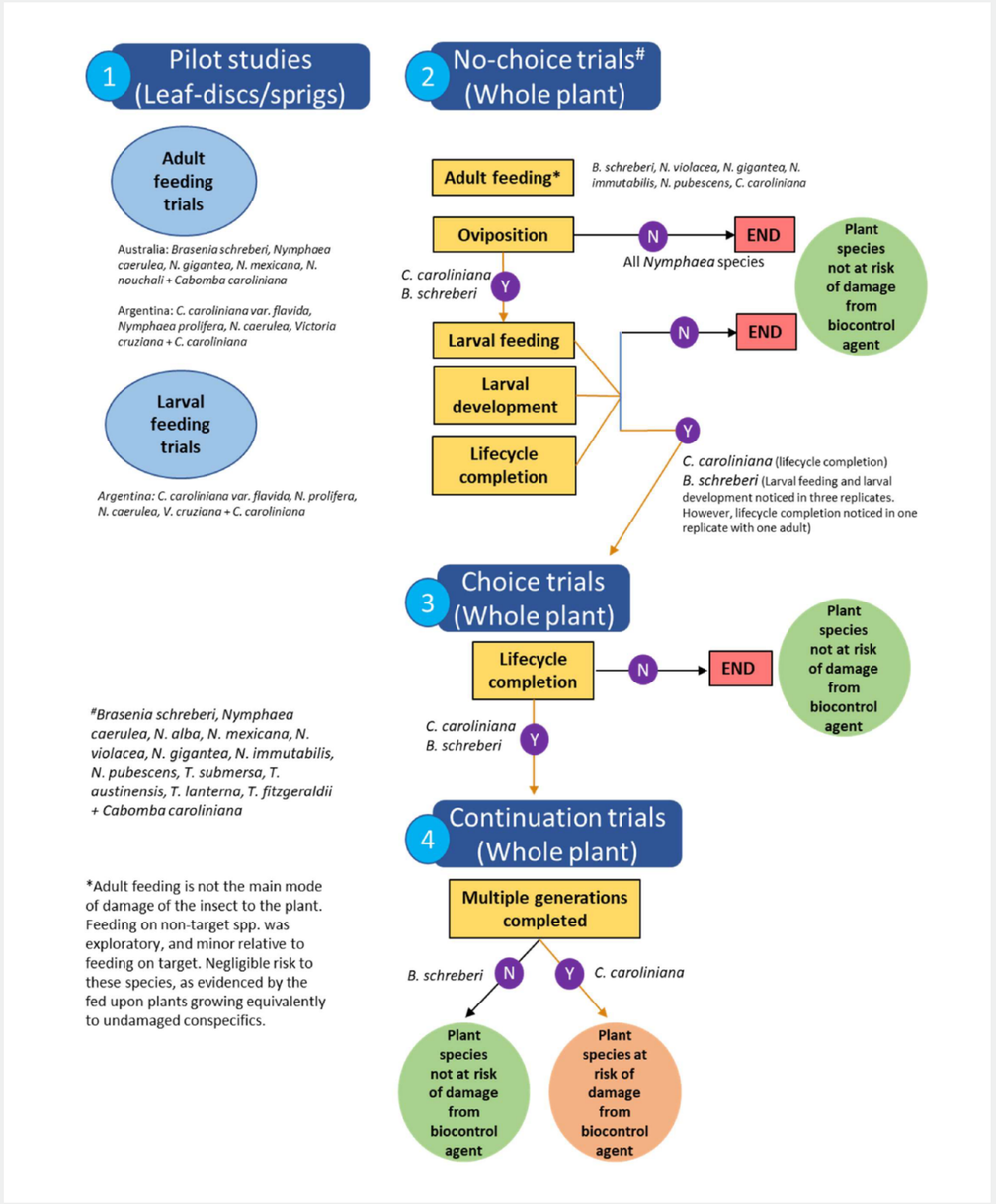


Figure 32. Decision tree outlining the range of host-specificity testing completed on the cabomba weevil, *Hydrotimetes natans*.

Project outcomes

- Source *C. caroliniana* from lakes/creeks and clean to get rid of pests, algae, fine sediment and bacteria; this hygiene step will boost the quality of the plant stock which will aid successful rearing of the weevil.
- Maintain a stock of *C. caroliniana* in 1-ton tanks, and replenish with additional, frequent (4 to 6 weeks) field collections.
- Set-up *C. caroliniana* in smaller 'Nally' bins (Nally; Viscount Plastics, Sumner Park, Queensland, Australia); this system requires:
    - Reverse osmosis water with nutrients,
    - Water pump, shade / shade cloth mesh cover to limit sunlight (C. caroliniana and the weevil prefer share with limited light).
- Introduce ~30 weevils into Nally bins with *C. caroliniana* (5 clusters, each with 6 sprigs); leave 2 to 14 days for egg laying depending on the need. Retrieve / take weevils out; keep them to setup a new array of rearing Nally bins.
- For the exposed *C. caroliniana* ('Breeding Tank') that is now free of adult weevils: Add new, clean *C. caroliniana* into the Nally bin every 2–3 weeks depending on larval feeding and availability of plant material for developing larvae.
- Maintain the 'Breeding Tanks' for the period of larval and pupae development through to adult (40–50 days; Figure 34).
- Set up new tanks with weevils already retrieved and newly emerged weevils. Frequent exposure of *C. caroliniana* to weevils is required to increase the colony number.

If approved for release by DAWE, further refinements of this mass-rearing protocol outside the confines of the quarantine facility to enable water asset managers to set-up their own colonies in larger tanks, in place of Nally bins.

Selection of nursery sites

Pending approval of release being obtained from DAWE, the cabomba weevil will be released at two primary nursery sites that have been selected for initial establishment of weevil populations in the field: Lake MacDonald (Noosa Shire, Queensland) and (Kingfisher Lagoon, Ross River, Townsville). These infested sites have been selected because they are among the larger infestations of the weed in Australia. Additional infested sites in the NT and Victoria have been identified as well, for the next phase of establishment of nursery sites. The mass-rearing protocol (Figure 34) will also enable landholders to maintain colonies of the weevil on farm dams impacted by *C. caroliniana*.

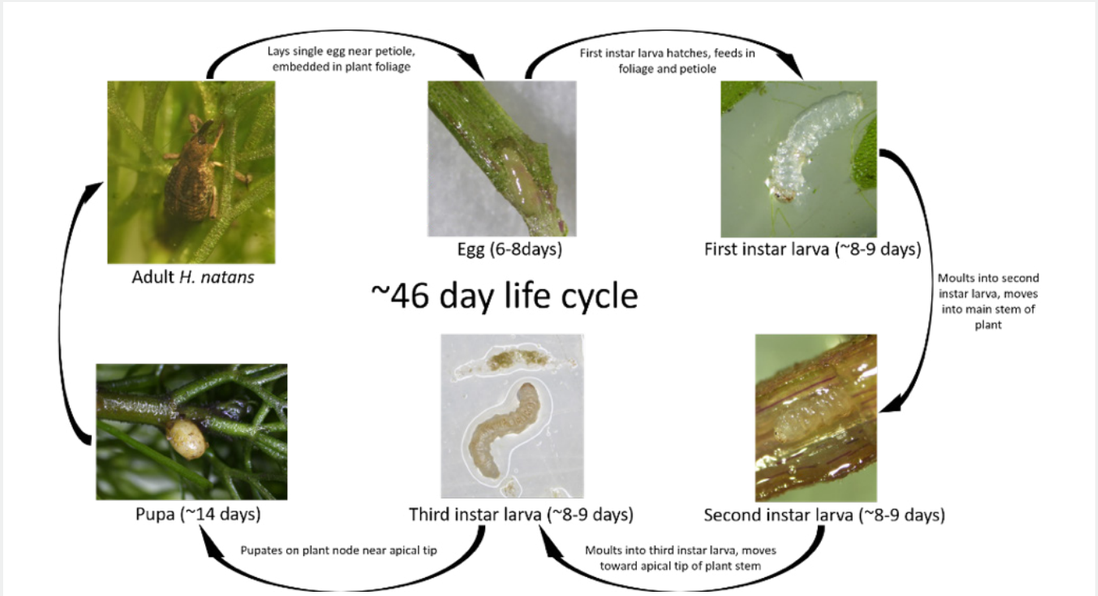


Figure 33. Life cycle of the cabomba weevil, *Hydrotimetes natans* on *Cabomba caroliniana*

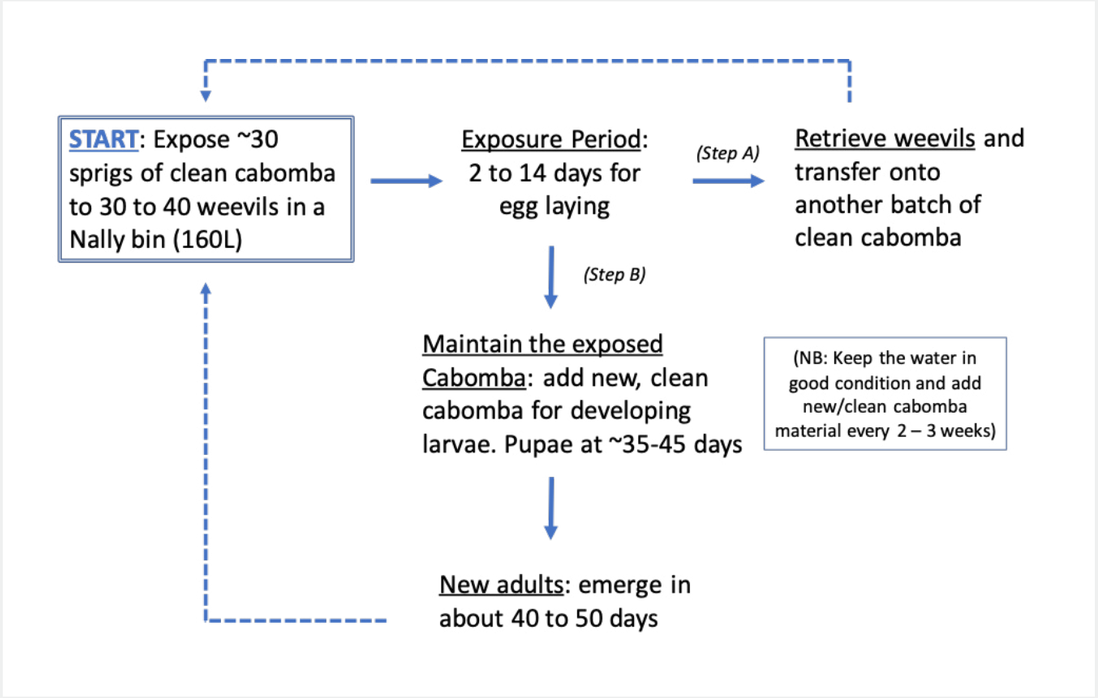


Figure 34. Schematic of the mass-rearing protocol developed for the cabomba weevil *Hydrotimetes natans* on *Cabomba caroliniana*.

Project outcomes

Output 5(e) - Develop an Integrated Weed Management (IWM) program for cabomba

Through ongoing discussions with key water asset managers (e.g. SEQwater) and our research, we have distilled context specific IWM guidelines for *C. caroliniana*. These are summarisedin the Table 2. Context-specific integrated management options for *Cabomba caroliniana*, below.

Table 2

Develop an Integrated Weed Management (IWM) program for *cabomba caroliniana*

Situation	Management goal	Current tools	Future tools*
Large potable water reservoir	**early eradication**	hand weeding by divers	spot treatment using Flumioxazin with carrier
	containment	Hand weeding through divers, e.g. around boat ramps suction dredging installation of benthic blankets	Flumioxazin with carrier for spot treatment: low cost biocontrol for reduction in overall biomass
	maintenance	suction dredging (e.g. to clear recreational areas harvesters/cutters	biocontrol for reduction in overall biomass; integrate with other tools to protect critical areas large scale treatment with Flumioxazin, with or without carrier; integrate with biocontrol to reduce amount of cabomba that needs to be treated
Large irrigation reservoir	**early eradication**	hand weeding by divers	Flumioxazin with carrier for spot treatment
	containment	Hand weeding through divers, e.g. around boat ramps suction dredging installation of benthic blankets	Flumioxazin with carrier for spot treatment biocontrol for reduction in overall biomass
	maintenance	suction dredging (e.g. to clear recreational areas harvesters/cutters	biocontrol for reduction in overall biomass; integrate with other tools to protect critical areas large scale treatment with Flumioxazin, with or without carrier; integrate with biocontrol
Medium sized lakes and impoundments	**early eradication**	hand weeding by divers	Flumioxazin with carrier for spot treatment
	containment	restrict water activities (swimming, fishing, boating)	biocontrol for reduction in overall biomass
	maintenance	harvesters/cutters water level manipulation carfentrazone	biocontrol for reduction in overall biomass large scale treatment with Flumioxazin, with or without carrier; integrate with biocontrol
farm dams	**early eradication**	hand weeding by divers	Flumioxazin with carrier for spot treatment
	containment/ maintenance	restrict water activities (eel fishing) shading draw down benthic blankets carfentrazone	Flumioxazin with or without carrier
Slow flowing large Rivers	**early eradication**	hand weeding by divers	Flumioxazin with carrier for spot treatment
	containment	restrict water activities (swimming, fishing, boating) Hand weeding through divers suction dredging installation of benthic blankets (depending on flow) carfentrazone (in still areas)	biocontrol for reduction in overall biomass Flumioxazin with carrier
	maintenance	harvesters/cutters (in still areas) carfentrazone (in still areas)	biocontrol for reduction in overall biomass large scale treatment with Flumioxazin, with carrier; integrate with biocontrol

Project outcomes

Situation	Management goal	Current tools	Future tools*
Small creeks	**early eradication**	hand weeding by divers	Flumioxazin with carrier for spot treatment
	containment/ maintenance	restrict access physical removal with diggers shading carfentrazone	biocontrol for reduction in overall biomass large scale treatment with Flumioxazin, with or without carrier; integrate with biocontrol

*pending future registration of the herbicide Flumioxazin for drinking water applications or ability to take the water system temporarily off the grid during treatment; pending approval for release given by DAWE for the cabomba weevil.

3.1.3 Fleabane (Conyza spp.)

Output 6(a) Undertake a literature review on taxonomy and distribution of fleabane and known natural enemies of the weed in the Introduced and native ranges.

Taxonomy

Conyza is mostly a New World genus in the tribe Conyzinae of the family Asteraceae (or Compositae), the largest of all plant families (c. 25,000 species worldwide). There are three main species of *Conyza* in Australia – *Conyza bonariensis* (flaxleaf fleabane), *Conyza canadensis* (Canadian fleabane) and *Conyza sumatrensis* (tall fleabane). *Conyza bonariensis* is the most widespread, occurring in all states and territories, followed by *C. sumatrensis* and *C. canadensis*. There are four other *Conyza* species present in Australia, but their distribution is limited – *C. bilbaoana*, *C. parva*, and *C. primulifolia* (PlantNET 2020). It is noteworthy that the curator of the Australian National Herbarium has doubts as to the circumscription and recognition of *Conyza bilbaoana* in Australia.

Conyza bonariensis has the narrowest leaves at the rosette stage when compared to other *Conyza* species (Thébaud and Abbott 1995). It has a more compact stature, with many short branches and bearing large capitula, while *C. canadensis* is essentially a single-stemmed taxon with few long branches and with small and elongated capitula. *Conyza bonariensis* is a genetic allopolyloid (arose through hybridisation; hexaploid (2n=54)) and strictly semelparous (has a single reproductive episode before death) (Thébaud and Abbott 1995). It is self-compatible and seems not to be pollinated by insects (Thébaud et al. 1996).

Evidence of genetic variation in several morphological traits of *C. bonariensis* was found in a common garden experiment conducted by Thébaud and Abbott (1995) in Europe. Hybrids of unknown fecundity, originating from crosses between *C. bonariensis* and *C. canadensis* and between *C. bonariensis* and *C. sumatrensis*, have been reported in Europe (McClintock and Marshall 1988). However, a subsequent isozyme survey of five *Conyza* species in Europe failed to find intermediate individuals (Thébaud and Abbott 1995). Zelaya et al. (2007) highlighted that loss of vigour in *Conyza* hybrids is apparently a common trait. They speculated that ploidy differences may be a significant barrier determining successful hybridisation between *Conyza* spp., e.g. more compatible hybrids would be expected from crosses between allopolyploids such as *C. sumatrensis* and *C. bonariensis* compared to crosses with the diploid (2n = 18) *C. canadensis*. The extent of genetic diversity in *C. bonariensis* and existence of hybrids in Australia are unknown.

Distribution

Conyza bonariensis is present in all states of Australia, occurring predominantly in temperate and Mediterranean coastal regions, and with restricted distributions in semi-arid to arid central regions (GBIF.org 2nd November 2018) (Figure 6). It is native to warm temperate South America, (Michael 1977) (Figure 35). It is considered widespread in Argentina, Uruguay, Paraguay and Brazil, and has been recorded in coffee plantations in Colombia and Venezuela (Mangolin et al. 2012).

Natural enemies

A comprehensive review of the literature was undertaken in late 2016 to identify potential agents already recorded on *C. bonariensis* in the native range, South America, and determine if any of these were already present in Australia. At the time of the review, three fungi and two insects had been recorded on *C. bonariensis* in Australia. In the native range in South America and nearby areas, Central America and southern USA, a total of 19 fungi, including several rust fungi, and 16 insects have been recorded on *C. bonariensis*. The likely specialists of interest for biocontrol were the various rust fungi, including *Aecidium conyzae colombiensis*, *A. erigerontis*, *Caeoma cyclostoma*, *Coleosporium erigerontis*, *Micropuccinia spegazzinii*, *Puccinia cnici-oleracei* (synonym *P. doloris*), *P. conyzella* and *Uredo erigerontis*, provided they are demonstrated to be autoecious (without alternate hosts). Among the insect species, two gall flies, *Trupanea bonariensis* and *Eutreta rhinophora* (Tephritidae), were identified as likely specialists. (Appendix 9, Appendix 10)

Project outcomes

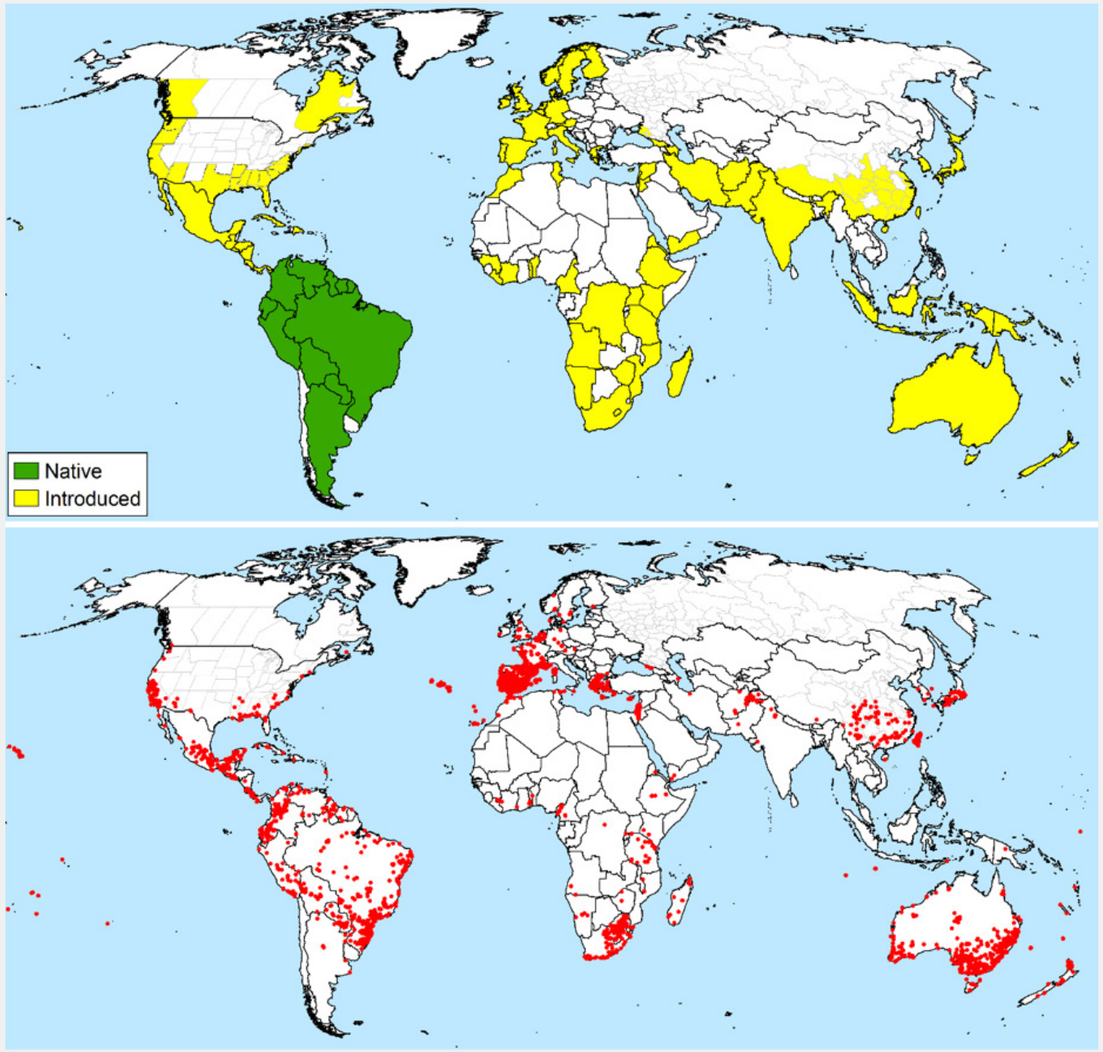


Figure 35. Global distribution of *Conyza bonariensis*. (a) Administrative level distribution assigned to native or introduced status at the national or provincial level (when not widely distributed across a whole country), modified from Scott et al. (2016) and updated to reflect (b) point distribution data records from GBIF.org (2nd November 2018).

Output 6(b) - Define goals for management of fleabane

Between May and July 2017, the online platform SurveyGizmo™ was used to survey key stakeholders in the grains industry affected by *C. bonariensis* and *Sonchus oleraceus*, about the impacts and desired management goals for these weeds. Both weeds were included in the same survey because they are problems in the similar areas and land uses. A total of 60 responses were received; 51 complete (85% answered all questions), and 9 partial responses. Respondents identified as either agricultural landholders (55.2%) or agricultural land managers, agronomists or extension officers (48.3%).

Respondents indicated that most of the agricultural impacts listed for these weeds were relevant as four of the six impact statements received > 50% response (respondents were able to select more than one impact statement) (Figure 36). The leading impact statement was that these weeds are "difficult to control" (79.2%) followed by "reduces stored water supplies in fallow" (66%).

Eighty-seven percent of respondents indicated that they felt that a new tool such as biocontrol would be useful in the management of fleabane and sowthistle (11.1% neutral, 1.9% disagreed). Fallow, roadsides and fence lines were considered by most respondents to be areas in which biocontrol could contribute to management as they received >50% responses.

The respondents selected the following top four management objectives to which biocontrol needs to contribute to be considered successful were:

- Decrease weed management cost and effort,
- Reduce herbicide inputs required,
- Decrease the need for follow-up control, and
- Reduce the occurrence of new infestations.

The latest review on the costs of weed to the grain industry (Llewellyn et al. 2016), states that *C. bonariensis* and *S. oleraceus* are high impact summer fallow weeds responsible for revenue losses of $43.2 and $4.9 million per year respectively. These two cropping weeds are also responsible for $3.6 (*C. bonariensis*) and $1.3 (*S. oleraceus*) million annually in additional herbicide costs due to the development of herbicide resistance in populations these species. The economic and chemical inputs required to control these weeds are set to increase if herbicide resistance increases in frequency and distribution. Based on the outputs of the management objectives' survey, these fiscal impacts serve as a baseline against which the economic and management success of any introduced biocontrol agents can be judged in the future.

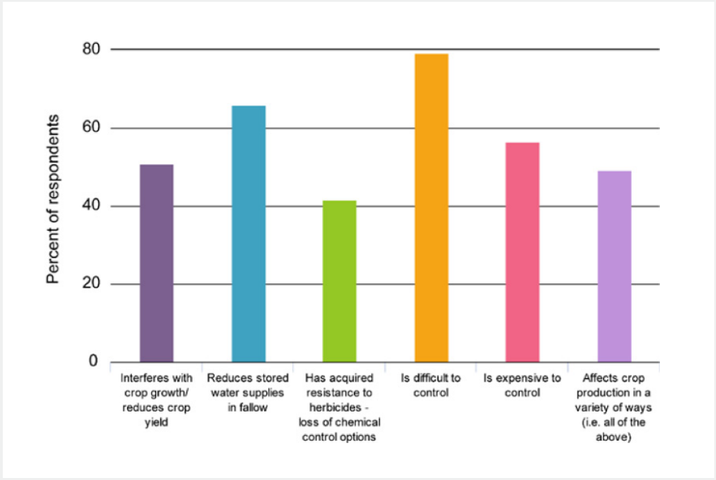


Figure 36. The percent of respondents who selected the various impact options for *Conyza bonariensis* (flaxleaf fleabane) and *Sonchus oleraceus* (sowthistle) provided in the survey.

Project outcomes

Output 6(c) - Nominate fleabane as a biocontrol target

The project prepared the documentation to support the nomination of *C. bonariensis* as a target for biocontrol. The documentation was submitted to the IPAC (now EIC), by the Queensland Department of Agriculture and Fisheries in May 2017 and endorsed by the Committee in November 2017 (Rafter and Morin 2017, Appendix 11).

Output 6(d) - Conduct genetic analysis on samples of fleabane from different regions in Australia and the native range.

A total of 375 putative individuals of *C. bonariensis*: 239 individuals from 18 sites in Australia, 60 individuals from 8 sites in Brazil, 9 individuals from 1 site in Argentina and 67 herbarium specimens from the Americas were analysed with Diversity Arrays Technology (DArT)seq. DArTseq Single Nucleotide Polymorphism (SNP) data for these samples consisted of 100,629 loci which was reduced to 18,110 loci following filtering of DArT parameters.

Due to challenges with collecting fresh specimens in the native range for this study, the dataset comprised samples from herbarium specimens. This is a newly emerging approach to supplement sampling in cases where it is difficult to collect fresh material from the field. It is noteworthy that 50% of the 137 samples from putative *C. bonariensis* herbarium specimens processed were successfully extracted and sequenced.

Phylogenetic trees using the whole data set were generated using both Geneious (https://www.geneious.com/geneious/) and SplitsTree (http://splitstree.org/) softwares. These trees indicated the presence of two groups of samples, with no evidence of hybridisation between them. A further phylogenetic tree was generated in Geneious using a reduced dataset comprising three randomly selected plants from each Australian site where fresh material was collected and all other samples in the dataset. We also excluded from this analysis the six West Mackay samples previously found to cluster with Group 1, as this indicated that sampling at this site was performed on more than one *Conyza* species (Figure 37).

Group 1 comprised samples primarily from South and Central America, with just one sample from the United States (close-up figure of Group 1 available on request). Group 2 comprised all Australian samples and the remaining 18 United States samples as well as samples from Brazil and a small number of other samples from South and Central America (Figure 38). Group 2 thus corresponded to *C. bonariensis*, as it is known in Australia. In contrast, Group 1 indicated that several of the herbarium samples included in the analysis had been misidentified as *C. bonariensis*. Furthermore, the analysis revealed that not all fresh samples collected from putative *C. bonariensis* plants at the same sites in Brazil were in the same group, indicating that plants from more than one *Conyza* species were sampled by our collaborators. This analysis indicated that *C. bonariensis* collected in Australia were more closely related to herbarium samples from Chile, Costa Rica and Guatemala, followed by a sample from Bahamas and a subset of samples from the United States (Figure 38).

Figure 37. Phylogenetic tree of a reduced DArTseq dataset of putative *Conyza bonariensis* samples produced using Geneious.

The Bayesian clustering program STRUCTURE and the Poppr R package were subsequently used to assess the extent of population genetic structure among samples in Group 2. Since this group was dominated by Australian samples, we undertook a different approach that firstly determined how many genetic clusters (*K*) occurred within the invaded Australian range without any prior knowledge of population affinities. The analysis showed that two genetic clusters were most likely present within Australia and that two populations from Western Australia (Mocardy; H and Oakabella; O) were distinct to the other Australian populations (Figure 39). Assignment of native range samples revealed that they were all admixed and that some samples in the United States had higher levels of admixture with the genetic cluster that characterised the two different Western Australian populations. The levels of admixture in native range samples makes it challenging to determine where Australian samples have originated from, although there is a suggestion that the two Western Australian populations have come from the United States.

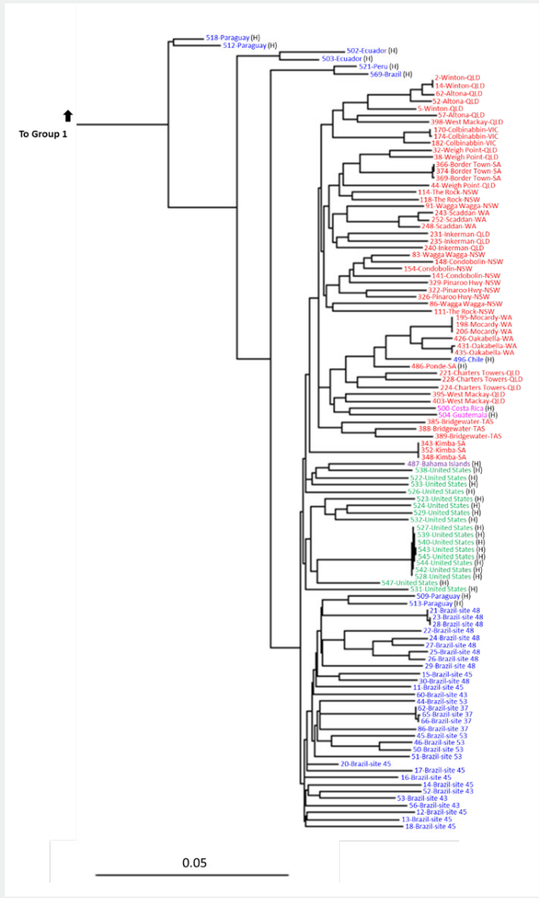


Figure 38. Close-up of Group 2 from the phylogenetic tree presented in Figure 37, which has been relabelled. Samples names are comprised of ID number and country (site name and state indicated for Australian samples, and site number indicated for field-collected samples from Brazil). Colour-coding: blue = South America, pink = Central America, purple = Caribbean, green = North America (USA); red = Australia. Samples that originate from herbarium specimens are indicated by H.

Project outcomes

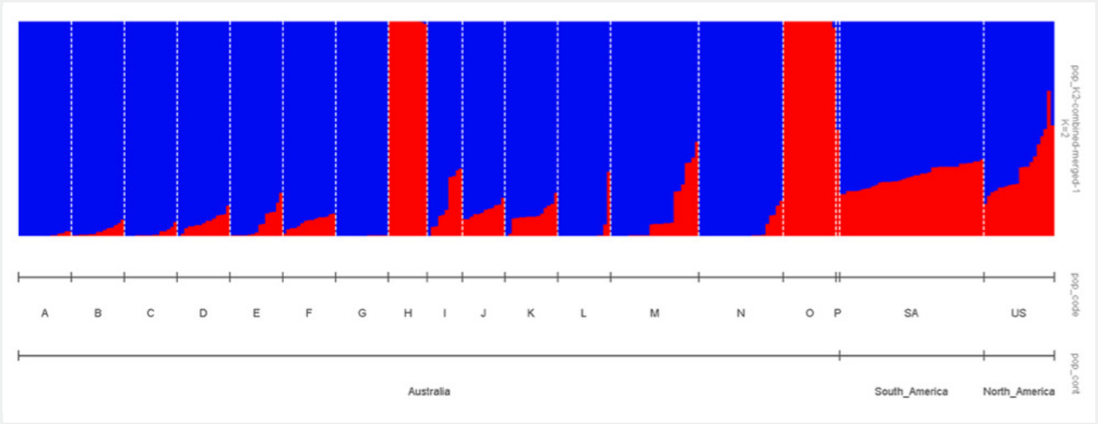


Figure 39. Bar plot representation of results from the STRUCTURE analysis of samples from the Group 2 only, which represents *Conyza bonariensis* as it is known in Australia. Genotypes are best described by two genetic clusters (blue and red).

Code	Site name	State
A	Wintoon	Queensland
B	Weigh Point	Queensland
C	Altona	Queensland
D	Wagga Wagga	New South Wales
E	The Rock	New South Wales
F	Condobolin	New South Wales
G	Colbinabbin	Victoria
H	Mocardy	Western Australia
I	Charters Towers	Queensland

Code	Site name	State
J	Inkerman	Queensland
K	Scaddan	Western Australia
L	Pinaroo Highway	South Australia
M	Kimba	South Australia
N	Border Town	South Australia
M	Bridgewater	Tasmania
N	West Mackay	Queensland
O	Oakabella	Western Australia
P	Ponde*	South Australia

*herbarium specimen

Output 6(e) - Undertake bioclimatic models to identify optimal locations and conduct native range surveys and host-specificity tests for potential biocontrol agent(s) and import at least one potential agent in quarantine.

Bioclimatic modelling

The approaches taken for the bioclimatic modelling of *C. bonariensis* were like those used for *L. ferocissium* outlined above; Match Climates and Compare Locations models developed using the CLIMEX package. An experiment was also conducted to measure the growth rate of *C. bonariensis* at different temperatures. Results are presented in Figure 40 to Figure 42.

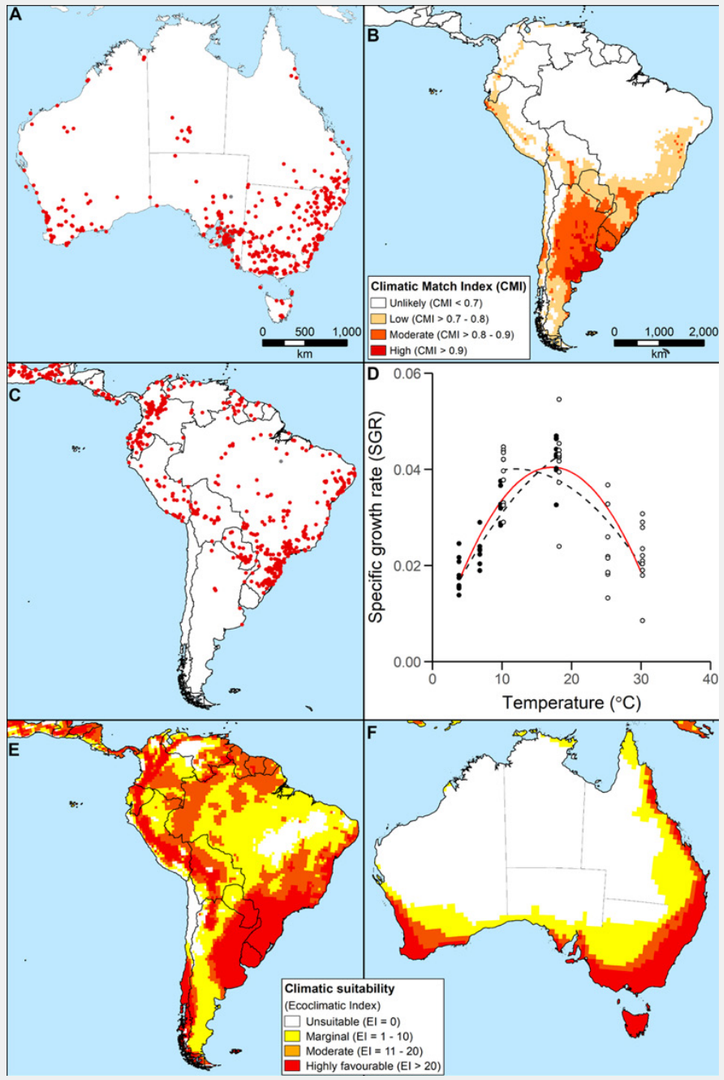


Figure 40. *Conyza bonariensis*' known distribution in (A) Australia and (C) the native range of South America. Preserved and living specimen records (red points) projected atop observational records (grey points) (GBIF.org 2nd November 2018). (B) CLIMEX Match Climates model, as projected for South America. (D) Temperature response curve. Filled and unfilled points indicate two separate experimental runs, with associated fitted polynomial models as dashed black lines. Final fitted polynomial model incorporating both datasets shown as a red line. Projected climatic suitability from the CLIMEX Compare Locations model projected for South America (E) and Australia (F).

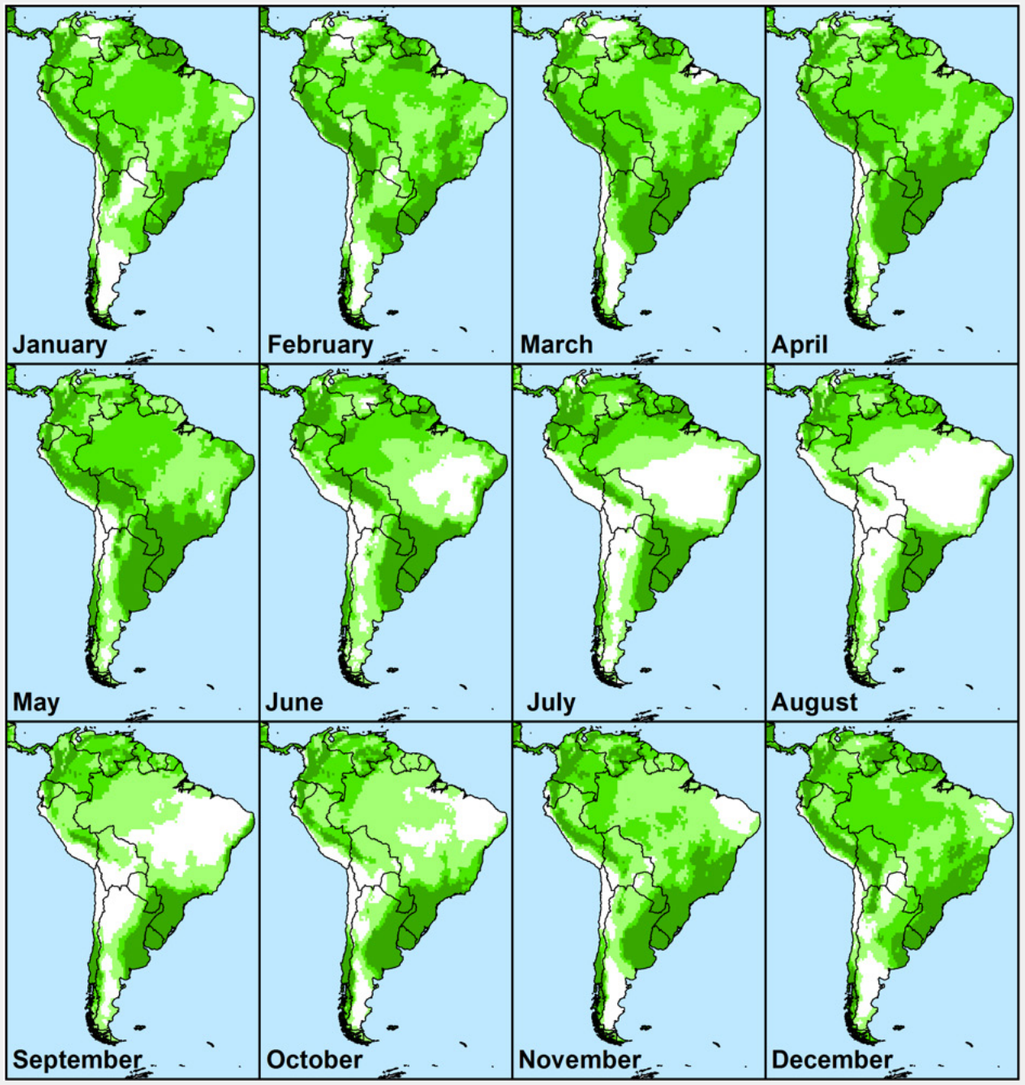


Figure 41. Monthly Growth Index values in native range for guiding when and where to survey for natural enemies on *Conyza bonariensis*. Values are averaged across five years from 2012 to 2017. Surveying is recommended within areas in which the Ecoclimatic Index is most suitable, indicating potential for year-round survival. Increased intensity of green colour indicates higher climatic suitability.

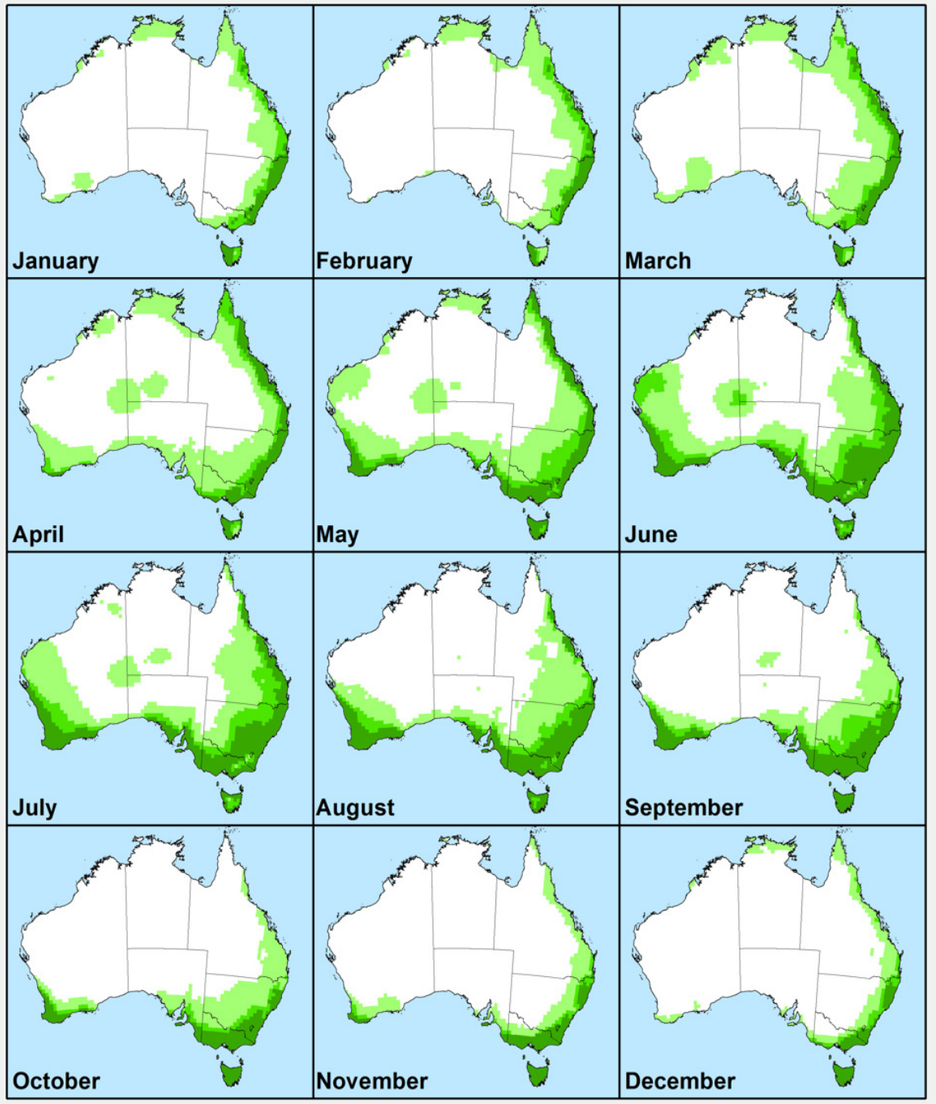


Figure 42. Monthly Growth Index values in Australia for guiding when and where to release biocontrol agents on *Conyza bonariensis*. Values are averaged across five years from 2012 to 2017. Agents would only be deployed in areas in which the Ecoclimatic Index was most positive, indicating potential for year-round survival. Increased intensity of green colour indicates higher climatic suitability.

Project outcomes

Native range surveys

Pathogens

Surveys for pathogens on *Conyza* species were performed in different departments of Colombia between November 2017 and May 2019. Surveys concentrated on Colombia because several rust fungi had only been recorded from *Conyza* sp. in this country in South America. A total of 136 *Conyza* sp. samples with disease symptoms were collected across all surveys during that period. High morphological diversity in *Conyza* species was observed in the field and it was difficult to categorically identify plants to species level. Consequently, plant tissue was collected from representative specimens and DNA extracted and sequenced to obtain a more reliable identification. Although some collections were made on *C. bonariensis*, most were made on *C. sumatrensis*. Collaborators in Colombia had the opportunity to present a few of their *Conyza* sp. herbarium specimens to Dr John Pruski was visiting the plant herbarium in Medellín during winter 2019. Dr Pruski is an expert taxonomist on the Asteraceae family based at the Missouri Botanical Garden in St. Louis, MO, USA. He identified each of the five herbarium specimens presented to him as either *C. sumatrensis* var. *leiotheca* or *C. sumatrensis* var. *sumatrensis*.

Disease symptoms observed during the field surveys ranged from chlorotic or necrotic lesions on leaves or stems, mildew and rust. Several fungi were recovered from the symptoms, including *Basidiophora entospora*, *Oidium* sp., *Cercosporella* sp., *Septoria* sp., *Wentiomyces* sp., *Diaporthe* sp. (confirmed with sequencing) and *Alternaria* sp. (Figure 43). Two rust fungi were also found: a species with *aecia* on *Conyza* sp. and the microcyclic species identified as *Puccinia cnici-oleracei*. The former rust fungus was later confirmed in a cross-inoculation study to be a heteroecious, macrocyclic rust species, with *Cyperus* sp. as its main host and *Conyza* sp., thus, unsuitable for biocontrol.

Insects

Surveys for insects have been undertaken on *Conyza* species in Argentina, Paraguay, Brazil, Colombia and the southern USA (Louisiana, Texas, and Alabama) between November 2017 and February 2020. These surveys have identified herbivorous insects from some 14 families (Agromyzidae; Cecidomyiidae; Cerambycidae; Curculionidae; Lixidae; Membracidae; Miridae; Mordellidae; Pseudococcidae; Pterophoridae; Tephritidae; Tingidae; Tortricidae; Coccidae) associated with *Conyza* spp. in the native range. Some 35 species/morphospecies have been identified to date, and among these the most promising are two species of gall flies (*Trupanea bonariensis* that forms stem galls; an unidentified fly that forms leaf-blister galls), a weevil (*Lixus* sp., a stem- and root-feeder) and two species of moths (both unidentified, both leaf rollers). These species have all been recorded in Argentina and Brazil, in areas of strong bioclimatic similarities to where *C. bonariensis* occurs as a weed in Australia. Several of the insect species in the native range are new to science and are in the process of being identified by taxonomic experts.

Host-specificity tests for potential biocontrol agents

The proposed list of non-target species for host-specificity testing of candidate biocontrol agents for *C. bonariensis* was submitted to DAWE in December 2018 for posting on their website for feedback (Hunter et al. 2018, Appendix 12).

Pathogens

A cross-inoculation experiment was performed in Colombia to obtain an initial indication of the specificity of *P. cnici-oleracei*. The experiment included accessions of *P. cnici-oleracei* recovered from *Conyza* sp. and *Emilia sonchifolia*, which were growing in proximity at the same site. Both *Conyza* (=*Erigeron*) and *Emilia* species are recorded as hosts of *P. cnici-oleracei*, although the fungus on *Emilia* is also referred to as *Puccinia emiliae* by some authors (Farr and Rossman 2020). Results demonstrated that the rust accessions were capable of only infecting the host species they originated from. Plants of *Conyza* sp., but not of *E. sonchifolia*, developed disease symptoms when exposed to rust-infect *Conyza* sp., and vice versa. Based on these results, we concluded that the fungus from *Conyza* sp. was probably highly specific and thus decided to refer to it as *P. cnici-oleracei* (ex. *Conyza*).

Comprehensive host-specificity testing in quarantine began in February 2019 and only one experiment remained to be performed. The test list comprises a total of 50 closely related, non-target species in the subfamily Asteroideae of the family Asteraceae that occur in Australia (ornamental, weed and native). Most species have been tested in at least two separate experiments using different accessions of each plant species, with *C. bonariensis* plants used as positive controls in all experiments.

Our results thus far showed that *P. cnici-oleracei* (ex. *Conyza*) is highly host specific to *C. bonariensis*. The fungus successfully developed and produced telia only on the nine Australian accessions of *C. bonariensis* tested. While *Conyza* sumatrensis and *Bidens pilosa* developed necrotic flecks and in some instances a few large necrotic blotches, the fungus never produced any telia on these species. Chlorotic flecks developed on one accession of *Calendula officinalis*, and a few, rare pin-sized telia were observed on one replicate of *Eschenbachia leucantha*. Inoculation of *C. bonariensis* using these pin-sized telia did not result in any infection. All other non-target plant species tested did not develop any visible symptoms and were rated as either immune or highly resistant based on microscopic examinations of the development of the fungus on these species.

Figure 43. Some of the fungi associated with symptoms observed on *Conyza* sp. during field surveys in Colombia. A: *Basidiophora entospora*, B: *Oidium* sp., C. *Cercosporella* sp., D. Cercosporoid fungus E: *Septoria* sp., F: *Periconia* sp., G: *Wentiomyces* sp., H: *Diaporthe* sp., I. *Alternaria* sp., J: *Puccinia cnici-oleracei*, K: *Puccinia cyperi* (aecium). Photos Universidad Nacional de Colombia, Medellín.

Insects

Field observations of host-specificity have been made on insects recorded to date. As part of these field surveys, assessments were made on co-occurring Asteraceae species (including species in the genera *Eupatorium*, *Chromolaena*, *Ageratrum*, *Senecio*, *Bidens* and *Baccharis*) to see if the insects being recorded on *Conyza* species are also found on these species. *Trupanea bonariensis* that forms stem galls, an unidentified fly that forms leaf-blister galls, a stem- and root-feeder weevil *Lixus* sp. and two species of moths (both unidentified, both leaf rollers) show promise for biocontrol. They cause significant damage and are seldom seen on co-occurring species in the Asteraceae in the field. Colonies of these species have been established in Brazil to elucidate their biology prior to importation into a quarantine facility in Australia to undergo detailed host-specificity testing (as part new Rural R&D for Profit project (AgriFutures Australia Project number: PRJ-12377; 2019-2022)).

Importation of potential agents in quarantine

Pathogens

Puccinia cnici-oleracei (ex. *Conyza*) was deemed the most promising candidate biocontrol agent to investigate. The necessary export permit was obtained from the relevant Colombian authorities. Concurrently, a permit to import the fungus in the CSIRO quarantine facility in Canberra was obtained from DAWE. The fungus was imported on 20 November 2018. A single-telium isolate was generated from the material imported and a culture established in quarantine.

Insects

Trupanea bonariensis, the stem gall forming tephritid fly, was imported in a quarantine facility in Australia in November 2019 and February 2020. Colonies of this fly are still in the process of being established as a precursor to detailed biological studies and host-specificity testing. This work will be undertaken as part of the new Rural R&D for Profit project (AgriFutures Australia Project number: PRJ-12377).

Output 6(f) - Pending risks to non-target plants are acceptable, submit application to the Commonwealth regulators seeking approval to release at least one potential agent. Upon receiving approval, release biocontrol agent(s)

Host-specificity testing with the rust fungus *P. cnici-oleracei* (ex. *Conyza*) is expected to be completed by the end of April 2020. A draft of the release application has been prepared. Provided results of the last experiment do not identify unacceptable risks with this candidate agent, the application for release will be submitted to DAWE.

Output 6(g) - Explore options for integration of biocontrol with other management techniques

Conyza spp. (incl. all three exotic *Conyza* spp.) and *S. oleraceus* are similar in terms of their impacts in grain production systems. So, the options for integration of biocontrol with other management techniques are somewhat analogous.

Both weeds are principally managed through chemical and cultural control techniques in agricultural/cropping systems during the growing season (Wu 2007; Widderick 2014; Widderick & van der Meulen 2016; Widderick et al. 2004). This involves a combination of chemical control using post emergence herbicides of two herbicides (double-knock; either as a mix or applied sequentially) for in-crop control, and the use of a residual herbicide in fallows. The use of this single tactic has resulted in herbicide resistance in both weeds. Cultural tactics include decreased row spacing for in-crop weed management (Wu & Walker 2004), and the strategic use of tillage for burial of weed seed to below 2 cm depth in fallows (Werth & Walker 2007); the latter disrupts the benefits of minimum tillage farming.

Chemical approaches can manage both weeds effectively at a cost, but there is an opportunity for integration with biocontrol to possibly reduce costs and to preserve the utility of the effective herbicides by delaying resistance. Biocontrol agents (e.g. like *Puccinia cnici-oleracei* (ex. *Conyza*) being developed as part of this project) could serve as chronic stressors to the weed in fallows and outside cropped areas and limit reproductive output of the weed, thereby reducing the risk of seedbank build-up in fallows and also the risk of spreading into fields from surrounded non-cropped areas.

The utility of biocontrol suppressing weed performance in unmanaged contexts (i.e. beyond crop fields and fallows) could further limit the rate of in-crop incursion of weed seeds within the growing season.

As for *L ferocissimum*, integration of biocontrol with chemical and cultural control tactics will require coordination among land managers and consultants recommending/deploying management tactics for *Conyza* spp. and *S. oleraceus*.

3.1.4 Sowthistle (*Sonchus oleraceus*)

Output 7(a)-Undertake a literature review on taxonomy and distribution of sowthistle and known natural enemies of the weed in the introduced and native ranges

Taxonomy

Sonchus is a cosmopolitan genus and currently includes 55-60 species (Thompson 2007, 2015a). Most *Sonchus* species are biennial or perennial with woody roots or rhizomes. Morphologically, species of *Sonchus* are characterised by their simple, glandular or eglandular hairs, basal and cauline leaves, cymose capitulescence, pedunculated capitula, multiseriate involucral bracts that are reflexed at maturity, yellow flowers, homomorphic achenes that are unbeaked and compressed, pappus of partially persistent almost smooth bristles of two types (Thompson 2007, 2015a). Within Australia, there is one native *Sonchus* species, S. hydrophilus, and three naturalised species: *S. oleraceus*, *S. asper* and *S. asper* var. *asper* (Thompson 2007, 2015a).

A recent study has demonstrated that climatic conditions may have driven rapid adaption of *S. oleraceus* in its introduced ranges in Australia and New Zealand (Ollivier et al. in press). Differences in 20 traits (relating to growth, resource acquisition, reproduction, phenology and defence) amongst 14 populations of the herbaceous plant *S. oleraceus L.* (Asteraceae) across its native (Europe and North Africa) and introduced (Australia and New Zealand) ranges were investigated in a glasshouse experiment. Introduced *S. oleraceus* plants possessed higher leaf and stem dry matter content, greater number of leaves and were taller at first flowering stage than plants from the native range.

Sonchus oleraceus belongs to the subtribe Hyoseridinae (synonym Sonchinae) in the tribe Cichorieae, subfamily Cichorioideae in the family Asteracea (Killian et al. 2009). The genus *Launaea* is closely related to *Sonchus*. *Launaea sarmentosa* is the only species of this genus that is native to Australia (Thompson 2015b). *Reichardia* is the next genus related to *Sonchus* and *Launaea*. It is a small genus of approximately eight species that is native to the Mediterranean and two species of this genus, *R. tingitana* and *R. picroides* have naturalised in Australia (Thompson 2015c). *Hyoseris* and *Aposeris* are the last two genera in the subtribe Hyoseridinae. Based on recent taxonomic revisions (Kilian et al. 2009; Thompson 2015d), there are no species of *Hyoseris* which currently occur in Australia. There is no record of *Aposeris foetida*, the sole species in this genus, occurring in Australia

Distribution

Sonchus oleraceus has a very broad global geographic distribution and can be found in temperate, tropical and subtropical climates (Kilian et al. 2009) (Figure 44). It is native to Europe, Macaronesia (Madeira Islands, Canary Islands), North Africa (Algeria, Egypt, Libya, Morocco, and Tunisia) and South-Western Asia (CABI 2020). The greatest diversity of Sonchus is regarded to be in the Western Mediterranean (Mejias & Andres 2004).

Figure 44. Graphical representation of the worldwide distribution of *Sonchus oleraceus* (common sowthistle) (CABI 2020).

Project outcomes

In Australia, *S. oleraceus* is widespread and found in all States and Territories but appears to be most prevalent in the southern half of the continent. There are more than 33,000 records of *S. oleraceus* in the Atlas of Living Australia (ALA 2017) (Figure 8).

Natural enemies

The comprehensive review of the literature we undertook revealed that 23 fungi and 7 insects are recorded on *S. oleraceus* in Australia. In Europe, 22 fungi and 75 insects, different to those reported in Australia, have been recorded as infecting and feeding on *S. oleraceus*, respectively. Among these fungi found on *S. oleraceus* in the native range, *Bremia lactucae* (species specific strains if they exist), *Bremia sonchi, Septoria sonchifolia* and *Entyloma bullulum* were singled out as those with the most promise as potential biocontrol agents. The specialist insects T*ephritis dilacerata*, *Contarinia schlechtendaliana*, *Cystiphora sonchi*, *Botanophila sonch*i and *Aceria sonchi*, were also of interest because of their potential restricted host range. (Appendix 13, Appendix 14)

More recently, a PhD study affiliated with this project combined literature and field surveys, to document 17 phytophagous arthropod species, mostly generalists of exotic origin, able to feed and develop on *S. oleraceus* in Australia (Ollivier et al. in press; Appendix 15). The capitula/flower heads were the most damaged plant part while stems were relatively free from insects, except aphids.

Output 7(b) - Define goals for management of sowthistle

Since both *S. oleraceus* and *C. bonariensis* are weeds affecting cropping systems, a combined survey of key stakeholders in the grains industry on the impacts and desired management goals for these weeds, was performed. See results above in *Output 6(b) - Define goals for management of fleabane.*

Output 7(c) - Nominate a sowthistle biocontrol target

The project prepared the documentation to support the nomination of *S. oleraceus* as a target for biocontrol. The documentation was submitted to the IPAC (now EIC), by the New South Wales Department of Primary Industries in August 2017 and endorsed by the Committee in November 2017 (Hunter and Ireland 2017, see Appendix 16).

Output 7(d) - Conduct genetic analysis on samples of sowthistle from different regions in Australia and the native range

We have analysed a large genomic dataset to determine the origins of *S. oleraceus* introductions into Australia in order to guide field surveys for natural enemies with potential for biocontrol. Detailed results from this study are available from CSIRO.

Leaf material of *S. oleraceus* was collected from 27 sites (6-18 plants per site) across its native range in Europe and North Africa as well as 17 sites (9-15 plants per site) across Australia (Figure 45). Each leaf was subsampled and forwarded to Diversity Arrays Technology (DArT) in Canberra for DNA extraction, quantification and genotyping. The Single Nuceotide Polymorphism (SNP) dataset from DArT consisted of 33,959 loci. The SNP dataset was filtered using a call rate threshold > 95%, leaving us with 11,405 loci. We removed monomorphic loci (as a result of removal of individuals), outliers based on individual's observed heterozygosity to eliminate potential mixed genomes and duplicate individuals, leaving 2883 SNP markers and 547 individuals.

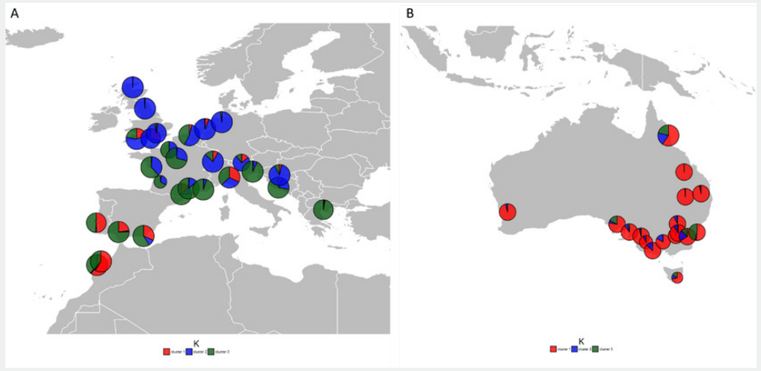


Figure 45. Geographic map of sampling locations of *S. oleraceus* in A) native range (Europe/North Africa) and B) introduced range (Australia). Pie charts show STRUCTURE results for K=3 considering all sampling locations (Fig. 26). Circle sizes represent relative number of samples per population.

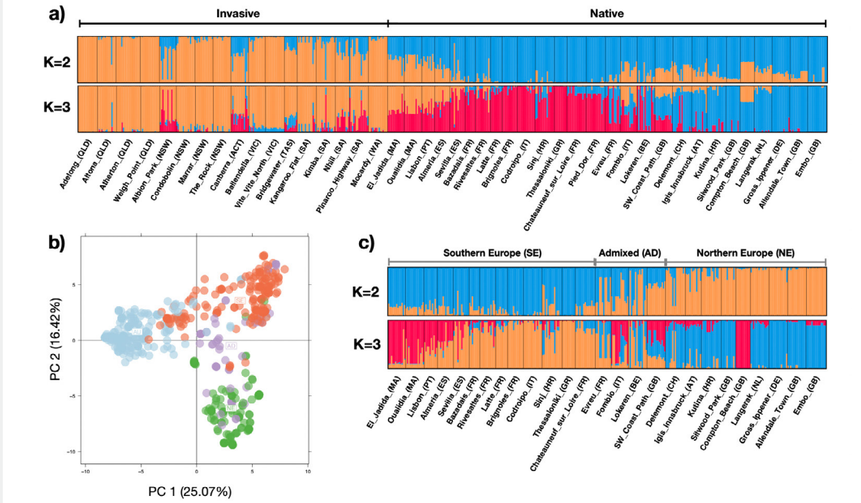


Figure 46. STRUCTURE output and PCA results for 2,883 unlinked loci for sampled populations of *Sonchus oleraceus* across the native (Europe/North Africa) and introduced (Australia) ranges. (A) STRUCTURE output for K=2 (optimal clustering level) and K=3 considering all sampling locations. (B) Principal component analysis considering all sampling locations; Northern Europe (green), Southern Europe (red), Admixed zone (purple) and Australia (light blue), based on STRUCTURE output considering only the native range for K=2 (optimal clustering level). (C) STRUCTURE output for K=2 (optimal clustering level) and K=3 considering only sampling locations of the native range (Europe/North Africa).

Our STRUCTURE analysis found low levels of genetic structure within the native and introduced range of *S. oleraceus* indicating a clustering level of *K=2* (Figure 45, Figure 46), according to *ΔK* method. The analysis clearly separated the native (Europe/North Africa) and introduced (Australia) ranges of *S. oleraceus*. There were some exceptions to this, showing mixed assignments in Southern Europe and North Africa (Spain, Portugal and Morocco) as well as two populations in the introduced range (ACT and NSW). The analysis also showed that for *K=3* (second highest *ΔK* value) the native range separates into two genetic clusters: Southern Europe and Northern Europe, revealing subpopulation structure in Europe (Figure 46c).

The PCA analysis confirmed the patterns found by STRUCTURE showing a separation of the large genetic clusters in Europe/ North Africa as well as the genetic cluster in Australia (Figure 46b). The PCA explained 41.49% of the total genetic variance for the two first principal components (PC) showing genetic variability among the native and introduced ranges. The first PC differentiates Europe/North Africa and Australia, while the second PC separates the native range into Northern and Southern Europe (the latter including North Africa samples) showing partial overlap with Admixed zone sampling locations.

To test multiple invasion scenarios of *S. oleraceus* into Australia we used an approximate Bayesian computation framework (ABC). Evolutionary relationships among the sampled populations were also evaluated with the software TreeMix. We simulated invasion scenarios from non-admixed and admixed source populations of their native range including multiple introductions to Australia from Europe (including North African populations). We selected the most likely invasion scenario using the abcrf R package, which uses a novel approach based on random forest (RF) machine learning algorithms. For detail on methods and results see Encinas-Viso et al. (in preparation; Appendix 25). The ABCRF analysis shows that the most likely invasion scenario of S. oleraceus into Australia was an initial introduction from the Northern Europe cluster and a secondary, more recent introduction from Southern Europe/North Africa cluster. The TreeMix and STRUCTURE analyses also clearly supported the scenario of multiple introductions from different regions of the native range and post-introduction admixture.

Project outcomes

Output 7(e) - Undertake bioclimatic models to identify optimal locations and conduct native range surveys and host-specificity tests for potential biocontrol agent(s) and import at least one potential agent in quarantine.

Bioclimatic modelling

As for *L. ferocissium* and *C. bonariensis,* Match Climates and Compare Locations models were developed for *S. oleraceus* using the CLIMEX package. The growth rate of *S. oleraceus* at different temperatures was also measured in an experiment. (For further information, contact CSIRO) Results are presented in Figure 47 to Figure 49.

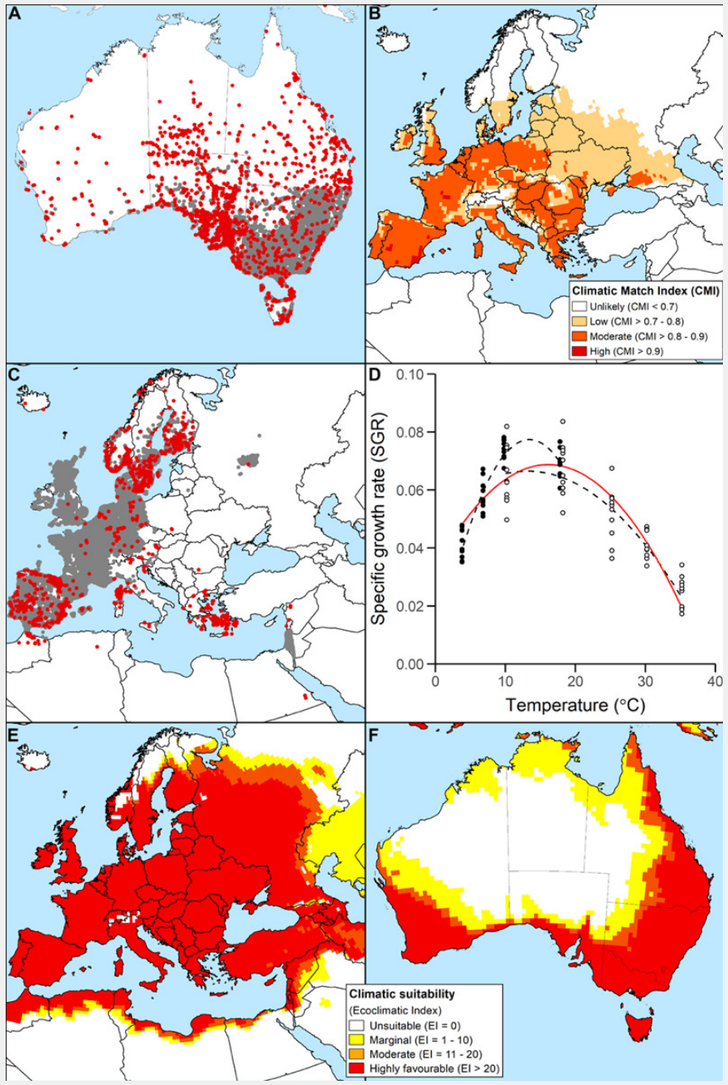


Figure 47. *Sonchus oleraceus.* Current distribution in (A) Australia and (C) the native range of Europe. Preserved and living specimen records (red points) projected atop observational records (grey points) (GBIF.org 24th July 2018a). (B) CLIMEX Match Climates model, as projected for Europe. (D) Temperature response curve. Final fitted polynomial model incorporating both datasets shown as a red line. Projected climatic suitability from the CLIMEX Compare Locations model projected for Europe (E) and Australia (F).

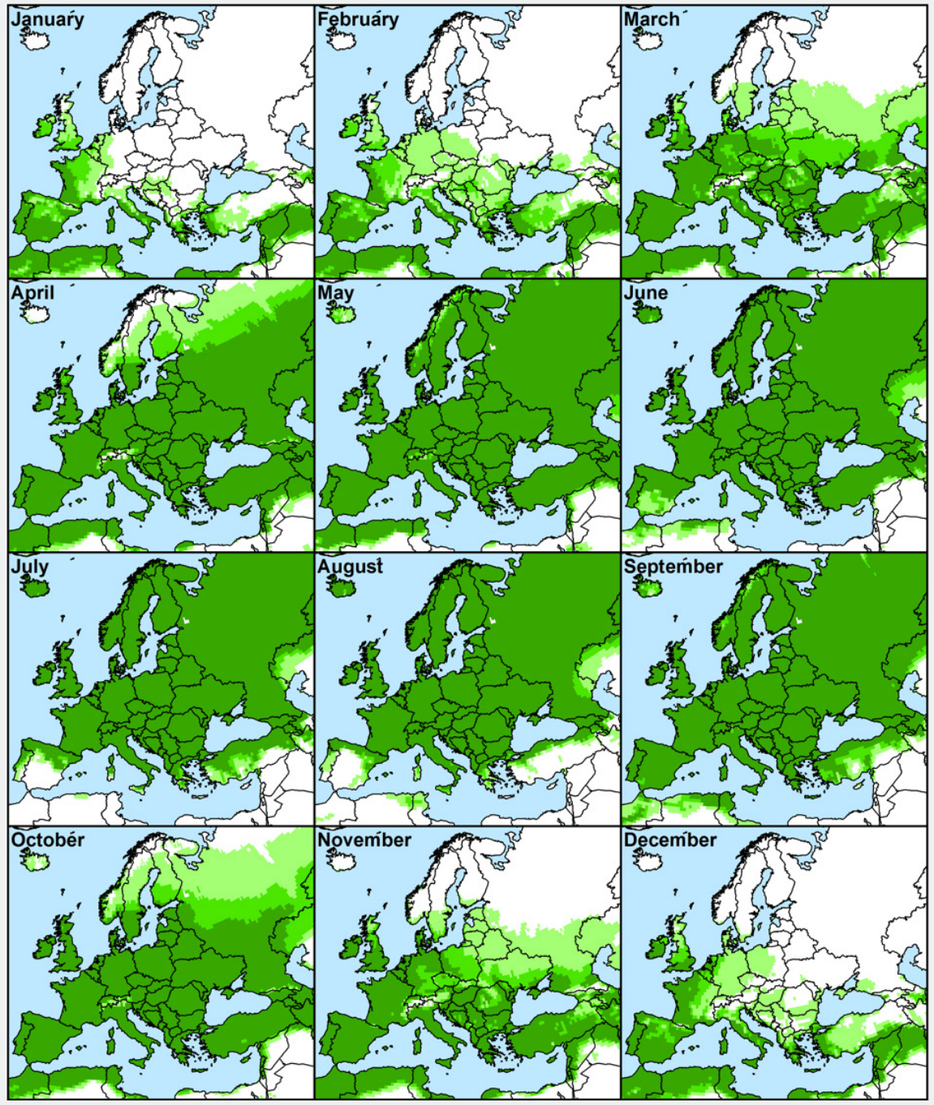


Figure 48. Monthly Growth Index values in native range for guiding when and where to survey for natural enemies on *Sonchus oleraceus.* Values are averaged across five years from 2012 to 2017. Surveying is recommended within areas in which the Ecoclimatic Index is most suitable, indicating potential for year-round survival. Increased intensity of green colour indicates higher climatic suitability.

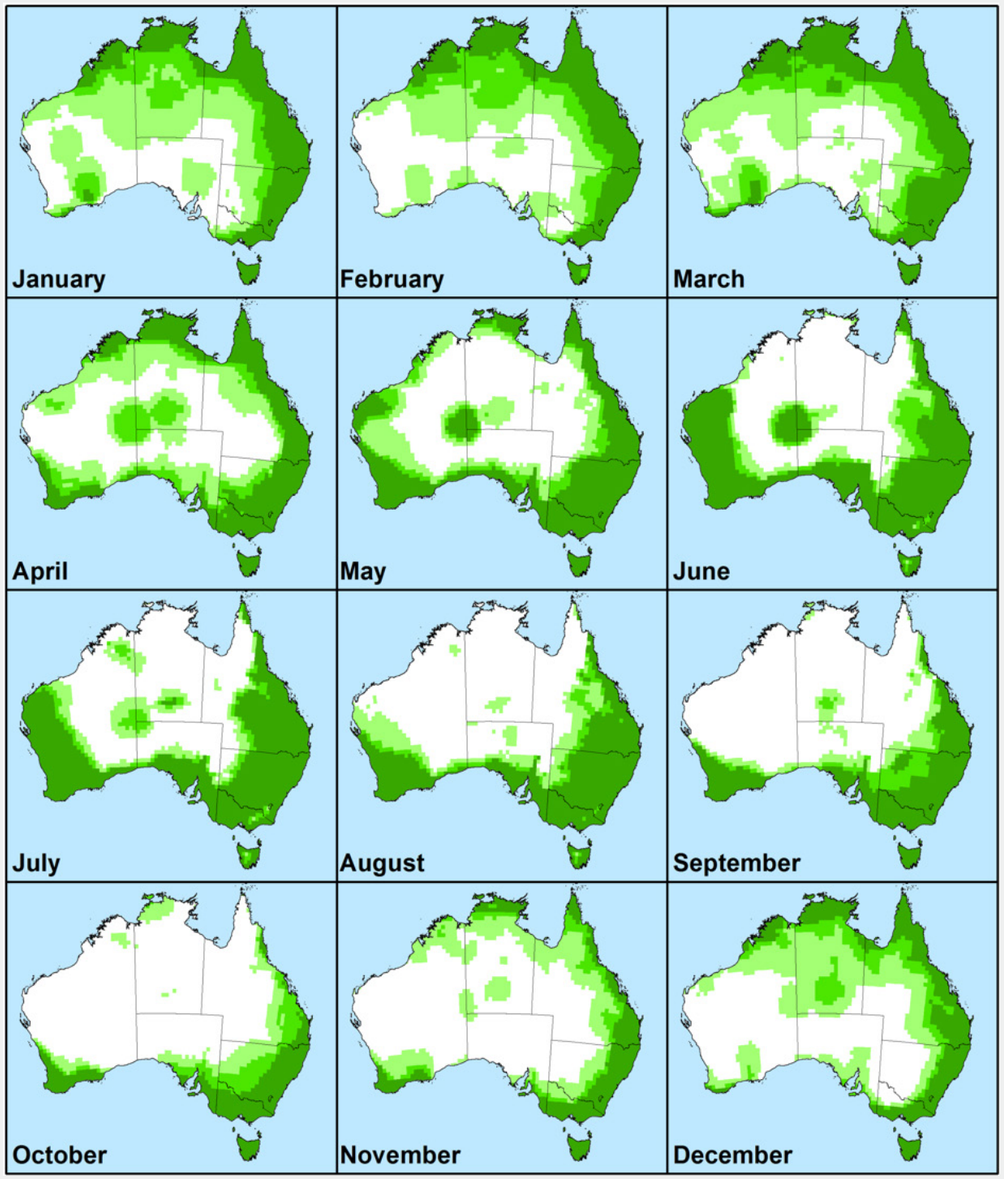


Figure 49. Monthly Growth Index values in Australia for guiding when and where to release biocontrol agents on S*onchus oleraceus*. Values are averaged across five years from 2012 to 2017. Agents would only be deployed in areas in which the Ecoclimatic Index was most positive, indicating potential for year-round survival. Increased intensity of green colour indicates higher climatic suitability.

Native range surveys

Native range surveys were conducted between March 2017 to March 2020, mainly in spring and autumn. The selection of areas surveyed was mostly based on parallel bioclimatic modelling that identified northern Africa and the Southern European edge of the *S. oleraceus* range as most climatically similar to regions where the species is a problem in Australia. A genetic analysis also identified this broad region as one of the likely sources of *S. oleraceus* populations in Australia. Surveys were thus concentrated in southern Portugal, southern France, northern Italy, the Balkans and Greece, with a particular attention in Morocco and southern Spain. Surveys were also performed in the Canary Islands, in the Macaronesian region off the northern African west coast. Short surveys were also conducted in northern France, Belgium, the Netherlands and Germany. Some sites were visited more than once across the years, especially in Southern Spain, Morocco and within the vicinity of Montpellier, southern France. In the latter, surveys were conducted regularly during the year to gather phenology data on the natural enemies present. (For further information, contact CSIRO).

Pathogens

Of the many fungi recovered from disease symptoms during the surveys, 10 were classified as pathogenic once their identification was confirmed. Examination of morphological characters, supplemented with sequencing, was performed to confirm their identity. The rust fungus, *Miyagia pseudosphaeria*, which already occurs in Australia, was found in several of the regions surveyed. Another rust fungus, *Coleosporium sonchi*, was found at one site in the Netherlands and four sites in Brittany (France). Prior to sequencing, the fungus could not be morphologically identified because teliospores were not present. A downy mildew morphologically identified as *Bremia* sp. was found at several sites and is in the process of being sequenced for species identification. A powdery mildew, *Golovinomyces sonchicola* or *G. cichoracearum*, and the leaf spot fungus *Alternaria sonchi*, were found to be common. Sequences of another leaf spot fungus, originally identified as *Ascochyta* sp. using morphological characters, were found to be highly similar to that of *Didymella rosea*. Two leaf spot fungi, morphologically identified as generalist *Phoma* species, were molecularly identified as *Didymella glomerata* (or *Didymella fabae* or *Phoma* sp.) and *Didymella* sp. Other leaf spot fungi isolated were *Ramularia helminthiae* and *Septoria sonchi*.

Insects

Fifty-eight phytophagous insect species, across seven orders, but primarily *Diptera* and *Lepidoptera*, and one mite species were collected on *S. oleraceus* during the surveys (For further information, contact CSIRO) Identifications were obtained by a combination of morphological and molecular approaches. Most of the species collected were polyphagous or oligophagous species (n = 38 (65%) and n = 2 (3%), respectively). Only a few insects specialized on the genus *Sonchus* were found (n = 2 (3%)).
For three species, the host range was unknown due to the lack of identification to species level or the lack of information within the literature. Many of the species were ectophagous sucking and chewing insects (31% and 25%, respectively), while the endophagous guilds were dominated by mining insects. The most damaged part of the plant was the flower heads, with 39 % of the recorded species possibly using it as feeding resource. No severe damage was observed on roots and only few species appeared to be associated with this feeding niche. Four insect species offered the most promise as potential biocontrol agents based on their identities, data from the literature and the damage on *S. oleraceus* observed in the field. The fruit fly *Tephritis formosa* (Diptera: Tephritidae) was commonly found and infested capitula of *S. oleraceus* and *S. asper* during the survey. Another fruit fly, *Campiglossa producta* was only collected in the Canary Islands (Spain). The syrphid fly *Cheilosia latifrons* (Diptera: Syrphidae), for which larvae mined the stems and the root-crown, was commonly found around Montpellier. The species was also collected in northern Spain. The leaf gall midge, *Cystiphora sonchi* (Diptera: Cecidomyiidae) was widespread across the sites surveyed.

Host-specificity tests for potential biocontrol agents

The proposed list of non-target species for host-specificity testing of candidate biocontrol agents for *S. oleraceus* was submitted to DAWE in December 2018 for posting on their website for feedback (Hunter and Morin 2018, see Appendix 27). Initial host-specificity testing with fungal pathogens and insects with potential for biocontrol in Australia was performed at the CSIRO European Laboratory in Montpellier, France.

Pathogens

Six pathogenic fungi recovered from *S. oleraceus* were tested: *Alternaria sonchi*, *Bremia* sp., *Coleosporium sonchi*, *Didymella rosea*, *Ramularia helminthiae* and *Septoria sonchi* (Lesieur et al. Appendix 19). These tests included *S. oleraceus* as a positive control and two closely related species that are native in Australia; S*onchus hydrophilus* and *Actites megalocarpus*. All fungi infected and caused disease symptoms on all three plant species, thus showing no promise for the biocontrol of *S. oleraceus* in Australia (Figure 50).

Project outcomes

Insects

An abridged phylogenetically based test list comprising ten plant species was used for initial screening of the specificity on the first insect candidate agent found, the gall midge, *C. sonchi*. Results of no-choice (Table 3) Choice tests showed that the midge could develop species in the same sub-tribe as *S. oleraceus*, including the two native species *S. hydrophilus* and *A. megalocarpus*.

More details in Lesieur et al. Appendix 19). Based on results from these tests, a decision was made to concentrate initial testing of the other promising candidate insects on these two important native species.

The three other insect species tested, *T. formosa*, *C. producta* and *C. latifrons* were found to oviposit, develop and complete their life cycle on *S. oleraceus* and the two native species (Table 4).

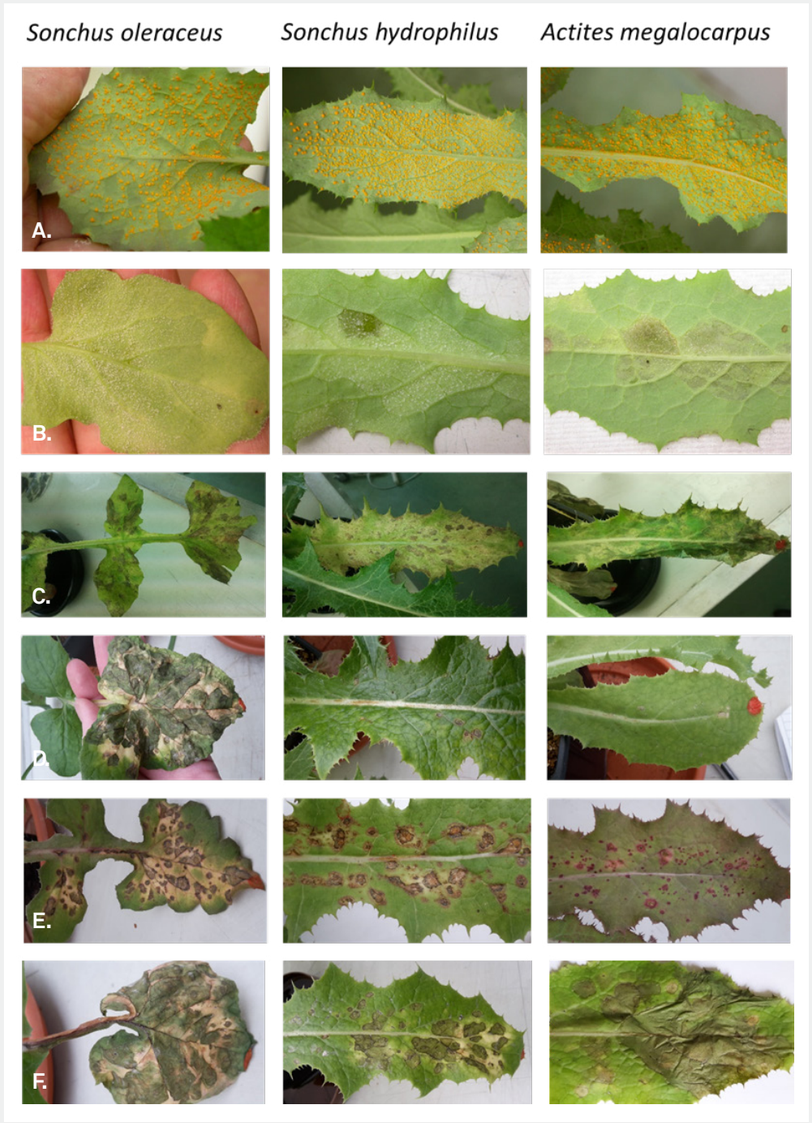


Figure 50. Disease symptoms observed during initial host-specificity tests on *Sonchus oleraceus* and closely related species inoculated with the following fungi:
A. *Coleosporium sonchi*,
B. *Bremia* sp.,
C. *Septoria sonchi*,
D. *Didymella rosea*,
E. *Alternaria sonchi*,
F. *Ramularia helminthiae*.

Table 3

Results of no-choice host range tests for Cystiphora sonchi.

Sub-tribe / Species	Importance in Australia	Infested plants / Tested plants	Proportion of infested leaves mean (± SE)*	Galls per plant mean (± SE)	Adults emerged mean % (± SE)
Hyoseridinae					
Sonchus oleraceus French origin	Invasive / Weed	6/6	0.75 (0.13) a	105.00 (36.63) a	87.90 (3.19) a
Sonchus oleraceus Australian origin	Invasive / Weed	6/6	0.93 (0.05) a	168.67 (50.73) a	90.10 (1.93) a
Sonchus asper	Invasive / Weed	5/6	0.74 (0.07) a	113.60 (30.72) a	85.18 (7.51) ab
Sonchus hydrophilus	Native	6/6	0.75 (0.07) a	188.67 (76.35) a	66.01 (7.56) b
Actites megalocarpus	Native	6/6	0.88 (0.03) a	214.17 (39.14) a	82.92 (1.49) ab
Reichardia tingitana	Invasive / Weed	6/6	0.55 (0.13) a	66.00 (32.68) a	1.27 (0.85) c
Lactucinae					
Lactuca sativa	Crop	0/6	-	-	-
Lactuca serriola	Invasive / Weed	0/6	-	-	-
Hypochaeridinae					
Helminthotheca echioides	Invasive / Weed	0/6	-	-	-
Cichoriinae					
Cichorium endivia	Crop	0/6	-	-	-
Cichorium intybus	Crop	0/6	-	-	-

The sub-tribes have been placed within the table to reflect their phylogenetic relatedness.
** Number of infested leaves / number of leaves exposed to the midges at the beginning of the test. Differences among the host plants were compared using ANOVA followed by Tukey's multiple comparison test. Means followed by different letters within columns indicate a significant difference ($p < 0.05$).*

Table 4

Results of host-specificity testing with three candidate insect agents.

Candidate	Plant Species	Infestation (±SE)		Survival (±SE)	
		No-choice test	Choice test	No-choice test	Choice test
T. formosa	*S. oleraceus*	39.26% (± 4.72) a	13.41% (± 2.41) a	94.01% (± 1.54)	93.62% (± 2.24) a
	S. hydrophilus	nt	nt	nt	nt
	A. megalocarpus	21.41% (± 4.98) b	3.58% (± 0.97) b	97.04% (± 1.77)	83.06% (± 5.41) b
C. producta	*S. oleraceus*	37.32% (± 4.89) a	48.99% (± 5.02) a	92.59% (± 2.48)	97.71% (± 1.25)
	S. hydrophilus	nt	nt	nt	nt
	A. megalocarpus	18.93% (± 4.12) b	31.64% (± 4.54) b	95.56% (± 3.11)	92.74% (± 3.81)
C. latifrons	*S. oleraceus*	-	1.50 (± 0.65)	66.7%	-
	S . hydrophilus	-	5.50 (± 2.86)	83.3%	-
	A. megalocarpus	-	1.88 (± 0.74)	66.7%	-

In addition to the above, host specificity tests focussed on candidate pathogen and insect agents, novel methodologies were developed to understand how multiple trophic levels may interact in the native vs invaded range to influence the efficacy of weed biocontrol (Ollivier et al. 2020; Appendix 19).

Infestation: *Tephritis formosa* and *Campiglossa producta* = percentage of infested capitula; *Cheilosia latifrons* = the total number of eggs laid per plant (only possible in choice test); *Cystiphora sonchi* = the number of galls per plant. **Survival:** T. *formosa* and *C. producta* = the percentage of adults emerged / total number of individuals produced (i.e. dead larva, dead pupa and adults emerged) per capitula; *C. latifrons* = the percentage of successful development from eggs to pupal stage (only possible in no-choice test); *C. sonchi* = the percentage of galls that resulted in an adult. For every candidate agent, within column means followed by different letters are significantly different within test plant species. nt = not tested.

Output 7(f) - Pending risks to non-target plants are acceptable, submit application to the Commonwealth regulators seeking approval to release at least one potential agent. Upon receiving approval, rear and release biocontrol agent(s)

None of the fungal pathogens and insects found on *S. oleraceus* in the native range, for which initial host-specificity testing was performed, are specific enough to be pursued further as possible biocontrol agents in Australia. While there may be additional natural enemies that could be found to have potential for biocontrol, this scenario is unlikely considering the considerable survey efforts undertaken in this project.

Discussions with GRDC (the principal co-investor for work on *S. oleraceus*) have commenced to explore meaningful ways forward for research on biocontrol of other grains weeds in the new Rural R&D for Profit project (AgriFutures Australia Project number: PRJ-12377).

Output 7(g) - Explore options for integration of biocontrol with other management techniques

Options for integration of biocontrol with other management techniques were jointly explored for *Conyza* spp. and *S. oleraceus* since they have similar impacts grain production systems. See text above in *Output 6(g) - Explore options for integration of biocontrol with other management techniques.*

3.1.5 Mother-of-millions (*Kalanchoe delagoensis*)

Output 8(a) Conduct native range surveys and host specificity tests on potential biocontrol agent(s)

The initial plan for the mother-of-millions biocontrol project was to import two potential biocontrol agents into Australian quarantine and conduct all the host range testing here. Two potential agents (*Rhembastus* sp. and the phyllode-feeding wasp, *Eurytoma bryophylli* from Madagascar) were not found during the life of this project despite repeated field visits over the life of the project.

Madagascan permitting requirements stipulated that there had to be an official relationship with the University of Antananarivo (UoA) and that a post-graduate student needed to be included in order to export any species. A post-graduate student was appointed who, played an important role in securing multiple export (insects) and import (plants) permits from Madagascar, and was been able to conduct open field host range studies using test plant species of interest to Australia.

The stem-boring weevil, *Osphilia tenuipes* and a new species of root-feeding beetle, *Bikasha* sp., were collected during native range surveys Cultures were established at UoA and the containment facilities in Orange and Brisbane where host specificity testing was undertaken.

Osphilia tenuipes

Impact trial

The impact trials conducted in Madagascar on various classes of plants very clearly showed that *O. tenuipes* has a significant impact on K. delagoensis. For example, the >30cm<40cm size class of plants had significant reductions in wet and dry weights, number of phyllodes, and number of bulbils (daughter plantlets located on phyllode tips) as a result of *O. tenuipes* feeding damage (Figure 51). This same trend was also observed for the <10cm, >10cm<20cm and >20cm<30cm size classes.

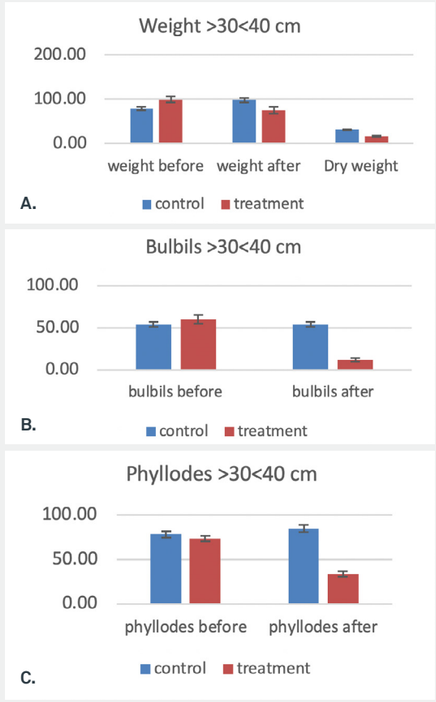


Figure 51. *Osphilia tenuipes* impact on *Kalanchoe delagoensis* (a) wet and dry weights, (b) number of phyllodes, and (c) number of bulbils (daughter plantlets located on phyllode tips) for the >30cm<40cm size class of plants.

Project outcomes

Closed field multiple-choice trial
These trials were completed in a 4x4x1.5m field cage (Figure 52). Species included in the trial were *K. delagoensis* (MoM), *K. blossfeldiana*, *K. spathulata*, *Kalanchoe daegremontiana*, *Kalanchoe prolifera*, *Kalanchoe pinnata*, *Kalanchoe miniata* and *Echeveria* sp. The plants were arranged randomly in eight radiating lines. One hundred adults were released at the centre of the cage, and observations were made every other day to record the position of the adults. After 15 days, the plants were dissected to record feeding damage, oviposition probes, eggs and larvae for each test species. *Kalanchoe pinnatum*, *K. miniata* and *Echeveria* sp. were unaffected by the weevil in all three replicates (Figure 53). The Madagascan ornamental, *K. blossfeldiana*, received significantly less feeding damage and oviposition probes than the control (MoM), however, the number of eggs and larvae recorded on this species were similar to the control. Similarly, *K. spathulata* received significant levels of feeding, oviposition probes, eggs and larvae (Figure 53). These two species, as well as the other two Madagascan test species (*K. prolifera* and *K. daegremotiana*) were all included in open field multiple-choice trials.

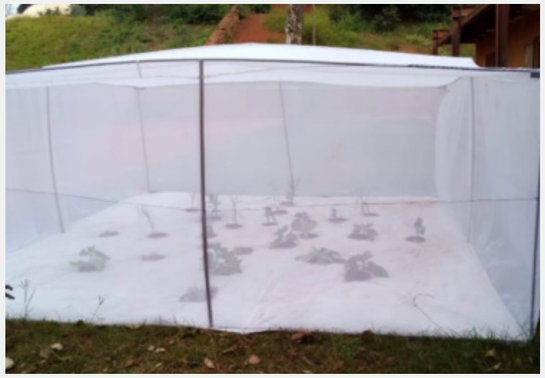
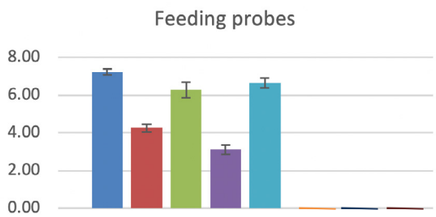


Figure 52. Closed field multiple-choice trial setup at UoA, used to investigate the host range of *Osphilia tenuipes*.



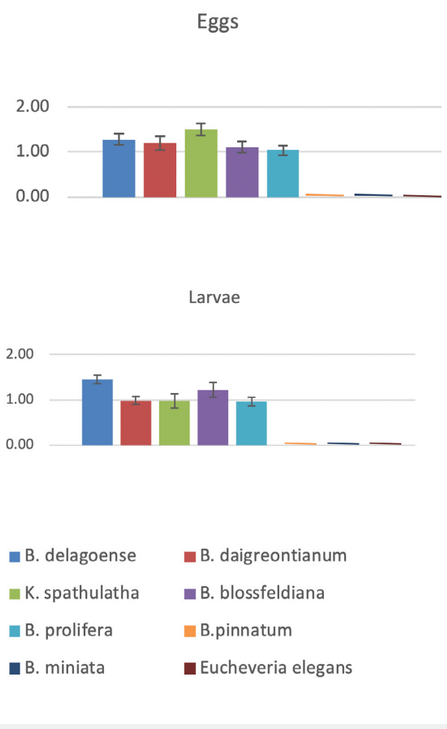


Figure 53. Closed field multiple-choice trial data for *Osphilia tenuipes*.

Open field multiple-choice trial
These trials were conducted in a large, open multiple-choice arena (7 x 7 m) in Madagascar (Figure 54) Species included in the trial were *K. delagoensis* (MoM), *K. blossfeldiana*, *K. spathulata*, *K.daegremontiana*, *K. prolifera*, *K. pinnata*, *K. miniata* and *Echeveria* sp. The plants were randomly arranged at one-meter intervals on eight radiating lines in the arena. One hundred adults were released at the centre of the arena and their position was checked and recorded every other day over a 15-day period. At the conclusion of the trial, all plants were dissected and feeding damage and oviposition were recorded. Feeding damage and oviposition were recorded on the following species: *K. delagoensis* (MoM), *K. blossfeldiana*, *K. spathulata*, *K. daegremontiana* and *K. prolifera* (Figure 55). As observed in the closed field multiple-choice trial, no feeding or oviposition was recorded for *K. pinnatum*, *K. miniata* and *Echeveria* sp. The species of interest for *Australia*, *K. spathulata* and *K. blossfeldiana*, both had significantly less feeding damage than *K. delagoensis*, however, oviposition by the weevil was similar for all three species (Figure 55).

Figure 54. *Osphilia tenuipes* open field multiple-choice trial.

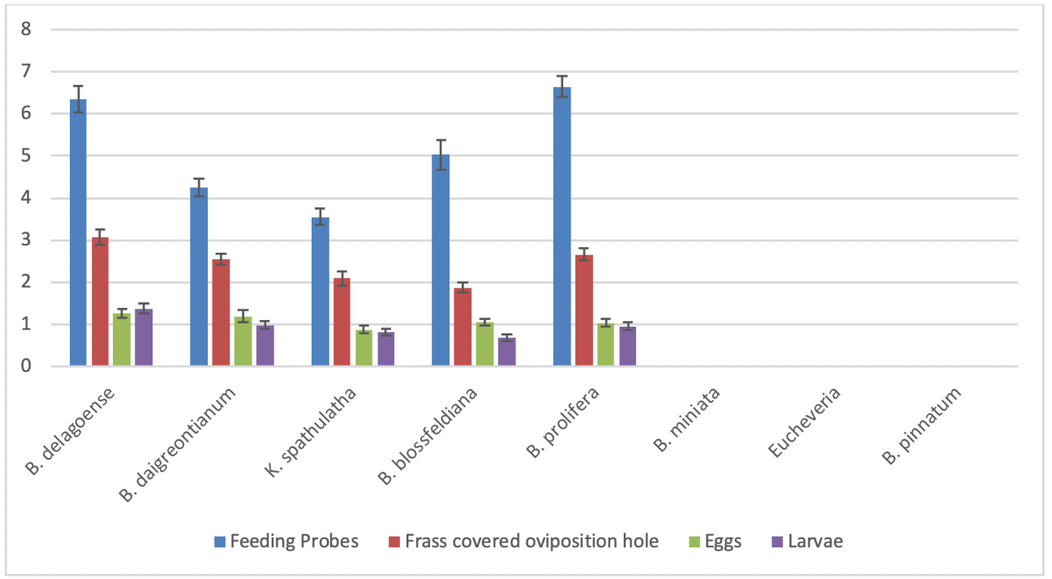


Figure 55 . Closed field multiple-choice trial data for *Osphilia tenuipes*.

Project outcomes

Bikasha sp. (Australia)

Field surveys were conducted in Madagascar and a root-feeding weevil, *Bikasha* sp., was imported into the quarantine at ESP to assess its potential as a biological control agent in Australia (Figure 56).

Figure 56. From top to bottom: Adult *Bikasha* sp., adult feeding damage on phyllodes and larval feeding damage to the roots of *Kalanchoe delagoensis*.

In adult feeding trials, beetles fed on all 19 species to which they were exposed (Table 5). In larval feeding trials, larvae completed development on 14 of the 15 species tested (Table 6). In potted plant trials, development was completed on 15 of the 19 species tested (Table 7).

Table 5

Adult feeding trial data for Bikasha sp.

Species tested	Adult feeding (Y/N)
K. delagoensis	Y
K. diagremontianum	Y
K. delagoensis x *K. diagremontianum* hybrid	Y
K. blossfeldiana	Y
K. beharensis	Y
K. fedtschenkoi	Y
K. humilis	Y
K. pinnata	Y
K. sexangularis	Y
K. synsepala	Y
K. tetraphylla	Y
K. tomentosa	Y
Crassula sieberiana (native)	Y
Crassula sp. (poss. South African origin)	Y
C. tetragona	Y
C. multicava	Y
C. ovata	Y
Sedum adolphii	Y
S. rubrotinctum	Y

Table 6

Plant species that supported development to adult when eggs were placed on root masses in small containers.

Species	% Adults eclosed
Kalanchoe delagoensis	26
Kalanchoe diagremontianum	28
K. delagoensis x *K. diagremontianum* hybrid	29
K. blossfeldiana	36
K. beharensis	65
K. fedtschenkoi	13
K. humilis	19
K. pinnata	50
K. sexangularis	66
K. synsepala	1
K. tetraphylla	43
K. tomentosa	22
Crassula sieberiana (native)	0.16
Crassula sp. (poss. South African origin)	0
C. tetragona	13

Project outcomes

Table 7

Plant species that supported development to adulthood when sexually mature adults were placed on potted plants for six days to oviposit.

Species	n	Completes development	Total number adults out
Kalanchoe delagoensis	13	yes	78
K. diagremontianum	5	yes	122
K. diagremontianum x *K. delagoensis* hybrid	6	yes	30
K. blossfeldiana	5	yes	58
K. beharensis	5	yes	38
K. fedtschenkoi	6	yes	76
K. humilis	3	yes	2
K. pinnata	5	yes	155
K. sexangularis	5	yes	254
K. synsepala	5	yes	1
K. tetraphylla	5	yes	27
K. tomentosa	5	yes	20
Crassula sp. (poss. South African origin)	5	no	0
C. tetragona	6	yes	12
C. sieberiana (native)	5	no	0
C. multicava	5	no	0
C. ovata	5	no	0
Sedum rubrotinctum	5	yes	77
S. adolphii	5	yes	32

No-choice trials
Replicated no-choice trials were completed, focussing on the Australian native, *K. spathulata*, and varieties of the commercial species, *K. blossfeldiana*. Significantly more feeding damage and larvae were recorded from the controls (MoM) than on both *K. spathulata* and *K. blossfeldiana* (Figure 57). While this data is encouraging, research findings (see Table 5, Table 6, Table 7) on the development of this species on a range of Crassulaceae, including several Australian natives, is less encouraging.

Adult paired-choice trials (Madagascar)
Ten unsexed adult beetles were placed in cages with a control (*K. delagoensis*) and single test species (*K. spathulata* or *K. blossfeldiana*). After 15 days, the adults were removed, and the plants were dissected to assess feeding and beetle life stages present. Significantly less adult feeding was recorded for *K. spathulata* and *K. blossfeldiana* compared to the controls (*K. delagoensis*) (Figure 58). However, similar amounts of eggs and larvae were found on all species.

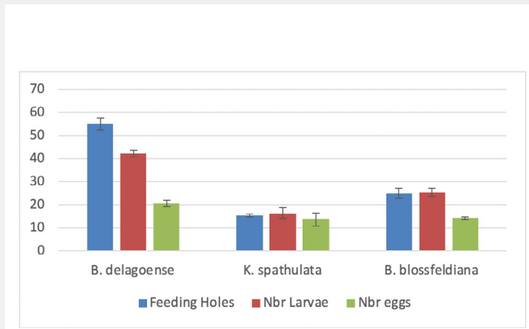


Figure 57. *Bikasha* sp. no-choice trial data from Madagascar.

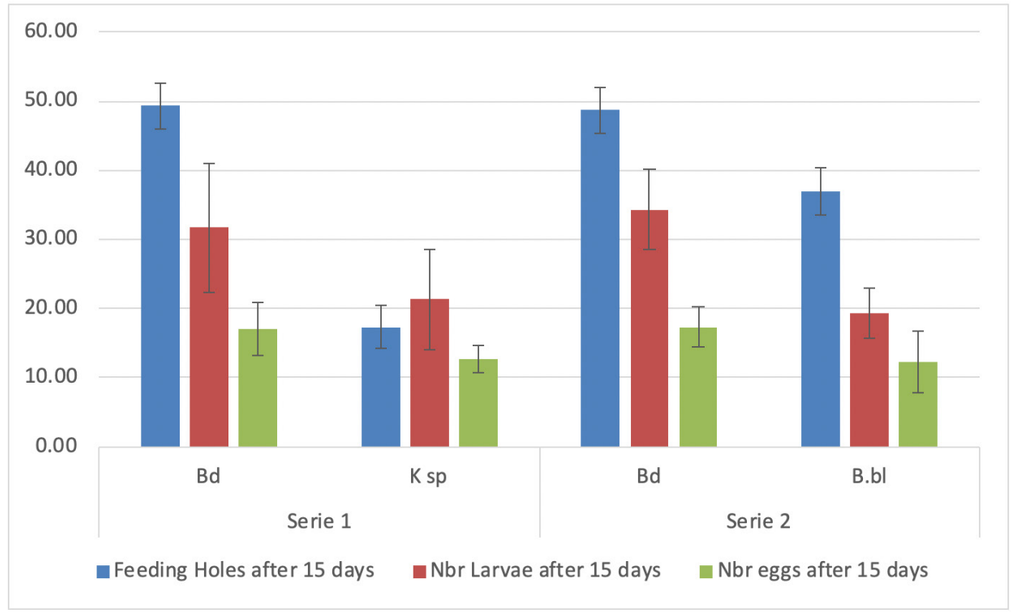


Figure 58. *Bikasha* sp. paired-choice trial data from Madagascar.

Project outcomes

Impact trials
This replicated trial was conducted in Madagascar using sleeved *K. delagoensis* plants. Four size classes of plants (Class 1: <10 cm; Class 2: > 10<20 cm; Class 3: > 20<30 cm; Class 4: >30<40 cm) were exposed to 10 adult *Bikasha* sp. over 30 days.

Parameters measured at the beginning and end of the trial included plant wet and dry weight, stem diameter, number of phyllodes and number bulbils. *Bikasha* sp. had a significant impact on three (wet weight, number of phyllodes and number of bulbils) of the five parameters measured (Figure 59).

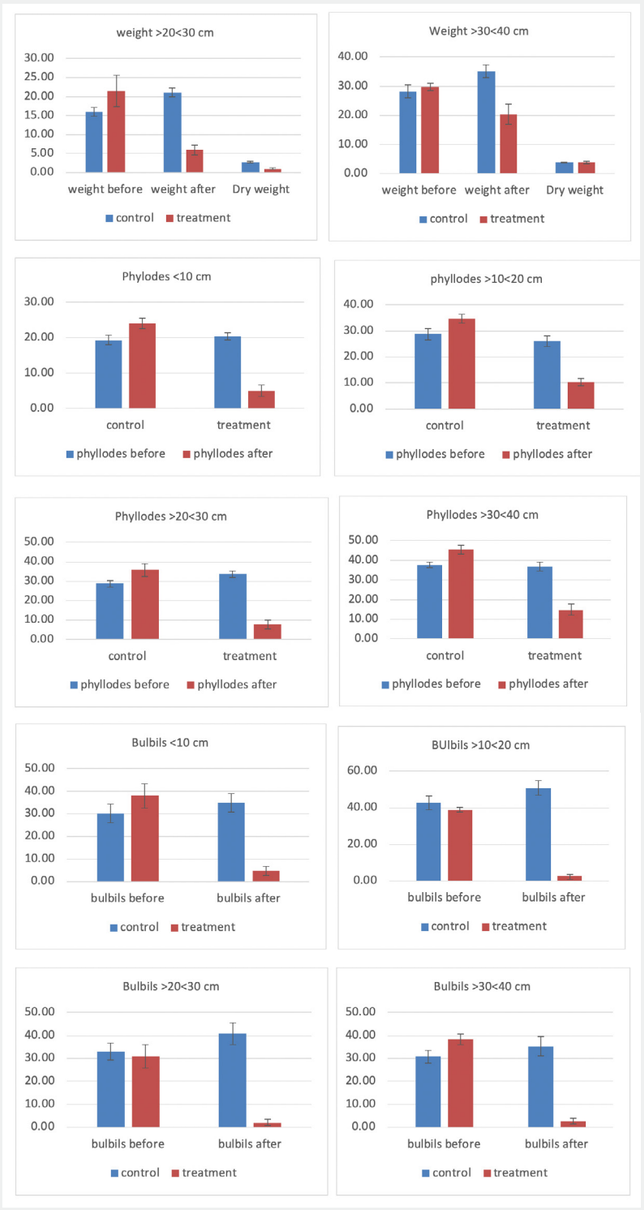


Figure 59. Trial examining the impact of Bikasha sp. on *Kalanchoe delagoensis*.

Output 8(b) - Import suitable biocontrol agent(s)

The stem-boring weevil, *Osphilia tenuipes*, was imported as planned in 2016 and a culture was established at the containment facility at Orange. A new species of root-feeding beetle, *Bikasha* sp., was however discovered and cultures were established at UOA and the containment facility in Brisbane.

Output 8(c) - Develop threshold studies, degree-day and Climex models for potential biocontrol agent(s).

Osphilia tenuipes

Developmental threshold trial and Degree-day modelling (CLIMEX)
A developmental threshold trial was conducted in Australian quarantine (OAI). *Osphilia tenuipes* eggs were reared through to adults at five constant temperatures (22.8, 25.6, 27.7, 30.1 and 32.5 °C) on cut stem material of *K. delagoensis*. Average egg to adult developmental times at these temperatures were 49.7, 45.6, 44.1, 38.5 and 38.6 days respectively (Table 8). At test temperatures below 22.5 (15, 17.5 and 20°C) and above 32.5 (35°C) incomplete development was recorded, presumably due to cold or hot stress under these constant conditions.

Due to the high levels of variability in the developmental data (note high SD values), a developmental threshold value could not be calculated, and therefore a degree-day model could not be run in CLIMEX. To address the high variability in the data, future trials will focus on less handling of the various life stages. This could be addressed by conducting the trials on whole plants (instead of cut stems in petri dishes) and not measuring the developmental time of each life stage, but instead focusing on the total developmental time from eggs to adult. Future trials could also attempt a higher number of replicates to reduce data variability.

Output 8(d) - Establish and conduct field experiments In NSW and QLD. Monitor release sites for establishment, dispersal and impact of suitable biocontrol agent(s).

Four mother-of-millions field study sites were established in Australia – two in NSW (Wee Waa and Turrawan) and two in Queensland (Dalby and Inglewood) - see Figure 60. These localities were chosen as long-term monitoring sites for several reasons. The first is that they are representative of *K. delagoensis* infestations in the core invaded range. The second is that they represent different affected land use types e.g. agriculture, travelling stock reserves, environmental conservation.

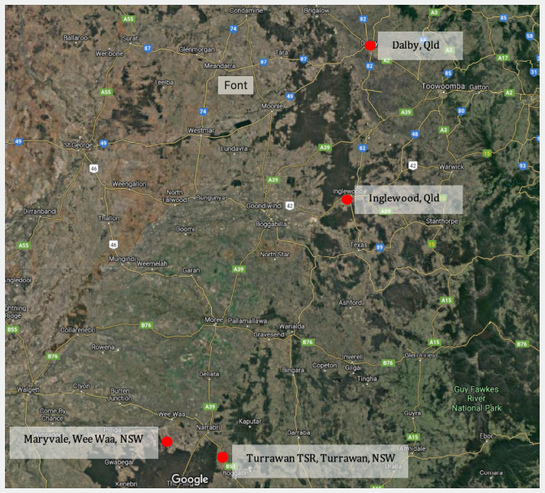


Figure 60. Pre-release field monitoring sites for *Kalanchoe delagoensis* in NSW and Qld.

Table 8

Developmental time from egg to adult for *Osphilia tenuipes* at five constant temperatures.

Stage	Mean duration (days) ± SD at indicated temperature				
	22.8°C	25.6°C	27.7°C	30.1°C	32.5°C
Males	50.0 (6.0)	45.5 (3.7)	42.9 (6.0)	38.8 (7.4)	39 (9.3)
Females	53.7 (5.9)	48.4 (6.0)	45.3 (2.8)	43.0 (0)	41.5 (4.9)
Combined	49.7 (10.3)	45.6 (4.7)	44.1 (4.6)	38.5 (6.5)	38.6 (6.9)

Field site monitoring

Four mother-of-millions field study sites were established and monitored in Australia – two in NSW (Wee Waa and Turrawan) and two in Queensland (Dalby and Inglewood). Interestingly, no viable seeds were recorded from the NSW sites in the first year of sampling. This was also observed for the Qld sites (which also had no recorded flowering) (Figure 60). The Turrawan site in NSW had the highest viable seedbank with an average of 461.4 seeds/m-2, followed by the Wee Waa site (222.8 seeds/m-2). Plant life stages reflected the same pattern for the NSW sites, which were also on average higher than the Qld sites. The data from these studies has established a good pre-release database of the weed in Australia. If a biocontrol agent is found and approved in the future, these sites could be used to establish long-term monitoring sites to evaluate impact.

Output 8(e) - Pending risks to non-target plants are acceptable, submit application to the Commonwealth regulators seeking approval to release at least one potential agent. Upon receiving approval, rear and release biocontrol agent(s).

Although *Bikasha* sp. significantly impacts the growth of *K. delagoensis*, it is clear from the testing conducted in both Australia and Madagascar that the beetle is not host specific and is highly unlikely to be approved for release by the Department of Agriculture, Water and the Environment. As a result, the culture in the containment facility in Brisbane will be destroyed at the completion of this project. As there is no additional funding, further work by QDAF exploring biological control opportunities will not be undertaken. The Madagascan PhD student will complete some development/continuation trials for his research, after which work will cease on this agent in Madagascar.

Output 8(f) - Update best practice manual.

Not completed as no agents approved for release.

Site	Dead plants (m-2)	Non-flowering plants (m-2)	Seedlings (m-2)	Flower heads (m-2)	Viable seeds (m-2)
Wee Waa	30	28.4	165.6	5.2	222.8
Turrawan	26.0	44.0	247.2	6.4	461.4
Dalby	16.4	17.2	24.4	0	-
Inglewood	9.2	32.0	30.0	0	-

Figure 61. Life stages and viable seedbanks of *K. delagoensis* monitoring sites in NSW and Qld.

3.1.6 Ox-Eye Daisy

Output 9(a) - Nominate ox-eye daisy as a biocontrol target.

Ox-eye daisy was successfully nominated as a suitable species for biological control research on 6 February 2020 (EIC OOS 2020-01).

Output 9(b) - Import and rear suitable biocontrol agent(s) from Switzerland.

Only one species, *Dichrorampha aeratana*, was imported into and reared in Australian quarantine. However, a culture of a second insect, *Cyphocleonus trisulcatus*, was maintained in Switzerland.

Output 9(c) - Conduct host-specificity tests on potential biocontrol agent(s).

No-choice larval development trials

Five recently hatched larvae were transferred with a thin paintbrush onto the petioles of each of the potted test and control plants. The pots were kept for one day in the laboratory and then transferred to an unheated greenhouse (Switzerland) or sleeved and kept in a temperature-controlled containment facility (Australia). Plants were maintained for 4-5 months and then dissected for insect life stages. In Australian trials, *D. aeratana* larval development was only found on *L. vulgare* (Table 9). In Swiss trials, *D. aeratana* larval development was only found on *Cotula cotuloides* (Australian native), *Mauranthemum paludosum* (ornamental) and *Tanacetum parthenium* (ornamental) (Table 10 and Table 11). These species were all included in open field trials.

Table 9

Results of no-choice larval development tests for *Dichrorampha aeratana* conducted in Australia

Species	No. of plants infested	No. plants dissected	% larvae found/ plant (mean ± SE)	% plants with larvae
Leucanthemum vulgare (Australia)	15	15	74.7 (±0.2)	100.00
Brachyscome multifidi	5	5	0.0	0.0
Brachyscome aculeata	5	5	0.0	0.0
Lactuca sativa	5	5	0.0	0.0
Osteospermum ecklonis	5	5	0.0	0.0
Argyranthemum frutescens	5	5	0.0	0.0
Chrysanthemum indicum	5	5	0.0	0.0
Leptinella reptans	7	7	0.0	0.0
Leptinella filicula	7	7	0.0	0.0
Leptinella longipes	5	5	0.0	0.0
Calotis pubescens	5	5	0.0	0.0

Project outcomes

Table 10

Results of no-choice larval development tests with *Dichrorampha aeratana* conducted for North America and Australia in 2017/2018 and 2018/2019.

Plant Species	No. replicates	No. plants dissected	% plants with larvae	% larvae found/ plant (mean ± SE)
L. vulgare Banff 3 (AB)	4	2c	100.0	40.0 ± 0.0
L. vulgare Douglas (CO)	4	3c	100.0	80.0 ± 20.0
L. vulgare Wavey (BC)	8	7c	100.0	65.7 ± 29.9
L. vulgare NSW (AUSTRALIA)	5	4c	100.0	40.0 ± 8.2
L. ircutianum (Austria)	4	3c	100.0	40.0 ± 11.5
Anthemis tinctoria	7	7	0.0	
Artemisia absinthium	9	9	0.0	
Artemisia dracunculus[a]	2	2	0.0	
Artemisia spinenscens[a]	3	3	0.0	
Brachyscome aculeata[b]	3	3	0.0	
Cotula alpina[b]	7	2d	0.0	
Cotula australis[b]	11	11	0.0	
Cotula cotuloides[b]	12	8d	8.3	2.5 ± 2.0
Daucus carot[a]	10	10	0.0	
Erigeron compositus[a]	5	4d	0.0	
Mauranthemum paludosum	7	6d	33.3	6.7 ± 3.9
Tanacetum parthenium	7	2c	100.0	30.0 ± 10.0

[a] Plant species native to North America; [b] Plant species native to Australia;
[c] The remaining plants were kept for adult emergence in spring; [d] The remaining plants died and had no roots to dissect.

Table 11

Results of additional no-choice larval development tests conducted with *Dichrorampha aeratana* in 2019/2020.

Plant Species	No. plants Infested	% larvae found/ plant (mean ± SE)	% plants with larvae
Leucanthemum vulgare	13	47.7 ± 8.0	92.3
Brachyscome aculeata[a]	7	0.0	
Calotis pubescens[a]	7	0.0	
Cotula australis[a]	6[b]	0.0	
Cotula cotuloides[a]	7[c]	0.0	
Leptinella filicula[a]	7	0.0	
Leptinella longipes[a]	9	0.0	

Open field trial

The open-field test was set up in a native meadow in Delémont, Switzerland. Fifteen or 16 plants each of *C. cotuloides*, *M. paludosum*, *T. parthenium* and *L. vulgare* were randomly arranged within a 7 m × 7 m plot with a 1-m distance between plants (Figure 61).

Thirty mated, egg-laying females were released in the centre of the plot over a 10-day period. Thereafter, exposed plants were transferred to a field holding cage and dissected three months later to assess larval development. *Dichrorampha aeratana* larval development was only recorded on *L. vulgare* and *M. paludosum* (Table 12).

Figure 62. Test and control plants exposed in an open-field test with *Dichrorampha aeratana*. From top left to bottom right: Meadow setting for trial, *Leucanthemum vulgare*, *Mauranthemum paludosum*, *Tanacetum vulgare*, Cotula cotuloides (healthy plant and plant attacked by unknown herbivore).

Table 12

Results of open field multiple-choice trials conducted with *Dichrorampha aeratana* in 2019.

Plant Species	No. of plants set up	% larvae found/ plant (mean ± SE)	% plants with larvae
Leucanthemum vulgare	16	2.9 ± 0.7	80.0
Cotula cotuloides[a]	16	0.0	0.0
Mauranthemum paludosum	16	0.1 ± 0.1	6.3
Tanacetum parthenium	16	0.0	0.0

[a] *Plant species native to Australia.*

Output 9(d) - Undertake developmental threshold studies, degree-day and Climex models for potential biocontrol agent(s),

Dichrorampha aeratana

Developmental threshold trial and Degree-day modelling (CLIMEX)

To assist in the development of a degree-day model (in CLIMEX), the duration of the egg stage of rhizome-feeding moth (*Dichrorampha aeratana*, Table 13) was investigated at the constant temperatures of 15°C, 22.5°C and 25°C. At these temperatures, eggs hatched in 18.28, 6.52 and 7.65 days respectively (Table 13). Additional test temperatures of 27.5 and 30 degrees could not be assessed due an overheating event in the quarantine, resulting in the loss of the *D. aeratana* culture. Once a new culture of the moth can be re-imported, the developmental threshold trials will resume.

Table 13

Results of *Dichrorampha aeratana* egg hatch trials at constant temperature

Test temperature (°C)	n	Mean time to hatching (days)
15	25	18.24
22.5	23	6.52
25	24	7.65

As an alternative to modelling the number of generations of *D. aeratana*, a MAXENT model was developed to investigate the potential distribution of *L. vulgare* in Australia (Figure 62). The model indicated that there are still further geographic areas of Australia that are eco-climatically suited to the growth of *L. vulgare*, both within its currently invaded range and in areas not currently invaded (i.e. WA).

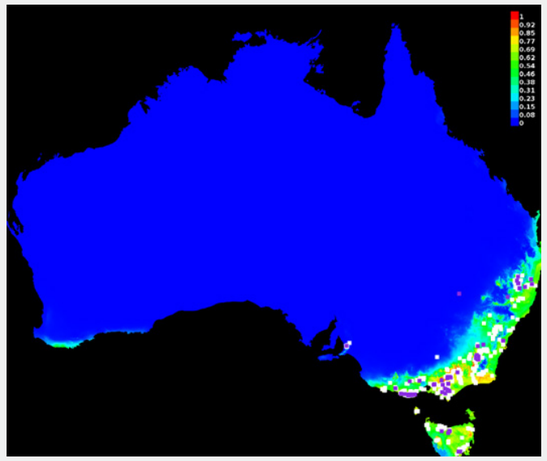


Figure 63. MAXENT model depicting the eco-climatic suitability (1 = highly suitable, 0 = not suitable) of Australia to the invasion of *Leucanthemum vulgare*.

Cyphocleonus trisulcatus

A rearing colony of the root-feeding weevil, *C. trisulcatus*, was established in 2019 to conduct host-range tests (Figure 63). Adults of *C. trisulcatus* were collected in CABI's garden during spring and summer and transferred to potted ox-eye daisy plants covered with gauze bags for egg laying. In addition, several potted ox-eye daisy plants that were left uncovered in CABI's garden during spring were covered with gauze bags in July and regularly checked for adult emergence. In total about 500 adults emerged and are currently overwintering in plastic cylinders placed in an incubator set at 2°C or on potted plants kept at ambient temperatures.

No-choice oviposition and larval development tests

Two egg-laying females were placed onto individually potted, gauze-covered test and control (*L. vulgare*) plants. After 4–8 days (depending on prevailing temperature) the females were retrieved from the plants. Three to eight weeks after the plants had been exposed to *C. trisulcatus*, the roots and rhizomes of all test plants and of a subset of the control plants were dissected, the soil was checked for larvae, and the total number of larvae found per pot was recorded. Larvae were only recorded from *L. vulgare* and *Tanacetum parthenium* (Table 14.).

Figure 64. *Cyphocleonus trisulcatus* adult (top) and larva feeding on *Leucanthemum vulgare* roots (bottom).

Table 14

Results of no-choice oviposition and larval development tests conducted with *Cyphocleonus trisulcatus* in 2019.

Plant Species	No. of replicates set up	No. of valid replicates	% larvae found/ plant (mean ± SE)
Leucanthemum vulgare	23	17	10.8 ± 1.5
Achillea ptarmica	8	7	0
Anthemis tinctoria	6	6	0
Artemisia absinthium	7	6	0
Artemisia dracunculus	7	5	0
Brachyscome aculeata[a]	7	6	0
Calotis pubescens[a]	3	3	0
Chamaemelum nobile	3	3	0
Cotula australis[a]	6	6	0
Cotula cotuloides[a]	3	1	0

Project outcomes

Table 14

Continued.

Plant Species	No. of replicates set up	No. of valid replicates	% larvae found/ plant (mean ± SE)
Glebionis segetum	3	1	0
Leptinella filicula[a]	6	3	0
Leptinella longipes[a]	7	4	0
Santolina chamaecyparissus	5	3	6
Tanacetum parthenium	6	4	0.3 ± 0.3

[a] Plant species native to North America; [b] Plant species native to Australia;

Output 9(e) - Pending risks to non-target plants are acceptable, submit application to the Commonwealth regulators seeking approval to release at least one potential agent. Upon receiving approval, rear and release biocontrol agent(s).

A release application for *D. aeratana* is currently being prepared and will be submitted in September 2020. Additional research is required for *C. trisulcatus*. Funding from the Environmental Trust of NSW has been secured in this regard, which will see further testing of the weevil in Switzerland and NSW from July 2020 onwards.

Output 9(f) - Establish and conduct field experiments In NSW, ACT and VIC. Monitor and release sites for establishment, dispersal and Impact of suitable biocontrol agent(s).

Not done as no agent approved for release.

Output 9(g) - Update best practice manual.

Not done as no agent approved for release.

3.1.7 Giant Rat's Tail Grass

Output 10(a) - Conduct genetic analysis of samples of Sporobolus species.

The genus *Sporobolus* is characterised by having single-flowered spikelets, one-nerved (rarely three-nerved) lemmas, fruits with free pericarps or modified caryopses, and ligules with a ciliate membrane or line of hairs. Species within *Sporobolus* generally inhabit dry or stony soils, saline or alkaline sandy soils, clay loam soils in grasslands, savannahs and along disturbed roadsides. Both herbarium-lodged samples and field-collected Sporobolus specimens were used in the genetic analysis study.

A minimum of four (where possible eight; Table 15) leaf samples were excised from each of the 23 *Sporobolus* species used in the Simon and Jacobs (1999) taxonomic revision. The S. latzii voucher specimen collected from the Northern Territory could not be destructively sampled, as it was the only specimen held at the BRI collection and the designated holotype for *S. latzii*. Approximately 12 mm of leaf material for *S. latzii* was sourced from the Northern Territory Herbarium. The DNA of five *Thellungia advena* (recently accepted as *Sporobolus* advenus by Australian Plant Census (APC), not accepted by BRI) samples were also extracted and tested. There have been so far unsuccessful destructive sampling requests for *Sporobolus anglicus* (C.E.Hubb.) P.M. Peterson & Saarela (*Spartina anglica*) from Victoria and Tasmania and for *Sporobolus schoenoides* (L.) P.M. Peterson (*Crypsis schoenoides*) (L.) Lam. from Western Australia. Field-collected *Sporobolus* material will be analysed before contract end date.

Table 15

Number of samples and specimen AQ number for each *Sporobolus* species tested and plastid markers used.

Species	G (rpl36-rps8)	H (rps16-trnK)	AQ Number
Sporobolus actinocladus (F.Muell.) F.Muell.	2	2	299144; 329154; 428186; 519914
Sporobolus advenus (Stapf) P.M. Peterson [Thellungia advena]	4	5	615146; 649076; 774862; 852563; 911860
Sporobolus africanus (Poir.) Robyns & Tour-nay	4	4	336985; 379917; 521559; 594929; 636916
Sporobolus australasicus Domin	3	3	299222; 361212; 387005; 469091
Sporobolus blakei De Nardi ex B.K.Simon	2	3	4485; 387004; 479748; 502736; 621729; 719568
Sporobolus caroli Mez	4	4	361200; 411832; 477082; 510212; 697830; 911788
Sporobolus contiguus S.T.Blake	3	2	306414; 331001; 361210; 635978
Sporobolus coromandelianus (Retz.) Kunth	4	4	329148; 425583; 452670; 469067; 914936
Sporobolus creber De Nardi	3	3	306449; 322022; 361227; 593682; 614225; 843729
Sporobolus disjunctus R.Mills ex B.K.Simon	5	4	361206; 432409; 520451; 574961; 787516
Sporobolus elongatus R.Br.	5	5	319570; 425200; 581565; 591164; 733302
Sporobolus fertilis (Steud.) Clayton	4	5	306697; 306825; 504597; 626780; 776586
Sporobolus jacquemontii Kunth	6	6	306465; 306833; 329126; 381528; 564838; 592063; 698258
Sporobolus latzii	1	1	521188
Sporobolus laxus B.K.Simon	4	3	361219; 407083; 411714; 502952; 841833; 851122
Sporobolus lenticularis S.T.Blake	6	5	306847; 306851; 306855; 516757; 616175; 842851

Project outcomes

Species	G (rpl36-rps8)	H (rps16-trnK)	AQ Number
Sporobolus mitchellii (Trin.) C.E.Hubb. ex S.T.Blake	6	6	306884; 306895; 382159; 425281; 429426; 619665; 912557
Sporobolus natalensis (Steud.) T.Durand & Schinz	5	5	396134; 426893; 459339; 564376; 970557
Sporobolus pamelae B.K.Simon	4	4	560347; 570589; 634188; 862678; 913655
Sporobolus partimpatens R.Mills ex B.K.Simon	2	3	169433; 299110; 337200; 570153; 832995
Sporobolus pulchellus R.Br.	2	2	306901; 306904; 316729; 317032
Sporobolus pyramidalis P.Beauv.	5	5	333813; 504575; 504586; 564743; 815992
Sporobolus scabridus S.T.Blake	6	6	306921; 306922; 306929; 306939; 544563; 697000; 907002
Sporobolus sessilis B.K.Simon	4	4	313979; 361208; 361214; 564742; 697009; 753577
Sporobolus virginicus (L.) Kunth	5	8	361252; 425726; 427205; 429427; 468598; 569708; 782358; 793235

Unfortunately, an accurate molecular phylogenetic tree for Australian *Sporobolus* was not possible at this stage as a definitive molecular marker for each species could not be determined Figure 64). Amplifiable DNA was only possible for 107 of the 132 samples collected from the Sporobolus collection. Further DNA extraction (including from the 145 S*porobolus* plants collected during the survey period), testing and analysis is continuing until contract end date of 15 June 2020.

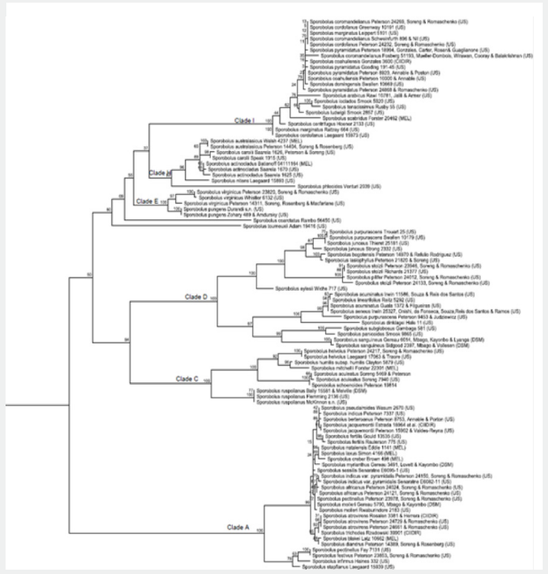


Figure 65. Preliminary molecular phylogenetic tree using the rps16-trnK spacer of Australian *Sporobolus* species distributed across six clades.

Table 16

Seven major clades strongly supported for the Australian *Sporobolus* species using nuclear (ITS) and plastid (*rps16-trnk*) analyses.

Clades	IID based on morphology (Simon and Jacobs 1999)	No. specimens matching	Percentage match
A	*Sporobolus africanu*	4	80
	Sporobolus blakei	4	67
	Sporobolus coromandelianus	3	60
	Sporobolus creber	6	100
	Sporobolus fertilis	2	40
	Sporobolus jacquemontii	5	72
	Sporobolus laxus	3	50
	Sporobolus natalensis	5	100
	Sporobolus pamelae	5	100
	Sporobolus pyramidalis	5	80
	Sporobolus scabridus	2	29
	Sporobolus sessilis	3	50
C	*Sporobolus latzii*	1	100
	Sporobolus mitchellii	2	29
D	*Sporobolus lenticularis*	2	33
	Sporobolus pulchellus	3	75
E	*Sporobolus virginicus*	6	75
H	*Sporobolus actinocladus*	2	50
	Sporobolus australasicus	3	75
	Sporobolus caroli	5	83
	Sporobolus contiguus	2	50
	Sporobolus coromandelianus	1	20
	Sporobolus fertilis	1	20
	Sporobolus lenticularis	3	50
	Sporobolus mitchellii	1	14
	Sporobolus partimpatens	4	80
	Sporobolus pyramidalis	1	20
	Sporobolus virginicus	3	38
I	*Sporobolus coromandelianus*	1	20
	Sporobolus scabridus	3	43
	Sporobolus sessilis	1	17
L	*Sporobolus advenus*	5	100
Unknown	*Sporobolus disjunctus*	5	80
	Sporobolus elongatus (*rpl32-trnL* sequence required)	5	
	Sporobolus jacquemontii	2	29

Project outcomes

Nuclear (ITS) and plastid (*rps16-trnk*) analyses to date has shown the Australian Sporobolus species to be grouped into at least seven major clades. Only four species (*S. advenus*. *S. creber*, *S. natalensis* and *S. pamelae*) contained amplifiable DNA for all sourced specimens and grouped into one clade. Nine species [*S. coromandelianus* (A, H, I); *S. fertilis* (A, H); *S. jacquemontii* (A, ?); *S. lenticularis* (D, H); *S. mitchellii* (C, H); *S. pyramidalis* (A, H); *S. scabridus* (A, I); *S. sessilis* (A, I) and *S. virginicus* (E, H)], contained samples that belonged to two or three clades suggesting that the voucher specimens used by Simon and Jacobs' (1999) *Sporobolus* revision were incorrectly identified morphologically. The endangered *Sporobolus* species, *S. pamelae,* listed in Schedule 2 of the Queensland Nature Conservation Act 1992, has been grouped in the weedy *S. indicus* complex clade A.

Further refinement and analysis will continue until the contract end date of 15 June 2020.

Figure 67. Rhodes University PhD candidate Guy Sutton surveying *S. pyramidalis* in Kaw-Zulu Natal, South Africa.

Output 10(b) - Conduct native range surveys and host-specificity tests for potential biocontrol agent(s).

Prior to conducting native range surveys within South Africa, climate-matching studies to determine the areas within South Africa that appear to be most suitable to conduct field exploration were conducted. These areas were based on the areas that the weedy *Sporobolus* spp. occur in Australia. The most suitable areas to survey were identified as coastal areas from East London north to the Mozambique border, as well as inland areas from Richards Bay north to the Zimbabwe border. This work has been published (Figure 65).

Over 2017-2019, field surveys were conducted in the most suitable areas identified in the climate matching study and involved sampling 135 sites where one or both of the two target species, *Sporobolus pyramidalis* and *S. natalensis* were present (Figure 66.).

Eighty-seven morphospecies were found on one or both of these species. A species accumulation curve showed that there would be little benefit in conducting more surveys within the delimited areas in the expectation of finding additional species on the two target *Sporobolus* spp. (Appendix 20).

Of the 87 morphospecies found, most were generalists or visitors to *Sporobolus* spp. and only six species (all endophagous species) were worth investigating further. No ectophagous species were found worth investigating further.

Field host range assessments were conducted on these six species, involving 47 non-target grass species growing sympatrically with the two target *Sporobolus* spp. Three of the endophagous species were found on other plant species numerous times and were rejected for further consideration.

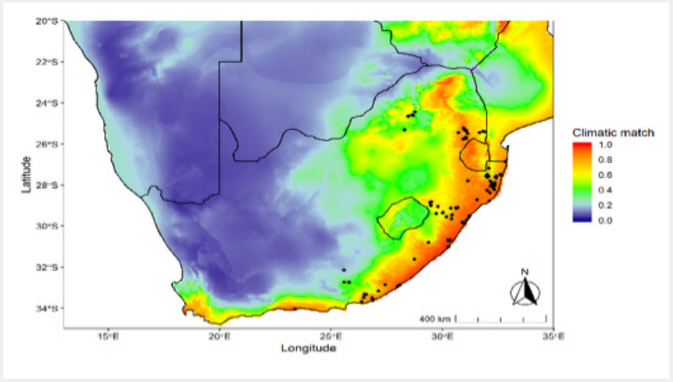


Figure 66. Climate matching map for *Sporobolus pyramidalis* and *S. natalensis* in the native-range in South Africa. Increasingly warmer colours indicate geographic regions that are more climatically-matched to weed infestations in Australia. Black, filled circles indicate individual sites where *S. pyramidalis* and/or *S. natalensis* were surveyed. Source: Sutton 2019.

Three stem-boring wasp species, two of which are thought to be *Tetramesa* species, and a *Bruchophagus* species were found to be present consistently on only the two target *Sporobolus* spp. (Figure 67, Figure 68, Figure 69).

In trials to determine the impact of each of the three wasp species on the two target *Sporobolus* spp., *Tetramesa* sp. A was considered the most damaging (Figure 69) and was prioritized for laboratory host specificity testing. *Tetramesa* sp. B was also damaging but not to the same extent. *Bruchophagus* sp. does little damage and appeared to interfere with the actions of *Tetramesa* sp. A. For this reason, *Bruchophagus* sp. A is viewed as a low priority and an undesirable candidate, compared to the other two species.

Based on field observations and preliminary host specificity testing on selected plants, closely related to the two target *Sporobolus* species, *Tetramesa* sp. A is deemed to be suitably host specific

Figure 70. Damage by *Tetramesa* spp. or *Bruchophagous* sp. on *S. pyramidalis*.

Figure 68. *Tetramesa* sp. A larva in stems of S. pyramidalis

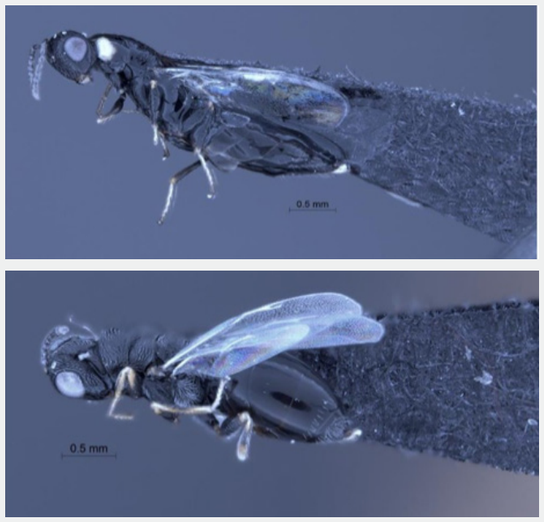


Figure 69. *Tetramesa* sp. A (top) and Bruchophagous sp. (bottom)

to warrant further investigation in detailed host specificity trials. Import permits are being organised to introduce the insect into the containment facility at the Ecosciences Precinct in Brisbane for more detailed host specificity testing (Figure 70). Here a wide range of native and economic grasses will be tested to determine if *Tetramesa* sp. A will attack and cause damage to any of the species.

Figure 71. Host specificity testing of *Tetramesa* sp. A

Project outcomes

Output 10(c) - Develop a rearing method for suitable biocontrol agent(s) identified in native range surveys.

All three species were taken into the laboratory and reared to facilitate host specificity testing. In laboratory-based host specificity testing to date, oviposition and subsequent larval development to adult occurred on only the two target *Sporobolus* species and none on any of the other 10 species tested to date. This supports field observations where Tetramesa sp. A was not observed on any other species.

Output 10(d) - Investigate Australian pathogens that could be effective and determine if additional potential biocontrol agent(s) attacks other *Sporobolus* species.

Pathogen survey

Nineteen pathogen surveys were carried out across 73 *Sporobolus*-infested sites in Queensland and northern New South Wales including Beechmont, Buchan Point, Bundaberg, Camooweal, Charters Towers, Clermont, Conondale, Dimbulah, Eton, Gin Gin, Julia Creek, Mackay, Mareeba, Miriam Vale, Mt Surprise, Taunton, Tewantin, Woodford and Yetman.

A total of 164 tussocks and symptomatic plant material belonging to 13 species (*S. actinocladus*, *S. africanus*, *S. caroli*, *S. coromandelianus*, *S. creber*, *S. fertilis*, *S. jacquemontii*, *S. laxus*, *S. mitchelli*, *S. natalensis*, *S. pyramidalis*, *S. scabridus* and *S. virginicus*) were removed from the field, labelled and transported to ESP. From this material, 136 plants displayed foliar disease symptoms that yielded almost 500 fungal isolates (Figure 71). Fast growing isolates that easily identified morphologically as common saprobic fungi were not retained, e.g. *Penicillium* spp.

The exploration stage for endemic GRT pathogens identified 44 genera (*Acremonium*, *Alternaria*, *Aureobasidium* , *Bipolaris*, *Chaetomium*, *Cladosporium*, *Claviceps*, *Codinaea* , *Colletotrichum*, *Curvularia*, *Darksidea*, *Didymella*, *Edenia*, *Fusarium*, *Hysteropatella*, *Kabatiella*, *Macalpinomyces*, *Magnaporthiopsis*, *Microdochium*, *Myrmecridium*, *Neopestalotiopsis*, *Neoroussoella*, *Neottiosporina*, *Nigrospora*, *Paecilomyces*, *Paraconiothyrium*, *Paraphaeosphaeria*, *Pestalotiopsis*, *Phaeoseptoriella*, *Phaeosphaeria*, *Phaeosphaeriopsi*, *Phoma*, *Pleosporales*, *Pyrenochaetopsis*, *Ramichloridium*, *Sclerotinia*, *Septoria*, *Setosphaeria*, *Sphaerellopsis*, *Stagonospora*, *Trichoderma*, *Tricothecium*, *Urohendersonia*, *Ustilago*, and *Xylariaceae*) on both Australian native and naturalised *Sporobolus* host plants. Many of the fungal genera contained multiple species of interest (Table 17). Nine of these genera (*Colletotrichum*, *Curvularia*, *Microdochium*, *Neopestalotiopsis*, *Paraphaeosphaeria*, *Pestalotiopsis*, *Phoma*, *Septoria* and *Stagonospora*) are known to contain fungal species pathogenic on grasses (Figure 72, Figure 73, Figure 74, Figure 75, Figure 76).

Figure 72. Endemic foliar pathogens found on Sporobolus natalensis.

To assist in the endemic pathogen prioritisation process, over 200 pathogens will be further sequenced using an additional four to five markers until the contract end date of 15 June 2020. Eighty percent of these pathogens appear to be new species, including seven novel genera.

Koch's postulate studies

Koch's postulate studies confirmed pathogenicity for three fungal isolates. Spores from the three novel species of fungi (*Microdochium* sp. BRIP 65649, *Pestalotiopsis* sp. BRIP 66615 and *Neopestalotiopsis* sp. BRIP 66617) inoculated onto GRT were removed, re-cultured and resulting spores re-inoculated (at 1x106 spores ml-1) onto new GRT seedlings. The three novel species of fungi (*Pestalotiopsis* sp. BRIP 66615, *Microdochium* sp. BRIP 65649, and *Neopestalotiopsis* sp. BRIP 66617) were found on hosts in the *Sporobolus* indicus complex. The identities of these isolates were confirmed through molecular analyses. Preliminary testing has shown that the three fungi play a role in GRT seedling mortality (Figure 77). GRT mortality was 72, 60 and 56% respectively for the three fungi seven weeks after inoculation. Trials will be repeated before the contract end date.

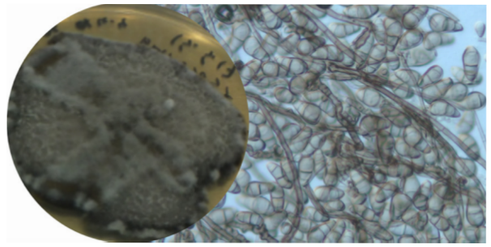


Figure 73. *Curvularia* sp. BRIP 69020 colony and spores.

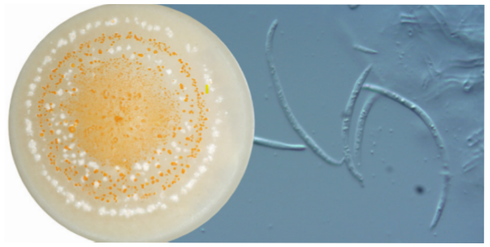


Figure 74. *Microdochium* sp. BRIP 68298.

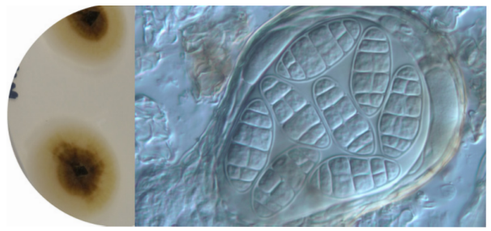


Figure 75. *Phoma* sp. BRIP 65632a colony and spores.

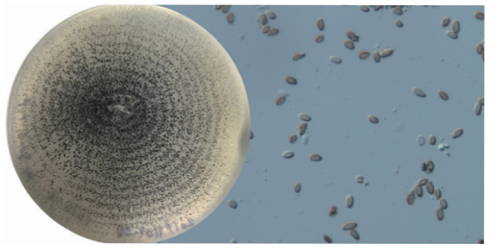


Figure 76. *Paraphaeosphaeria* sp. BRIP 66619 colony and spores.

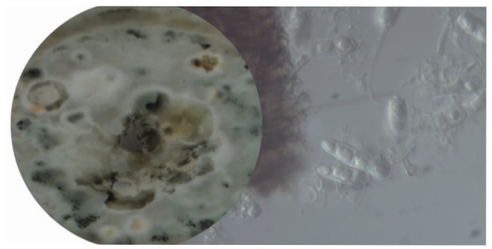


Figure 77. *Stagonospora* sp. BRIP 65638a

Table 17

Genera of endemic pathogens of interest found on Sporobolus host plants during the survey period of 2017-2020.

Genus (Family)	Number of Species	Genus (Family)	Number of Species
(Phaeosphaeriaceae)	7 (Novel genus)	*Magnaporthiopsis (Magnaporthace-ae)*	2
Alternaria	2	*Microdochium*	3 (Novel species)
Aureobasidium (Saccotheciaceae)	2 (Novel species)	*Myrmecridium schulzeri*	1
Bipolaris	1 (Novel species)	*Neopestalotiopsis*	5 (Novel species)
Claviceps (Clavicipitaceae)	1	*Neptunomyces (Didymosphaeria-ceae)*	1 (Novel species)
Colletotrichum	8 (Novel species)	*Pestalotiopsis*	2 (Novel species)
Curvularia	4	*Phaeoseptoriella (Phaeosphaeria-ceae)*	1 (Novel species)
Darksidea (Lentitheciaceae)	1 (Novel species)	*Phaeosphaeria (Phaeosphaeria-ceae)*	2 (Novel species)
Dictyochaeta (Chaetosphaeriaceae)	3 (Novel species)	*Phoma*	2 (Novel species)
Didymellaceae (Stagonosporopsis)	1	*Pyrenochaetopsis (Cucurbitaria-ceae)*	2 (Novel species)
Elsinoe (Myriangiales)	2	*Scytalidium (Hyaloscyphaceae)*	1 (Novel species)
Epicoccum (Didymellaceae)	1	*Septoria*	1 (Novel species)
Fusarium	1	*Stagonospora (Phaeosphaeriaceae)*	16 (Novel species)
Leptosphaerulina (Didymellaceae)	1	*Ustilago sporoboli-indici*	1

GRT leaf smut *Ustilago sporoboli-indici*

In 2017, Ustilago sporoboli-indici was found infecting *S. natalensis* in Australia, previously known only from South Africa on *S. pyramidalis* (Vitelli et al, 2017). The leaf smut produced black teliospores in sori in the leaves, leaf sheaths and stems which rendered infected shoots almost sterile (Figure 78).

The pathogen surveys during 2018-2020 uncovered further records of *Ustilago sporoboli-indici* on *Sporobolus natalensis*. The leaf smut has been found in Queensland regional areas of Bundaberg, Conondale, Childers, Gin Gin, Miriam Vale and Taunton, spanning a distance of greater than 350 km.

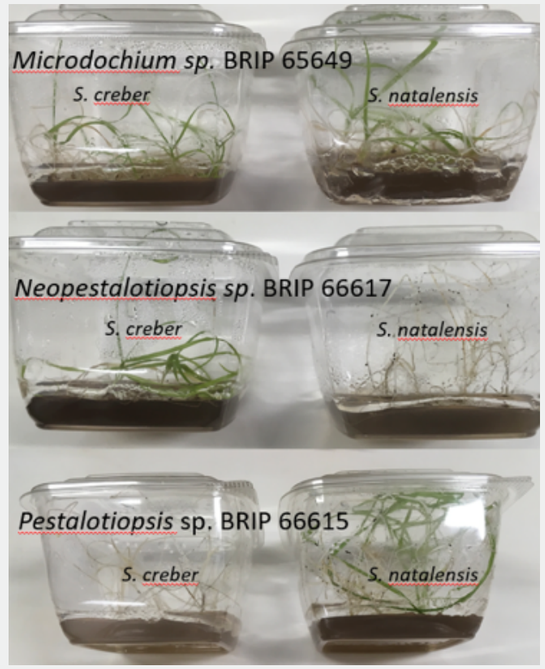


Figure 78. Effect of three novel species of fungi (*Microdochium* sp. BRIP 65649, *Neopestalotiopsis* sp. BRIP 66617 and *Pestalotiopsis* sp. BRIP 66615) on seedlings of *S. creber* and GRT (*S. natalensis*), seven weeks after inoculation.

Figure 79. *Sporobolus natalensis* stems (circled in red) with formation of black teliospores. Ruptured epidermis in the middle insert shows a powdery mass of blackish-brown spores when released that are stuck together. Insert on the right shows a leaf smut infected flower head that failed to elongate.

Output 10(e) - Establish and conduct field experiments at suitable locations.

The Conondale field trial demonstrated the benefits of adopting an integrated control approach, using a slasher, heavy grazing, targeted application of herbicides to GRT tussocks using wick wipers and the African GRT leaf smut *Ustilago sporoboli-indici* (Figure 79). Populations of GRT at the Conondale trial site have been reduced by almost 40% in three years. Slashing removes rank and dead growth from the previous season, crash grazing provided the height difference between the desirable pasture and GRT in readiness for herbicide (glyphosate/flupropanate) application, and the wick wiper provided the means to target individual GRT tussocks on a broad-acre scale (Figure 80). The addition of the GRT leaf smut at the trial site has meant that almost all (>95%) GRT flower-heads found are sterile. Feedback from landholders incorporating the use of weed wick wipers is supporting the research results but, with a viable soil seedbank of 8 to 10 years, control efforts will need to be sustained until endemic and classical biocontrol options are optimized. A field day was held at the trial site on June 19th 2019.

The use of flupropanate in field sites in central and southeast Queensland when applied both as a spot-application or as a broadacre application with a wick wiper is proving effective at managing GRT. At sites where the GRT leaf smut has established, surviving GRT tussocks post herbicide or mechanical treatments are not producing viable seed.

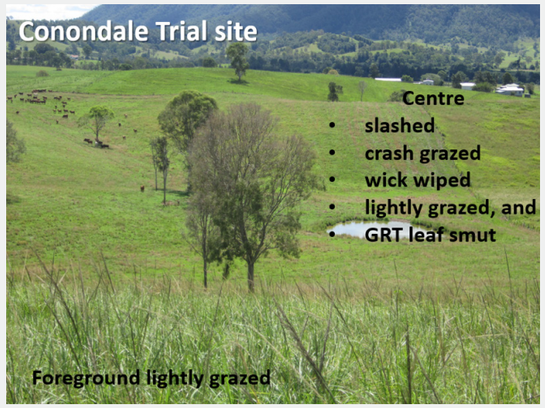


Figure 80. Integrated management trial site at Conondale, Queensland, examining the use of crash grazing, wick wipers and leaf smut in managing dense swards of GRT.

Figure 81. Wick wiping GRT grazed tussocks using a C-Dax Eliminator.

Additional trials were established in central Queensland, looking at ways to optimise flupropanate for the management of GRT in seasonally-waterlogged gilgais and alluvial flats:

1) GRT present in seasonally-waterlogged gilgais is difficult to control largely due to the rapid breakdown of flupropanate, which reduces efficacy, and the lack of suitable alternative selective herbicides. A spot application trial in 2018-19 investigated a range of herbicides including flupropanate and the timing of herbicide application to determine if GRT control could be improved in these areas. Spot application of flupropanate was highly effective in controlling individual giant rat's tail grass plants with plant mortality at or close to 100% compared to the untreated plants, which all survived. The untreated plants produced on average almost 90 seed heads per plot compared to no seed heads produced on any of the treated plants. Following the success of spot application of flupropanate in 2018-19, additional trials were established in October 2019 at two seasonally flooded areas in central Queensland. These treatments were 0.3ml flupropanate product (Taskforce)/tussock applied prior to the wet season. These treatments will be assessed in May/June 2020 following the wet season.

2) Controlling GRT on the seasonally waterlogged flats along creeks has proved problematic largely due to the rapid breakdown of flupropanate by hydrolysis which reduces efficacy resulting in rapid GRT regeneration. These areas are suited to the establishment of highly competitive pasture grasses such as *Urochloa decumbens* (Stapf) R.D.Webster (signal grass) which, if well managed, can out-compete GRT and provide considerable benefits to animal production. Assessments on GRT mortality were completed nine months after treatment in August 2019. Liquid flupropanate treatments 2 L/ha and 3L/ha had the greatest mortality with 97% and 98% respectively. Granular flupropanate when applied at 15 kg/ha and 22.5 kg/ha had mortality rates of 89% and 90%, which, although lower, were not significantly different from liquid flupropanate treatments. The glyphosate 3 L/ha treatment was significantly different from the other treatments with a mortality of 75.6%. Signal grass has not yet established at the site. This trial will continue to be monitored for a second year to determine flupropanate residual activity. A similar trial was established at nearby site in a gully adjacent to a creek in October 2019. The treatments for this trial were a non-treatment control and liquid flupropanate at a rate of 2 L/ha and 3 L/ha. Assessments will be completed in May/June 2020 following the wet season.

Output 10(f) -Pending risks to non-target plants are acceptable, submit application to the Commonwealth regulators seeking approval to release suitable native pathogens or at least one potential agent. Upon receiving approval, release biocontrol agent(s).

Not met as host specificity testing has not been completed.

Output 10(g) - Update the biocontrol and native pathogen sections of the best practice manual and communicate Information to farmers and land users.

The biocontrol component has not been updated as there is not a biocontrol agent released yet. Work is on-going.

3.1.8 Silverleaf Nightshade

Output 11(a) - Consult with Meat and Livestock Australia (MLA) on project RnD4Profit-14-01-040, Fast-tracking and maximising the long lasting benefits of weed biological control for farm productivity, in relation to the development of biocontrol's for silverleaf nightshade to build on their research efforts and avoid duplication.

A two-day meeting of the Meat and Livestock Australia (MLA) Rural Research and Development for Profit (RRnD4P) Program Round 1 was held in Melbourne on 5-6 September 2016. The meeting included representatives of MLA and AgriFutures, and silverleaf nightshade research leaders for the Round 1 and Round 2 projects (PIRSA and DJPR respectively). Consensus was reached on a strategy to avoid duplication and realise synergies between the two projects. MLA was nominated to lead development of the first agent for silverleaf nightshade, the leaf beetle *Leptinotarsa texana*, while AgriFutures was the lead for overseas exploration, selection, importation and testing of a second agent. The RIRDC Round 2 project therefore builds on, and is nested with, MLA Round 1 research. Liaison and coordination with MLA ceased when *Leptinotarsa texana* was found not to be host specific.

Output 11(b) - Select overseas survey area based on updated species distribution modelling and ongoing genetic analysis of Australian silverleaf nightshade populations.

A strategy of initiating host-specificity testing of prospective biological control agents in the native range was adopted for Round 2 to avoid expending time and resources importing agents with a low likelihood of being approved for release. This approach was warranted for biological control of silverleaf nightshade because apparently host-specific agents will sometimes utilise non-target species within the confines of laboratory cages. This occurred with the leaf beetle *L. texana*, which utilised eggplant *S. melongena*, certain potato *S. tuberosum* cultivars and some Australian native *Solanum* spp. in host-specificity experiments conducted at AgriBio (Lefoe et al. 2020a). Australian regulatory authorities rely heavily on the results of laboratory experiments and are unlikely to approve an agent that utilises important crops such as potato, even if other evidence supports field host-specificity. Conducting cage and/or field experiments in Argentina or USA prior to importation maximises the likelihood of only importing agents that could subsequently be demonstrated to be host-specific in Australian quarantine laboratory experiments.

A limitation of pre-importation testing in Argentina or USA is that only plants available in those countries can be tested. In most circumstances, testing of Australian native plants must be conducted at AgriBio, where an important collection of Australian *Solanum* is being grown and maintained. Also, while commercial crops such as potato *S. tuberosum*, tomato *S. lycopersicum* and eggplant *S. melongena* are readily available in Argentina and USA, cultivars known to be susceptible in cage tests and those important in the Australian market may not be available. Despite these limitations, pre-importation host-specificity testing of *Solanum*-feeding agents against available commercial *Solanaceae* crops is preferred because it can eliminate biological control agents that are unlikely to be approved in Australia.

Kwong (2006) also identified the origin of Australian populations of silverleaf nightshade as a key knowledge gap that could inform future natural enemy surveys. This knowledge gap was addressed in the RR&D4P Round 1 project, which found the main source of Australian silverleaf nightshade to be central USA (especially Oklahoma; Heap 2018). However, supplementary climate modelling conducted in RRnD4P Round 2 predicted Oklahoma to be a less suitable climate-match than Argentina (Figure 82; John Weiss pers. comm.).

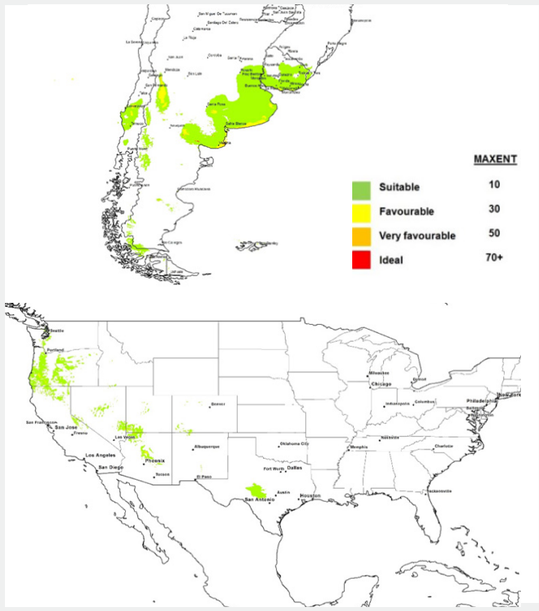


Figure 82 Maxent/ArcMap outputs of areas of Argentina (above left) and North America (above right) climatically matched to Australian silverleaf nightshade locations (Atlas of Living Australia 2018) (John Weiss pers. comm.).

Project outcomes

There is a conflict then, between prioritising areas of the native range that are climatically matched to south eastern Australia (Argentina) and areas with the closest genetic match to Australian populations of silverleaf nightshade (central USA). A further consideration was highlighted by Wapshere (1988), who argued that southern Texas and northern Mexico should be prioritised for natural enemy surveys because it is the region with the greatest diversity of phytophagous arthropods associated with silverleaf nightshade. For example, Goeden (1971) found silverleaf nightshade was largely free of insect damage in California, but damage increased dramatically as surveys moved eastwards toward south Texas. Additional considerations when prioritising native range surveys are the ease of surveying, collecting, screening and exporting prospective agents, and the presence of established and reliable in-country collaborators.

We therefore adopted a diversified strategy that implemented the recommendations of Kwong (2006) to survey new areas of Argentina, while also developing prospective agents in south Texas. In addition, we investigated whether the range of natural enemies in south Texas extended further north toward Oklahoma, with a view to making separate collections of the most promising North American agent from both States.

Output 11(c) - Conduct natural enemy surveys in target area and prioritise potential biocontrol agent(s).

Natural enemy surveys were conducted during the growing season in Argentina (spring-autumn or summer-autumn), and to a lesser extent in neighbouring Paraguay, during 2017-2018, 2018-2019 and 2019-2020. Observations of prospective agents were also made during *L. texana* field experiments in Texas, USA, in April 2017 and April 2018, with more extensive follow-up surveys across south Texas from December 2019 to February 2020.

2017-2018 natural enemy surveys, Argentina & Paraguay

Surveys were conducted from December 2017 to May 2018 (Appendix 23).

Since silverleaf nightshade is sometimes small, making it difficult to see from a moving vehicle, inspection stops were assigned according to herbarium records, or along roadsides, where the habitat was suitable for plant growth (Figure 82). Plant specimens from all locations were collected and pressed to confirm identification. Fruits and leaves were also collected in silica gel. The fruits were collected for cultivation and for cytogenetic analysis.

Arthropods were collected with pooters and placed in 70% ethanol for identification or placed alive in collection jars. Immature insects/arthropods were hand collected and reared in the laboratory to adulthood for identification or used to develop laboratory cultures. Diseased stems or leaves were collected for isolation and identification studies.

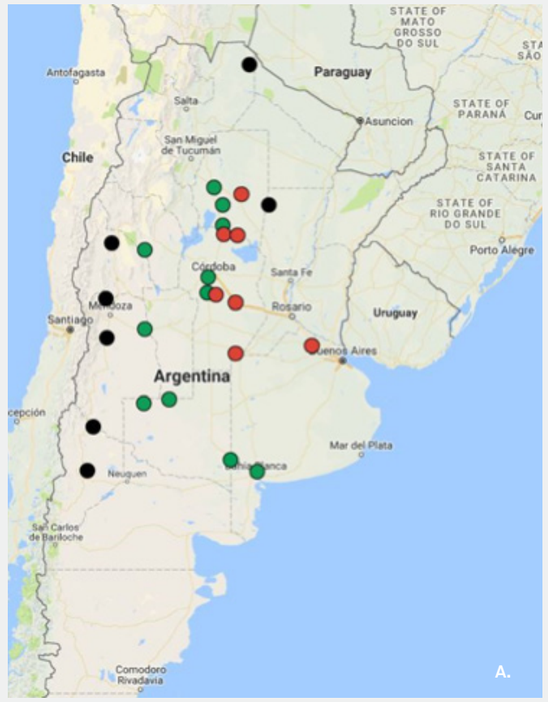
Silverleaf nightshade was found at approximately 30 sites distributed across Argentina in a wide range of habitats and latitudes. FuEDEI personnel sampled the plant from northern Argentina (Salta Province) to Sierra de la Ventana, Buenos Aires Province; and from Entre Ríos Province in the east to Uspallata, Mendoza Province in the west (57-70 Long W; 38-20 Latitude S); from sea level to 2000 meters above sea level.

Figure 83. Roadside patches of *S. elaeagnifolium*. (A) Uspallata, Mendoza province, western Argentina; (B) Tres Arroyos, Buenos Aires province, eastern Argentina.

Cytotype distribution

According to cytogenetic studies, populations that grow spontaneously in Argentina exhibit a euploid series (2x, 4x and 6x), while only diploids are found in the rest of the world, including Australia (Scaldaferro et al. 2012). The distribution pattern of different ploidy levels of *S. elaeagnifolium* is probably a response to environmental conditions, although historical factors have not been considered to date. Phylogeographic studies could make a significant contribution to interpreting the origin and timing of polyploidy. Scaldaferro et al. (2012) proposed that polyploidy in *S. elaeagnifolium* has multiple origins from different diploid populations.

Preliminary assessment of material from our survey suggested that hexaploidy was associated with more humid climates, while diploidy seemed to be associated with dry areas with hot summers and cold winters. Tetraploidy fell in the middle of the distribution and is also found in the south of the province of Buenos Aires (Chiarini pers. comm.; Figure 83). Further analysis of plant and insect collections is planned to determine whether there is any correlation between ploidy level and associated fauna. In addition, seeds from every site were collected and propagated for subsequent host-specificity testing. Germination rate was low in most of the cases (10-20%).



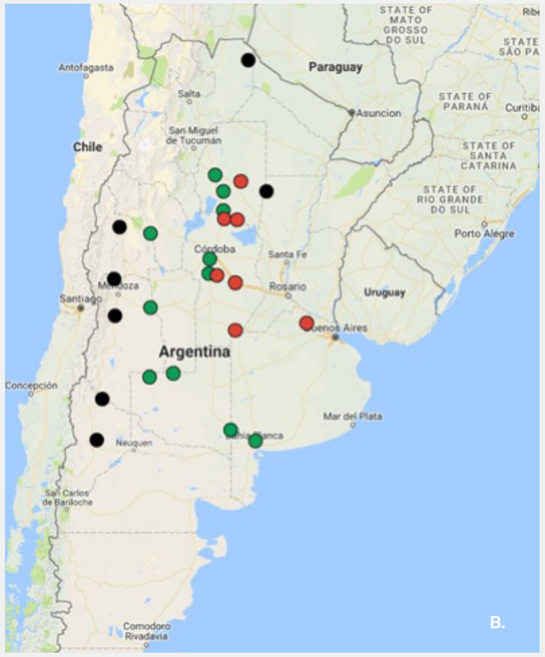


Figure 84. (A) Cytotype distribution of *Solanum elaeagnifolium* in Argentina adapted from Scaldaferro et al. 2012. Three cytotypes are naturally distributed: diploid (black circles), tetraploid (green) and hexaploid (red). (B) Collection sites from FUEDEI surveys in Argentina and Paraguay.

Natural enemies

Seventeen natural enemy species were found during the 2017-18 surveys (Appendix 23). The beetle *Gratiana cf. lutescens* was found in the southern, eastern and northern part of the plant's distribution in Argentina and Paraguay and was very common. Both larvae and adults caused extensive damage to the plant. Both life stages were difficult to see on leaves when population levels were low (Figure 84).

A Tingidae (possibly in the genus Gargaphia) was found in almost all locations. It produced characteristic and extensive damage on leaves (Figure 85), but it was also observed on the congeneric *Solanum sisymbriifolium*.

Other frequently observed natural enemies were gall-producers, noticeably Eriophyidae and nematodes (Figure 86). Mite galls of two types were observed; curly external leaf margins, and others resembling typical rust pustules (Figure 87), probably of the genus *Aceria*.

Project outcomes

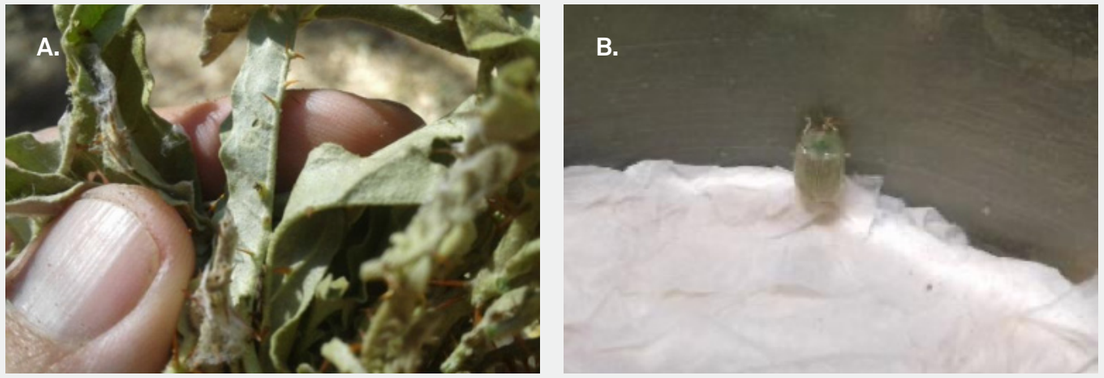


Figure 85. The tortoise beetle *Gratiana cf. lutescens*; (A) damage with second instar in foreground (arrow); (B) Adult.

Figure 86. Tingidae possibly *Gargaphia* sp. found on *S. elaeagnifolium*. (A) typical tingid damage; (B) nymphs congregated on the underside of the leaf.

Figure 87. Other natural enemies found on *S. elaeagnifolium* in the northern part of the survey area: (a) Coccidae; (b) galls formed by nematodes.

Apart from these herbivores, several species of adult Meloidae, in the genera Epicauta and Tetraonyx were observed producing extensive damage. However, these were considered not suitable for biological control as the larval stage is insectivorous.

Figure 88. Galls produced by Eriophyidae: (A-B) two types of galls produced by Acarii (possibly different species), (C) detail of one type of gall, and (D) eriophid mites (*Aceria* sp.) photographed under 400 x stereoscope.

2018-2019 natural enemy surveys, Argentina & Paraguay

2018-19 surveys used the previous season's results to target surveys more effectively. Surveys were conducted in southern Buenos Aires Province, Central and Northern Argentina. These regions were selected because of previous success collecting natural enemies, plant ploidy level, or climate suitability with south eastern Australia. There was a focus on diploids in southern areas of the distribution of the plant for most of the potential agents. The mite Aceria was collected from sites where specimens for identification were previously sourced. At every site, fruits, leaves, flowers and whole-plant specimens were collected for future genetic and morphological studies.

Natural enemies

Aceria aff. bicornis

In the field, plants attacked by the *Aceria* mite were distinguished by typical gall formations of buds, leaves and even stems (Figure 88). A mite specialist from La Palta Museum considered this species may be *Aceria* aff. *bicornis* (Trotter 1900), a mite that has been found in abundance on this weed in Argentina (Kwong and Sagliocco 2012). Specimens were sent to Denis Navia (Embrapa, Brasilia-Brazil) to confirm the identification.

Entire plants with galls were taken to the FuEDEI laboratory and conditioned for mite rearing and transfer to new plants.

Gratiana cf. lutescens

The beetle *Gratiana cf. lutescens* was found on most plants checked at all sites visited. Around 50 adults and 30 larvae were collected in southern Buenos Aires Province for host-specificity testing .

Rust

A pathogenic rust fungus was found on silverleaf nightshade near Merlo city (San Luis Province, central Argentina). The rust is apparently heteroic, which means that it needs another plant to complete its lifecycle. The rust is being studied by Dr Freda Anderson (CONICET-CERZOS, Bahía Blanca) to identify the species. Dr Anderson has stated that there are no records of rust on silverleaf nightshade in Argentina. The rust is unlikely to be useful for classical biological control, however the finding is significant because it demonstrates that the full suite of natural enemies of silverleaf nightshade is not yet described.

Corythaica passiflorae

The tingid *C. passiflorae* (Berg) (Tingidae) was found frequently during this survey. The geographic distribution of this tinged was previously recorded by Montemayor and Melo (2012). The insect was recorded on several host plants (Solanum melongena, *S. nigrum v. americanum*, *S. paniculatum*, *S. sisymbrifolium*, *S. argentinum*, *S. argillicolum*, *S. elaeagnifolium* and others) (Montemayor and Coscarón 2005; Montemayor and Melo 2012) and is not likely to be sufficiently host-specific for Australia.

Project outcomes

2019-2020 natural enemy surveys, Argentina & Paraguay

Data from natural enemy surveys conducted during 2019-2020 were still being assessed at the time of reporting.

An *Aceria* mite previously referred to as *Aceria* aff. *bicornis* in this report is now considered to be a new, undescribed species that is possibly host-specific on silverleaf nightshade. Reporting from this point will refer to this mite as *Aceria* sp. nov.

***Aceria* sp. nov. (Acari: Eriophyidae)**
Aceria sp. nov. was found at every site visited in 2019-2020 (Figure 89). At each site, mites were collected to confirm identification and to develop rearing protocols. For identification purposes, symptomatic leaves (with galls) were collected and put into vials with ethanol (96%). These were preserved in the freezer for further morphological and molecular analysis. Live mites for rearing and host-specificity testing were collected from Lavalle (Santiago del Estero Province) and Anillaco (La Rioja Province).

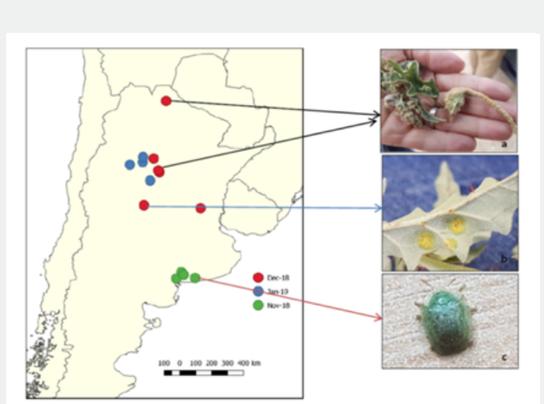


Figure 89. Exploration surveys conducted in Argentina for potential biocontrol agents against *S. elaeagnifolium*. (a) *Aceria* aff. *bicornis*, (b) rust, (c) *Gratiana* cf. *lutescens*

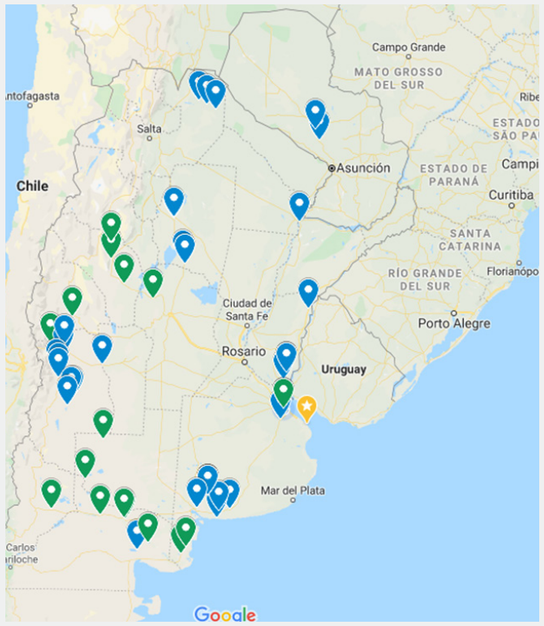


Figure 90. Locations where *Aceria* sp. nov. was collected during the 2019-2020 survey period.

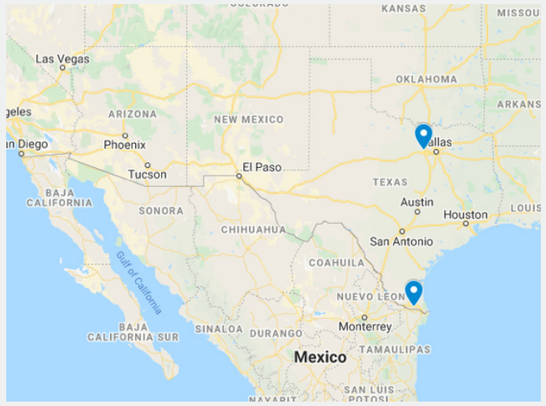


Figure 91. Locations of natural enemy observations in the USA in 2017 (Weslaco, south Texas and Fort Worth, north central Texas) and 2018 (Weslaco).

2017 and 2018 natural enemy observations, Texas, USA,

Greg Lefoe (Agriculture Victoria) recorded observations of potential silverleaf nightshade agents in Texas, USA, in April 2017 and April 2018 (Figure 90). This work coincided with native-range field studies of the leaf beetle *L. texana* (research into *L. texana* was part of the MLA-led RR&D4P Round 1 project but the opportunity was taken to commence assessments of other potential agents in Texas for the RR&D4P Round 2 project).

Apart from *L. texana*, the tortoise beetle *Gratiana* cf. *pallidula*, a tingid *Gargaphia* aff. *arizonica*, and the leaf galling nematode *Ditylenchus* cf. *phyllobius* appeared to be common and damaging to silverleaf nightshade (Figure 91). Black spots and yellowing leaves were also observed on silverleaf nightshade infested with *Gargaphia* aff. *arizonica*, which may be associated with a fungus from the genus *Pestalotiopsis* (Figure 91 and Figure 92).

The nematode *D. phyllobius* has previously been ruled out as a potential agent in Australia (Field, Kwong & Sagliocco 2009).

The host-range of some *Gargaphia* spp. is uncertain, although *G. arizonica* is purported to be specific to silverleaf nightshade (Kwong 2006). *Gargaphia arizonica* has a wide distribution in the USA (Wapshere 1988) and *G.* aff. *arizonica* was observed in south Texas and north central Texas (close to Oklahoma) in this study.

The tortoise beetle *Gratiana pallidula* is also widespread in Texas and may be the same as, or very closely related to, *G. lutescens* in Argentina. Genetic studies could shed light on the relationship between these *Gratiana* species.

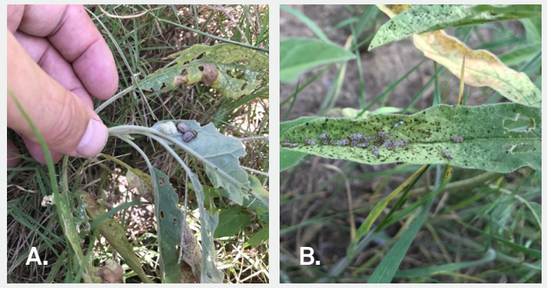


Figure 92. Weslaco, South Texas, 2017. (A) combined impacts of several potential agents are shown on this plant including *Leptinotarsa texana* larvae, disc-shaped feeding damage from *Gratiana* cf. *pallidula*, evidence of feeding by *Gargaphia* aff. *arizonica*, and a leaf gall possibly caused by the nematode, *Ditylenchus phyllobius*, (B) *Gargaphia* aff. *arizonica* on silverleaf nightshade. Black spots and leaf yellowing may be associated with a fungal pathogen. Note disc-shaped damage caused by *G.* cf. *pallidula*.

Figure 93. *Tingids Gargaphia* aff. *arizonica*. damaging *S. elaeagnifolium* at Fort Worth Nature Reserve, north central Texas, USA 2017.

Project outcomes

2019-2020 natural enemy surveys, Texas, USA

Periodic observations for the lacebug *G. arizonica* were made in December 2019 and January 2020 on field populations of silverleaf nightshade in Hidalgo county, Texas. No individuals of the lacebug were observed during those months. A more extensive survey was conducted in February 2020 at 160 distinct locations across Hidalgo county and 11 other south Texas counties (Figure 93, Table 18). Data on site condition was recorded at each location, and 3-5 plants of silverleaf nightshade were collected and stored in plastic bags. Bags were brought back to the lab at the end of each day and plants were inspected for herbivores.

Across all sites, we found over 20 types of insects on silverleaf nightshade (including individuals of *L. texana*, coccinelid beetle, unidentified weevil, mites, aphids, among others). Lacebugs (*Gargaphia* spp.) were observed at 30 of the 160 sampling locations in 7 of the 12 counties (Figure 94). Leaf beetles (*L. texana*) were detected in 51 sampling locations in 6 counties (Table 18). Adult lacebug specimens (47 total insects) are being prepared to submit for identification.

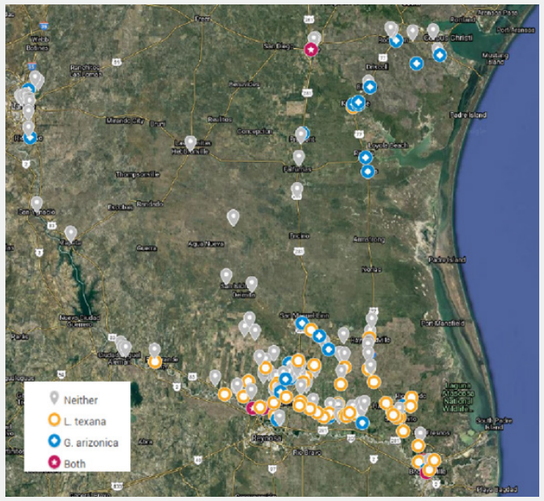


Figure 94. Silverleaf nightshade sampling locations in south Texas, USA, February 2020.

Table 18

Sampling results for lacebugs and leaf beetles in south Texas, February 2020.

County	Total Sites	*Gargaphia* spp. sites	*L. texana* sites
Brooks	2	0	0
Cameron	26	5	16
Hidalgo	78	12	30
Jim Hogg	3	0	0
Jim Wells	5	2	1
Kenedy	1	1	0
Kleburg	5	4	1
Nueces	10	4	0
Starr	8	0	1
Webb	11	2	0
Willacy	8	0	2
Zapata	3	0	0
Total	**160**	**30**	**51**

Figure 95. *Gargaphia* sp. collected by the University of Texas in February 2020

Conclusion

The diversified survey strategy we adopted, which encompassed new, climatically matched areas of Argentina and supplementary observations and surveys in Texas, USA, identified at least three promising candidate agents for Australia. Of these, the tortoise beetle Gratiana lutescens in Argentina (and possibly *G. pallidula* in the USA) was prioritised for testing due to its ability to damage silverleaf nightshade, wide distribution and apparent field host-specificity. Further studies were also initiated on Aceria sp. nov. to develop culturing methods and enable host-specificity experiments if *G. lutescens* was not suitable. Of the potential agents observed in Texas, USA, the tingid *G. arizonica* was prioritised due to its apparent field host-specificity and wide geographic distribution. As with *Aceria* sp. nov., further work is necessary to understand the biology and ecology of *G. arizonica*, to develop rearing methods, and to assess it suitability as a potential biocontrol agent in Australia.

Further analysis of plant and insect collections from Argentina is also planned to determine whether there is any correlation between silverleaf nightshade ploidy level and associated fauna, and any possible implications for agent collection and testing.

Output 11(d) - Conduct host-specificity tests on potential biocontrol agent(s) and (11e)

Revising the host-specificity test list – selecting crop and ornamental cultivars

New recommendations for selecting cultivars used in host-specificity testing were developed after observing *L. texana* utilise potato in our RnD4P Round 1 Project. We argued that damage to potato in our laboratory experiments was due to differences between the cultivars tested in Australia, and those previously tested in South Africa. Our findings from Round 1 testing of L. texana, and new recommendations for documenting and reporting cultivars developed in Round 2, were published in the peer-reviewed journal *Biological Control* (Lefoe *et al.* 2020a) (Appendices 31 & 32). We subsequently reviewed existing guidelines for cultivar selection and developed a new cultivar selection tool to address identified gaps (Lefoe *et al.* 2020b) (Appendix 22). This work resulted in a revised list of potato cultivars for host-specificity testing.

An abstract (below), manuscript (Lefoe *et al.* 2020b) (Appendix 22) and copy of a presentation to Australian biological control collaborators and regulators (Appendix 24) are provided.

Abstract

Classical biological control, using specialised natural enemies (biocontrol agents), is important for long-term, sustainable management of invasive species such as weeds. To be acceptable for introduction, new biocontrol agents must not damage crops, native plants or other non-target species. Host-specificity experiments inform risk assessment of new biocontrol agents by prioritising and testing non-target plant species. However, it was recently highlighted that current approaches may be inadequate for assessing risks to crop and ornamental species because susceptibility to damage can vary between cultivars of the same species.

We reviewed and documented current cultivar selection practice published in prominent biological control journals and government documents. In our review, most papers either did not mention cultivars of the crop or ornamental species being tested, or they provided incomplete descriptions of cultivars without explaining omissions. In most cases, if cultivars were listed then the criteria used to select cultivars were not described, were incorrectly applied or inconsistently applied. Only one of 29 papers fully described the method for selecting and prioritising cultivars and reported the results for each cultivar tested.

Project outcomes

To address perceived gaps, we elicited expert opinion and, combined with our assessment of best practice, developed a decision support tool comprising a process chart and criteria for prioritising cultivars for biological control host-specificity testing. We applied the decision tool to an important and complex host-specificity testing case study.

By applying our criteria and process chart to a case study we demonstrated how current gaps in cultivar selection practices can be addressed. From the thousands of potato cultivars grown world-wide, we selected a short-list of cultivars that is feasible to test, and which can be scrutinized and updated. We demonstrated how selections could be made through a collaborative and transparent process involving key stakeholders and risk bearers.

The decision tool has broad application in weed biological control risk assessment. We demonstrated that the decision tool is easy to use, can account for uncertainty, is adaptable to different species, and is suitable for both small and large cultivar groups irrespective of complexity. We argue that our approach, if adopted, will result in more transparent, defensible and reproducible cultivar selection practices leading to greater confidence in biological control risk assessments.

Host-specificity testing of two prospective agents from Argentina

Standard no-choice testing (Sheppard, van Klinken, & Heard, 2005) was conducted at the FuEDEI-Argentina laboratory in Buenos Aires. In each experiment six or eight replicates of *S. elaeagnifoium* and six or eight replicates of each non-target test species were presented as whole potted plants in insect cages.

***Gratiana* sp.**

Around 50 adults and 30 larvae were collected in southern Buenos Aires Provinces. These were enclosed in cages in replicated no-choice experiments with silverleaf nightshade *S. elaeagnifolium*, eggplant *S. melongena* and potato *S. tuberosum*.

***Aceria* sp. nov.**

Test plants (target and non-target species) were cleaned of insects and mites, and their leaves cut to encourage growth of new buds. Symptomatic *S. elaeagnifolium* plants with mites were collected near La Banda (Santiago del Estero Province, Northern Argentina), and transported to the FuEDEI laboratory in Buenos Aires. Entire plants with galls were maintained in the laboratory and conditioned for rearing. Mites were transferred to S. elaeagnifolium, eggplant *S. melongena* and potato *S. tuberosum* using one of two methods (1) galls from symptomatic plants were put in contact with new buds, or (2) mites were picked with a fine needle and directly placed onto test plants (Fig. 3.2.2.1). This procedure was repeated each day for five consecutive days to ensure establishment. In further experiments, plants with symptoms of *Aceria* were collected and taken to FuEDEI. Leaves with galls were put in contact with asymptomatic plants of *Solanum elaeagnifolium* and potato in a no-choice design. Leaves with galls were randomly attached to eight plants of *S. elaegnifolium* as control treatment and eight plants of potato in November 2019. Another set of experiments were conducted in the same way in December 2019 but using eggplant as a test plant. Plants were potted and enclosed in fine-mesh cages.

To confirm presence of mites on no-choice test plants (and field collected plants), symptomatic buds or galls and non-symptomatic buds were collected and placed in vials with 96% ethanol following a modified method of Zacharda *et al.* (1988) (Figure 95). Infested plant material (leaves, spurs and shoots with undeveloped leaves) were immersed in 80–96% ethanol in a covered beaker and shaken for 5–10 seconds. The plant material was then removed and the alcohol containing the preserved mites poured into a separating funnel. The mites, which settled on the bottom, were transferred to a microscope slide and counted under a dissecting microscope.

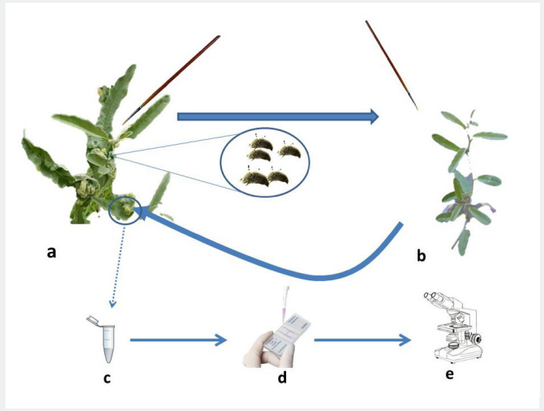


Figure 96. Schematic procedure to collect, inoculate and record *Aceria* sp. nov. (A) *S. elaeagnifolium* plants with galls (symptoms) are selected, mites are taken from galls with a fine brush or fine needle and (B) placed onto new buds of asymptomatic *S. elaeagnifolium* and test plants. After several weeks, symptoms appear on infested plants. (C) sections of galls and buds are collected in vials containing ethanol, which are shaken to separate mites from plant tissue. (D-E) mites are mounted on slides and counted with a dissecting microscope.

Results and Discussion

The South American tortoise beetle *G. lutescens* was initially prioritised for host-specificity testing in Argentina because surveys in that country found it to be widespread, damaging to *S. elaeagnifolium*, and apparently host-specific in the field. However, Gratiana cf. lutescens fed and laid eggs on *S. elaeagnifolium* and the crop species eggplant *S. melongena* and potato *S. tuberosum* in no-choice experiments in cages (data not shown). Off-target feeding damage to eggplant has occurred with other *Solanum*-feeding insects under no-choice conditions and may be resolved if other evidence of host-specificity is available (Olckers & Hulley 1994). However, damage to potato has ruled out *Gratiana* cf. *lutescens* as a suitable agent for Australia due to the crop's importance, and the difficulty of testing certain cultivars relevant to Australia. Further research into the South American *Gratiana* cf. *lutescens* was therefore discontinued.

The South American gall-forming mite, thought to be the previously recorded *Aceria bicornis*, is now considered to be an undescribed species *Aceria* sp. nov. *Aceria* sp. nov. was prioritised as a candidate agent for Australia when work on *Gratiana* cf. *lutescens* ceased. Initial studies of *Aceria* sp. nov. focused on engaging specialist support for molecular and taxonomic identification and developing inoculation and culturing methods. A modified inoculation method was applied to initiate host-specificity testing on *S. elaeagnifolium* and non-target test plants. Initial host-specificity testing results were considered inconclusive as two out of six control (*S. elaeagnifolium*) plants, and one out of six test plants (*S. tuberosum*) developed galls. However, it was considered probable that the *Aceria* sp. nov. culture was contaminated with another Aceria species, confounding the results of the experiments. New collections of *Aceria* sp. nov. were therefore made from infested *S. elaeagnifolium* and strict culture hygiene and isolation protocols were implemented. Preliminary results of the latest host-range experiments are very encouraging (Table 19); to date, galls have developed on silverleaf nightshade control plants (Figure 96 Figure 96. Silverleaf nightshade plants inoculated with *Aceria* sp. nov. at the FuEDEI-Argentina laboratory in Argentina.), but incipient symptoms only were detected in two replicates of eggplant (i.e. no development of true galls) (Figure 97).

Table 19

Interim results of host-specificity testing with *Aceria* sp. nov. Numbers refer to replicate plants.

Plant/Replicate		1	2	3	4	5	6	7	8
Control	Mites	+	-	+	+	-	-	+	+
	Galls	-	-	-	+	-	-	+	+
Potato	Mites	?	?	?	?	?	?	?	?
	Galls	-	-	-	-	-	-	-	-
Control	Mites	+	-	+	+	+	-	-	+
	Galls	+	-	+	-	+	-	-	+
Potato	Mites	?	?	?	?	?	?	?	?
	Galls*	-	-	-	-	-	-	-	-

? = still in process ** = no true galls formed to date*

Figure 97. Silverleaf nightshade plants inoculated with *Aceria* sp. nov. at the FuEDEI-Argentina laboratory in Argentina.

Figure 98. Incipient gall development in two replicates of eggplant, *Solanum melongena* inoculated with *Aceria* sp. nov., (A) upper leaf surface, (B) underside of leaf.

Output 11(e) - Obtain testing/import permits for suitable biocontrol agent(s) and develop rearing protocols and experimental design.

A potential barrier to biological control of silverleaf nightshade has been uncertainty surrounding the dual provincial and national permitting processes for exporting biocontrol agents from Argentina. However, FuEDEI have now advised that La Rioja Province has issued collection permits for *Aceria* sp. nov. for biological studies at FuEDEI and potential studies in Australia.

It is anticipated that the necessary provincial and national permits will now be issued in time for a September 2020 shipment of an *Aceria* sp. nov. to Australian quarantine. Once this is confirmed, import permits will be sought from Australian regulators in time for the September 2020 importation.

Output 11(f) - Conduct a silverleaf nightshade biocontrol workshop with stakeholders In NSW.

These were not undertaken as no agent was released.

3.1.9 Sagittaria

Output 12(a) - Conduct a preliminary study and genetic analysis of Sagittaria natural enemies to determine if distinct biotypes of agent(s) exist.

Abstract

Molecular barcoding detected differences between *Listronotus* (Coleoptera: Curculionidae) taxa that were collected from different host species within the Alismataceae family. These differences were observed across multiple collection sites, spanning a range of longitudes within the distribution of the biocontrol target weed, *Sagittaria platyphylla* in its native range in southern USA.

Specifically, *Listronotus sordidus* and *L. appendiculatus* were only collected from *Sagittaria* species and not from *Echinodorus* species despite small amounts of oviposition and development in laboratory trials with *L. sordidus*. Similarly, *S. latifolia* can support the development of *L. appendiculatus* in laboratory host-range experiments, but there was no evidence of *S. latifolia* being utilised as a host in the native range. Rather, the results from DNA barcoding indicate that the weevil larvae sampled from *S. latifolia* were consistently different from *L. appendiculatus* collected from *S. platyphylla* during native range surveys, in turn identical to our own laboratory cultures.

Molecular barcoding provided evidence that the fundamental host range of these weevil species from laboratory host-specificity testing may have overestimated the ecological host range in its native range. The physiological suitability of a plant species to support the lifecycle of an insect does not always predict that the plant will be utilised as a host in the field. Molecular barcoding is one way to illustrate and flag the presence of this phenomenon. Host-specificity testing that relies only on physiological suitability data will always be susceptible to over-estimating the risk of non-target attack by candidate agents.

Introduction

The fundamental host range of an insect is the suite of plant species that can physiologically support its life cycle. The ecological host range of that insect is the suite of plants that an insect utilises as a host in the native and/or introduced environment outside the lab. The fundamental host range of biological control agents determined under laboratory conditions almost always over-estimates the ecological host range (Hinz *et al.* 2014). One explanation for this is that no choice host testing trials do not allow insects to exhibit normal host finding behaviours.

In laboratory-based host-specificity testing two candidate biocontrol agents, *Listronotus sordidus* and *L. appendiculatus*, could develop on a range of plant species within Alismataceae in addition to the target weed, *Sagittaria platyphylla* (Steel *et al.* 2019). Trials designed to test host finding behaviours suggested that the range of plants that could support development of *L. sordidus* in the laboratory may be an overestimate of the host range if the agent was released. Native range studies can be used to investigate the ecological host range of a proposed agent by surveying for attack on the target weed and co-occurring related species. We used DNA barcoding of larval specimens to identify which plant species were being utilised by *L. sordidus* and *L. appendiculatus* in their native range of southern USA.

Methods

Larval samples were collected from plants within the Alismataceae family at sites across the native range of the target weed, *S. platyphylla*. DNA was extracted from each larval sample and two barcode sections were sequenced: COI and ITS. Barcode sequences were used to construct dendrograms showing how similar each sample was to each other. Clusters of samples in the dendrogram were allocated species names if they could be identified, or a taxon code if they could not.

If adult weevils were observed as present at a site they were collected and preserved in ethanol. Further details on the methods used to conduct the molecular study are provided in Appendix 39.

Results and Discussion

Mitochondrial barcode (COI) sequences were generated for more than 100 samples.

Dendrogram produced from sample sequences

The COI barcode sequences clustered into nine groups (Figure 99), three of which clearly aligned with the candidate biocontrol agents *L. appendiculatus*, *L. frontalis* and *L. sordidus*. Another group aligned best with *L. lutulentus*. The other groups were assigned taxon codes (such as L. Ssp1), because without morphological identification, they could not be assigned to a specific species. There were clearly two levels of molecular differentiation within the tree. Most of the groups differed from each other by more than 10% which strongly supports their status as separate species. Two groups (*L.* Ssp1 and *L.* Ssp2) were closely aligned to *L. appendiculatus* and *L. sordidus* respectively (highlighted in Figure 98), differing by approximately 4%. This suggests that these samples may be biotypes of *L. appendiculatus* and *L. sordidus*.

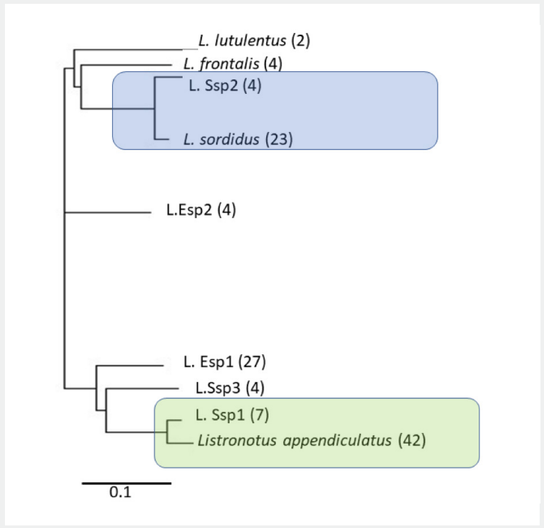


Figure 99. Dendrogram generated from 117 sequences using neighbour-joining. Subtrees collapsed to show only nodes at which samples clustered. Number of samples represented at each node in parenthesis. Clusters highlighted are taxa that are more closely related than others.

There was a clear host plant distinction between the *Listronotus* taxa sampled from the *Sagittaria* species and those sampled from *Echinodorus* species (Table 20). There was particularly strong evidence that *L. appendiculatus* and *L. sordidus* were never recorded from Echinodorus species, as DNA samples for these two weevils were from sites where both *Sagittaria* and *Echinodorus* species were present. There was also a clear delineation of host plants between *L. appendiculatus* and the taxon most closely related to it, LSsp1; the latter was only recovered from *Sagittaria latifolia* and *L. appendiculatus* was only detected on *S. platyphylla*. *L. appendiculatus* and LSsp1 appear to have distinct host plant species.

Table 20

Summary of *Listronotus* taxa collected from Alismataceae host plants in USA (from Steel et al. in prep. b).

Insect taxon	Host plant						
	***Sagittaria* species**				***Echinodorus* species**		
	S. platyphylla	*S. calycina*	*S. latifolia*	*S.* aff *brevi-rostra*	*E. berteroi*	*E. cordifolius*	*E. sp.*
L. appendicu-latus	y	y	n	-	n	n	n
L. Ssp1	n	-	y	-	-	-	n
L. frontalis	y	-	-	-	-	-	-
L. lutulentus	y	-	y	-	n	n	-
L. sordidus	y	y	y	-	-	n	n
L. Ssp2	y	-	y	-	-	-	-
L. Ssp3	-	-	-	y	-	n	-
L. Esp1	n	n	n	n	y	y	y
L. Esp2	n	-	n	-	-	y	y

y: insect taxon collected from this plant species; n: insect taxon was present but not collected from this plant species; insect taxon was not known to be present at a site with this species. Dotted line delimits insect taxa sampled from Sagittaria species from those collected from Echinodorus species.

Comparison to adult samples collected/observed
Adult specimens thought to be *L. appendiculatus* were collected from one site which had both *S. platyphylla* and *S. latifolia* present. The adult specimens collected from *S. latifolia* at this site were noticeably larger than the *L. appendiculatus* collected from *S. platyphylla* and also appeared to possess caudal processes missing from other *L. appendiculatus* specimens collected on *S. platyphylla*. This accords with the mitochondrial data that suggested *L. appendiculatus* is closely associated with another taxon collected from *S. latifolia* whilst *L. appendiculatus* was collected from *S. platyphylla* (Table 20).

Comparison with host-specificity data
In host testing *L. sordidus* performed equally as well on *S. calycina* as it did on *S. platyphylla* (Steel *et al.* in prep. a). *L. appendiculatus* developed equally well on *S. calycina* as *S. platyphylla* and laid many more eggs on *S. calycina* (Appendix 36). The results of host testing accords with the discovery through this molecular testing that *S. calycina* is also a host for both *L. sordidus* and *L. appendiculatus* in the native range.

Under laboratory conditions *L. appendiculatus* was able to oviposit and develop on *S. latifolia* but did not perform as well on this species as it did on its field hosts, *S. platyphylla* and *S. calycina* (Appendix 36). *S. latifolia* was referred to as a field host for *L. appendiculatus* in the literature (from Kwong 2016) but the molecular evidence suggests that the *Listronotus* taxon collected from *S. latifolia* in the field is distinct from that tested in the laboratory. If the two taxa are not different species, they are certainly biotypes predisposed to utilising different host species and likely on an evolutionary path to reproductive isolation. Molecular methods were able to distinguish between these two taxa, one of which was clearly more suitable for biocontrol of the two *Sagittaria* weed species, *S. platyphylla* and *S. calycina*. The *L. appendiculatus* cultures that were used for host testing were of the biotype associated with *S. platyphylla* and *S. calycina*.

Similarly, *Echinodorus cordifolius* was a poor physiological host for both weevils in laboratory trials both in Australia (Steel *et al.* 2019) and in South Africa (Martin *et a*l. 2018) and not found to be a host in native range sampling. Although small quantities of oviposition and development of *L. sordidus* was observed in these laboratory trials, the molecular work suggests that *Echinodorus* are unlikely to be secondary hosts in the field. This evidence is useful for interpreting laboratory-based host testing results where it is difficult to determine whether a plant species may be at risk of off-target damage in the field when small amounts of development are observed in the laboratory.

Conclusions

Molecular barcoding provided evidence that the fundamental host range of these two weevil species from laboratory host-specificity testing overestimated the ecological host range of these insects in their native range. The physiological suitability of a plant species to support the life cycle of an insect does not always predict that the plant will be utilised as a host in the field. Molecular barcoding is one way to illustrate and flag the presence of this phenomenon. Host-specificity testing that relies only on physiological data will always be susceptible to over-estimating the risk of an agent causing off-target damage.

Molecular approaches to field surveys can detect a greater host range (because the larvae can be present but cryptic) and can identify biotypes that may have different host ranges. Larval DNA samples should be taken during initial native range surveys across the target plant family to detect cryptic host use that has not been recorded in the literature. The barcoding approach allows researchers to ensure that they are testing a single biotype of the candidate agent, and that the biotype is the one associated with the target weed.

Output 12(b) - Develop extension material for new Sagittaria agents through the Biocontrol Portal of the Atlas of Living Australia.

The draft Sagittaria Biological Control Field Guide provides general guidelines for how to integrate biological control into the management of Sagittaria at national, regional and local scales. At the national level, the role of biocontrol is considered within the framework of the "Sagittaria Weeds of National Significance strategic plan (2012)" and identifies regions across Australia that could be targeted for biocontrol. At the regional scale, guidelines are provided on how to prioritise Sagittaria sites for biocontrol based on regional weed management plans, such as "The Sagittaria Control Zone of the Murray Darling Basin". At the local scale, a flow-chart is provided to assist in determining if the site is suitable for biocontrol and if so, which agents should be released at the site based on the habitat requirements of each agent. Finally, the manual provides instructions of how to conduct biocontrol agent releases.

The manual is currently in draft form (Appendix 40) and will be updated as each Sagittaria agent becomes available for release.

Output 12(c) - Import suitable biocontrol agent(s) and develop rearing techniques.

Colonies of all three weevil species were collected from multiple locations across the southern USA in collaboration with scientists from the US Army Corps of Engineers (Nathan Harms and Julie Nachtrieb) and Tennessee Department of Environment and Conservation (Alan Trently). Details of the weevil collection locations are provided in Table 21. Once imported into AgriBio's quarantine facility, rearing techniques were developed to produce viable colonies to enable host specificity testing to commence.

Table 21

Importation details for three *Listronotus* species collected from *Sagittaria platyphylla* in the southern USA.

Importation date	Import Permit/Quarantine Entry Numbers	Listronotus species	Collection details
29 Jan 2015	IP14015673	*L. sordidus* (30 adults)	Collector: Nathan Harms (U.S Army Corps of Engineers) Laboratory reared insects collected from two locations in the USA: TARA Wildlife Reserve, Vicksburg (MS) and LEARF, Lewisville (TX)), October 2014
29 Jan 2015	IP14015673 VA15035377	*L. appendiculatus* (64 adults)	Collector: Raelene Kwong: Sunk Lake, (TN) USA, Sep 2015. Lat 35.70962, Long -89.73801
26 Nov 2015	IIP15011407 VA15042193	*L. appendiculatus* (58 adults)	Collector: Julie Nachtrieb: Lewisville Aquatic Ecosystem Research Facility, (U.S Army Corps of Engineers), Lewisville (TX) USA, Aug 2015. Lat 33.0695, Long -96.95852
26 Nov 2015	IP15011407 VA15042193	*L. sordidus* (23 adults)	Collector: Alan Trently (Tennessee Department of Environment and Conservation): Reelfoot Lake (TN) USA, Sep 2015. Lat 36.46723, Long -89.31911
2 Oct 2016	IP 0000679656 NA16086004 (DOE)PWS2016-AU-001340	*L. appendiculatus* (43 adults)	Collector: Raelene Kwong USACE (TX) USA, Sep 2016. Lat 33.0695, Long -96.95852
2 Oct 2016	IP 0000679656 A16086004 (DOE)PWS2016-AU-001340	*L. appendiculatus* (48 adults)	Collector: Raelene Kwong Reelfoot Lake (TN) USA, Sep 2016 Lat 36.46723, Long -89.31911
2 Oct 2016	IP 0000679656 A16086004 (DOE)PWS2016-AU-001340	*L. appendiculatus* (48 adults)	Collector: Raelene Kwong Reelfoot Lake (TN) USA, Sep 2016 Lat 36.46723, Long -89.31911
1 Dec 2016	IP 0000679656 QA16051566 DOE)PWS2016-AU-001340	*L. sordidus* (30 adults)	Collector: Nathan Harms (U.S Army Corps of Engineers) Laboratory reared insects collected from two locations in the USA: TARA Wildlife Reserve, Vicksburg (MS) and LEARF, Lewisville (TX)), October 2014
1 Dec 2016	IP 0000679656 QA16051566 (DOE)PWS2016-AU-001340	*L. sordidus* (2 adults)	Collector: Raelene Kwong Yazoo Wildlife Reserve (MS), Sep 2016. Lat 33.12547, Long -91.00337
1 Dec 2016	IP 0000679656 QA16051566 (DOE)PWS2016-AU-001340	*L. appendiculatus* (4 adults)	Collector: Raelene Kwong Reelfoot Lake (TN) USA, Sep 2016. Lat 36.46723, Long -89.31911

Output 12(d) - Conduct host specificity tests on potential biocontrol agent(s)

Host specificity test list

The test list for determining the host specificity of the three *Listronotus* species included representative genera based on the molecular phylogeny of the Alismataceae family (Chen *et al.* 2012, 2013, see Figure 99), with an emphasis on Australian native species, species of economic importance and those that are likely to overlap biogeographically with the target weeds, *S. platyphylla* and *S. calycina*. Genera and/or species not present in Australia were omitted from testing. Two unrelated plant species were included. The native species, *Cycnogeton procerum* (R.Br.) Buchenau (Syn = *Triglochin procera*) (Juncaginaceae) was included because it commonly occurs in Sagittaria-invaded habitats and has emergent inflorescences with fleshy fruit. The crop species, *Oryza sativa* (rice) (Poaceae) was included because *S. platyphylla* and *S. calycina* are common weeds of rice crops in New South Wales.

Host specificity testing trials

The specificity of *L. appendiculatus*, *L. sordidus* and *L. frontalis* followed internationally accepted protocols as outlined by Sheppard *et al.* (2005). While both *S. platyphylla* and *S. calycina* were nominated as "targets" for biocontrol, for ease of testing only *Sagittaria platyphylla* was used as the designated "control" species. Hence, *S. calycina* was treated as a test species throughout the trials.

An overview of the host specificity test types (oviposition, larval development, continuation trials), test designs (no-choice, choice-minus-target), and whether excised plant parts or whole potted plants were used for each biocontrol agent species is provided in Figure 100. Further details of the specific methods used for each agent species is provided in Section 3 Project Outcomes, and associated appendices where relevant.

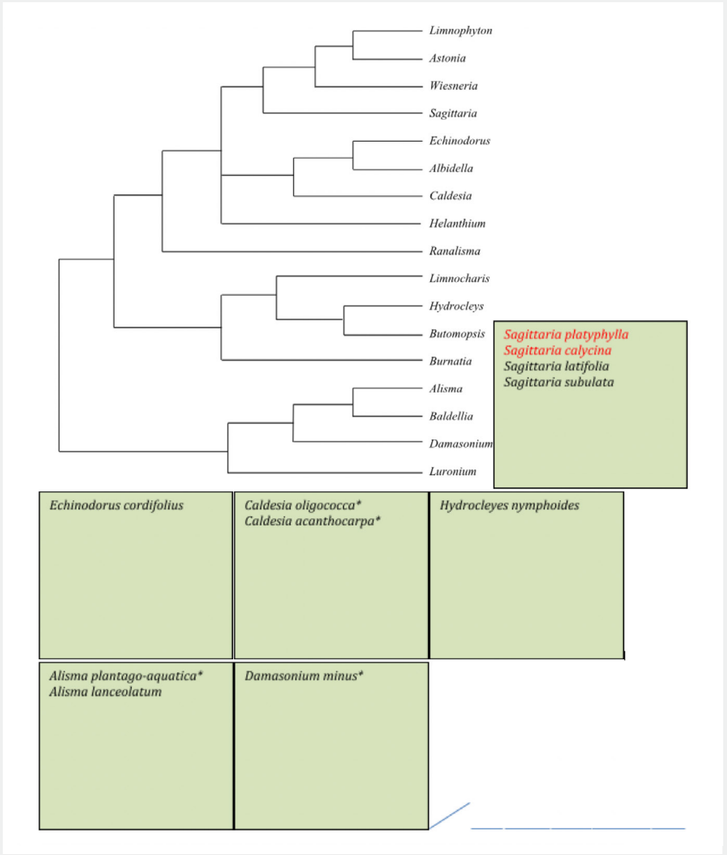


Figure 100. Molecular phylogeny of Alismataceae (from Chen *et al.* 2012, 2013), with the taxonomic relationships between *Sagittaria* spp. and other species used in the host range tests for *Listronotus appendiculatus* shown in boxes. Australian native species are indicated by an Asterix. The target weeds, *Sagittaria platyphylla* and *S. calycina* are highlighted in red text.

Project outcomes

Listronotus appendiculatus fruit-feeding weevil

The specificity of *L. appendiculatus* followed internationally accepted protocols as outlined by Sheppard et al. (2005), using a three-stage process. While both *S. platyphylla* and *S. calycina* were nominated as "targets" for biocontrol by *L. appendiculatus*, for ease of testing only *Sagittaria platyphylla* was used as the designated "control" species. Hence, *S. calycina* was treated as a test species for adult oviposition and larval development trials.

1. Container trial
In these trials, adults were placed in a testing arena (plastic container) with only one (**no-choice single species**) or two test species (**choice-minus-target**), but not with the target. The target species (i.e. *S. platyphylla*) was offered to adults in a separate container. For both no-choice and choice-minus-target trials, bouquets of cut foliage and flowers were presented to adults and assessed for oviposition as well as levels of foliage and fruit herbivory.

2. No-choice whole plant oviposition trials
In these trials, Australian native plant species for which some oviposition and egg hatch had occurred in the container trials, were subjected to no-choice whole plant oviposition trials on potted plants contained within gauze sleeves. In these trials, oviposition (egg laying) and survival to adults was assessed.

3. Whole plant larval development trials:
In these trials, a set number of mature eggs were placed on whole plants to assess the survival rate from egg to adult as well as to assess the damage caused by larval feeding on leaf petioles and flowering stems.

4. Continuation trial:
As adults had emerged from the native species, *Damasonium minus* in the larval development trials a continuation trial was conducted to assess the reproductive performance of these F_1 adults and hence, the ability of *L. appendiculatus* to maintain a viable population on *D. minus*.

Data collected from these three trials were then used to evaluate two key biocontrol agent risk factors: 1) the ability of *L. appendiculatus* to oviposit, survive and maintain viable populations on non-target plant species, and 2) the damage caused by adult and larval feeding on non-target species.

In adult no-choice whole plant trials, oviposition (less than four eggs per plant) were laid on the native species, *Alisma plantago-aquatica* and *D. minus*, while no eggs were laid on the remaining test species.

In no-choice whole plant larval development trials, adult emergence occurred on *S. latifolia* and *D. minus*, albeit at much lower levels than on *S. platyphylla* and *S. calycina*. No larval development was supported on the native species, *A. plantago-aquatica* or *Caldesia acanthocarpa*, or the ornamental species, *Echinodorus cordifolius*.

In the continuation trial, first generation adults reared from *D. minus* plants laid very few eggs and were unable to survive in sufficient numbers after subjected to a winter diapause treatment. The analysis of the population growth rate based on egg production and larval survival over two generations predicted that *D. minus* would be unable to maintain viable populations of *L. appendiculatus*.

Field studies in the native range indicated that the preferred hosts of *L. appendiculatus* were S. *platyphylla* and *S. calycina*. Molecular tools were used to confirm that *L. appendiculatus* did not utilise other closely related species such as *S. latifolia* or *Echinodorus* species growing near *S. platyphylla* or *S. calycina* plants. Furthermore, there was no evidence of biotype differences between L. appendiculatus collected from either *S. platyphylla* or *S. calycina*.

In summary, the results of the quarantine-based host testing and native range molecular studies demonstrate that *L. appendiculatus* has a high degree of specificity for the target weeds, *S. platyphylla* and *S. calycina* and that the risk of off-target damage to native and ornamental species in Australia is low. The native plant, *D. minus* was able to support the development of some larvae, however emerging adults showed low fecundity and survival, and was therefore a substantially inferior host for *L. appendiculatus*. The impact caused by larval feeding on *D. minus* fruit was minimal and unlikely to cause population-level impacts on this widespread species, itself a troublesome weed of rice crops. If approved for release, *L. appendiculatus* might cause some adult-feeding damage to the ornamental species, *S. latifolia* and *S. subulata* however these species are of minor value to the Australian horticulture industry and are banned for sale in states where the *Sagittaria* genus is declared noxious. The decision tree outlined in Figure 101 shows the level of risk likely for each test plant species based on the series of trials undertaken in this study.

Based on these positive results, an application for field release of *L. appendiculatus* for the biological control of *S. platyphylla* and *S. calycina* in Australia was submitted to DAWR on 13th September 2018 and is awaiting approval (as of 3rd March 2020). Further details on the methods and results of host testing are given in Appendix 25. DEDJTR Application for release of *L. appendiculatus*.pdf.

Test type	Test design	Method	*L. appendi-culatus*	*L. sordidus*	*L. frontalis*
Oviposition	INo-choice	*Container trial: adults exposed to plant parts (i.e. fruit, petioles, foliage and/or tubers) of individual test species.*	Yes	Yes	No
	No-choice	*LWhole plant trial: adults confined to potted test plants.*	Yes	Yes	Yes
	Choice-minus-target	*Container trial: adults exposed to multiple test plants within the container.*	Yes	No	No
Larval starvation / development	No-choice	*Larvae placed onto potted test plants and their survival to adults recorded.*	Yes	Yes	Yes
Continuation	No-choice	*Generation trial: assesses whether the agent can maintain successive generations with an increase in population growth rate on test plant species – only conducted for "at risk" test species.*	Yes	Yes	Yes (partially completed)

Figure 101. Overview of host specificity tests for three *Listronotus* species, candidate biocontrol agents for *Sagittaria platyphylla* and *S. calycina*.

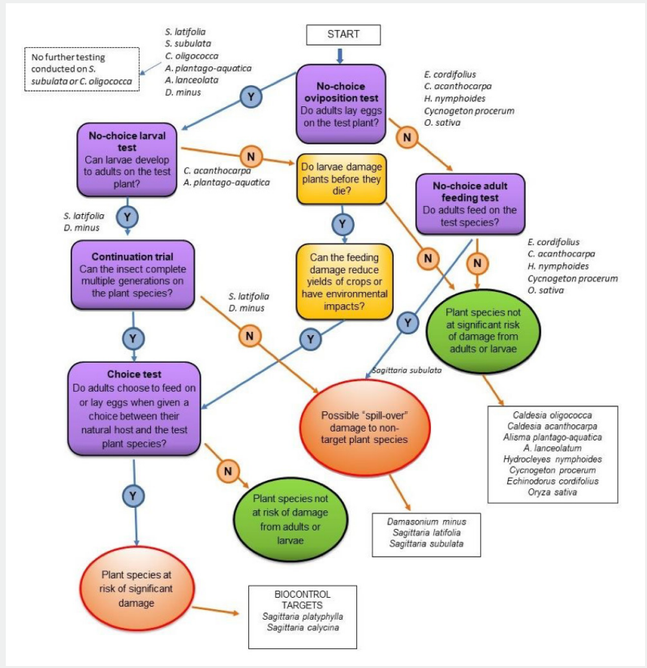


Figure 102. Decision tree used to determine the types of host specificity tests to be undertaken for *Listronotus appendiculatus* for the target species, *Sagittaria platyphylla* and *S. calycina*. The outcome for each test plant species is show in the rectangular boxes.

Project outcomes

Recommendations

1. Pending approval for the release of *L. appendiculatus*, new colonies of fruit-feeding weevils would be imported from the USA to boost the genetic stock of the culture currently held within quarantine.
2. After being released from quarantine, the mass production of weevils should commence in preparation for a national biocontrol implementation program.
3. Research should focus on assessing the impact of the fruit-feeding weevil on S. platyphylla and S. calycina reproduction and spread.
4. Techniques to integrate biological control with other management practices should be developed.
5. The draft Sagittaria Biocontrol Implementation Plan should be updated to incorporate new information and strategies to maximise the potential of biocontrol.

Further work (March -June 2020)

1. Complete life history studies and prepare a manuscript on the biology and host specificity of *L. appendiculatus*.

Listronotus frontalis: tuber-feeding weevil

Standard larval starvation protocols provided by Sheppard *et al.* (2005) were followed to assess the host specificity of *L. frontalis*. Four stages of no-choice tests involving egg transfer and adult oviposition were carried out. Follow up choice and longitudinal tests involving non target species at risk of off target damage are being carried out to supplement results from the no choice trials.

Trial 1. Larval development on whole plants
In these trials, freshly oviposited eggs were transferred onto test plants including the target weed and allowed to develop for approximately one month. The eggs were distributed equally between oviposition sites (crown close to the soil, outer petioles above the crown and the inside of the petioles) on each plant. Data were collected on number of larvae, pupae and adults that developed on each plant species.

Trial 2. Larval development on excised tuber and crown/container trial
Eggs were transferred onto tubers or crowns (for plants that do not produce tubers). Prior to inoculation, the tubers and crowns were placed in containers filled with moistened coco peat. Five containers, i.e. 25 eggs were set up for each test plant. Data were collected on number of larvae, pupae and adults that had developed on each plant species by assessment day.

Trial 3. Oviposition and larval development on whole plants
A single mating pair was confined on each test plant for two weeks, after which, mating pairs were removed from plants and the eggs oviposited allowed to develop. All females were checked for consistent oviposition, for at least on week, prior to being introduced onto test plants. At assessment time, data were collected on number of larvae, pupae and adults collected on each plant species.

Trial 4. Oviposition and subsequent larval development on priority native plants
Results from Trial 3 above, showed that *L. frontalis* could oviposit, and larvae could develop on several non-target species including some priority native species. Trial 4 was a repeat of Trial 3 focusing on the priority native species *A. plantago-aquatica* and *D. minus* that occur alongside the target weed in the field.

Results and Discussion

Results from the above no choice trials showed that *L. frontalis* larvae can damage and complete development on several non-target species including important native species (Figure 102, Appendix 37). These results highlight the risk of *L. frontalis*' attacking non-target plants in the field (Table 22). The likelihood of off-target attack was classified as low (when no larval development was recorded on a test plant), moderate (when inconsistent larval development was recorded) and high (when consistent complete larval development was observed on a species).

Based on this criterion, the likelihood *L. frontalis* attacking any of the non-Alismataceae species (*Eleocharis dulcis*, *Cycnogeton procerum* and *Oryza sativa*) was predicted to be low (Figure 102). Conversely, the likelihood of *L. frontalis* attacking tuber-producing congeneric species (*S. latifolia*, *S. subulata* and *S. sagittifolia*) was predicted to be high because complete larval development comparable to *S. platyphylla* was recorded. Lower levels of larval development occurred on *S. calycina*, presumably due to the small sizes of crowns of *S. calycina* plants tested and the absence of tuber production in this species.

Among other Alismataceae species tested, the likelihood of off-target damage was predicted to be low for *Echinodorus cordifolius* (did not support complete larval development), and *Caldesia oligococca* (occurs under submerged conditions unfavourable for *L. frontalis* oviposition and larval development in the field). The likelihood of attack of *Damasonium minus* was predicted to be moderate mostly because results on this species were inconclusive. *D. minus* plants tested were often overwhelmed by feeding larvae and died before larvae could complete development. A high likelihood of off-target attack was predicted for *Hydrocleys nymphoides* and *A. plantago-aquatica*. Both of these species supported complete larval development.

Recommendations

1. To improve the prediction of off target attack in the field, choice experiments should be conducted to assess whether *L. frontalis* adults can orient towards non target plants presumed to be at risk of off target damage in the field. Native plants such as A. plantago-aquatica and *D. minus* should be prioritised for these tests.
1. Continuation studies should be carried out to assess whether native plant species at risk of attack can sustain increasing populations of *L. frontalis* over successive generations.
1. Host utilization by *L. frontalis* was not consistent between young (less than 3 months old *vs* old 6 months old plants) (Appendix 26). The implications (to host specificity testing) of changes in plant resistance to herbivory as plants grow older should be investigated further. Often in host specificity studies, host utilization is determined by testing plants of a similar age, this poses a risk of underestimating the host range of a potential agent if plants are tested at the age when they are most resistant to herbivory.

Further work (March -June 2020)

1. Choice experiments to assess whether gravid *L. frontalis* females will orient towards *A. plantago-aquatica* and *D. minus* for oviposition.
1. Choice experiments to assess whether *L. frontalis* adults can migrate from completely damaged *S. platyphylla* plants to infest *A. plantago-aquatic*a and *D. minus* plants.
1. Continuation studies to assess weather *A. plantago-aquatica* can sustain increasing populations of *L. frontalis*.

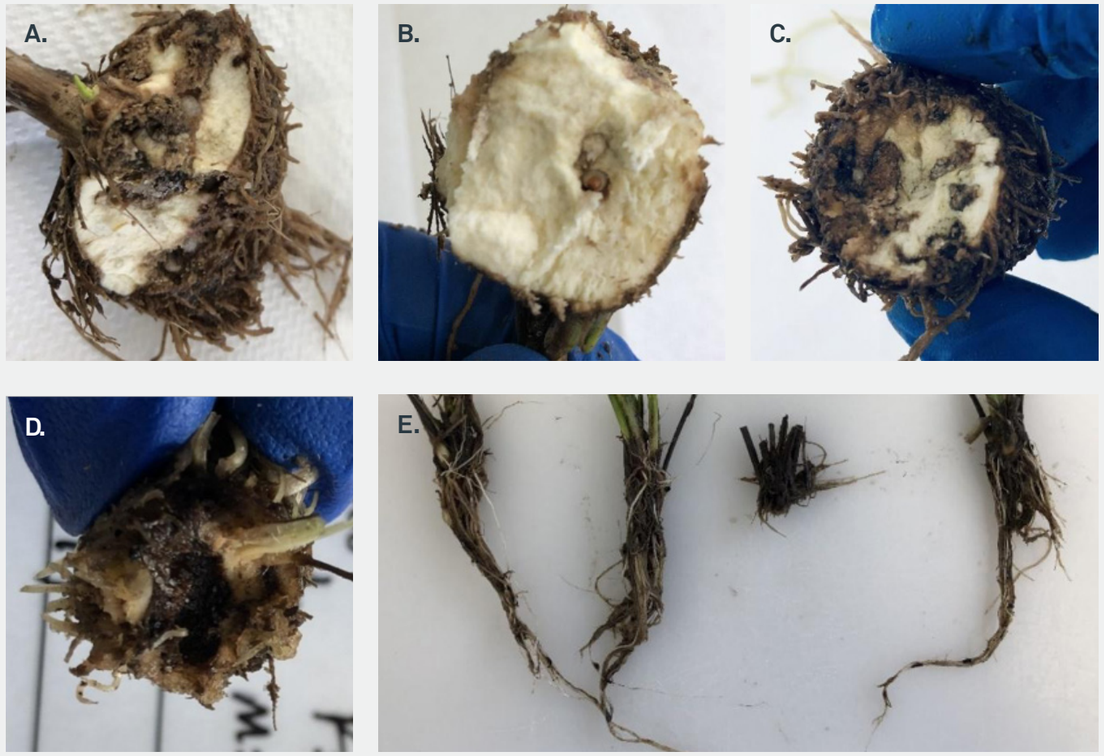


Figure 103. Examples of crown damage observed on priority native plants (A-C) *Alisma plantago-aquatica*, (D) *Damasonium minus*, (E) small sized *Damasonium minus* crowns that were often overwhelmed by *Listronotus frontalis* larval feeding.

Table 22

Summary of *Listronotus frontalis* host specificity results. The likelihood of off-target attack is predicted to be: low (no larval development occurred on test plant), moderate (inconsistent larval development presumed to have resulted from unnatural plant conditions), or high (consistent larval development occurred on test plant). + = positive observation, - = negative observation and NT = not tested.

plant species	no choice egg transfer and larval development						no-choice adult oviposition and larval development						Likelihood of field attack	
	whole plants (trial 1)			tubers & crown (trial 2)			priority plants whole plants (trial 3)			high priority native plants (trial 4)				
Congeneric species	larvae	pupae	adults	larvae	pupae	adults	larvae	pupae	adults	larvae	pupae	adults	larval development	field damage
Sagittaria platyphylla	+	-	-	-	+	-	+	+	-	+	+	+	H	H
Sagittaria calycina	+	-	+	-	-	-	+	-	-	NT	NT	NT	M	M
Sagittaria latifolia	+	-	+	-	-	+	NT	NT	NT	NT	NT	NT	H	H
Sagittaria cf. subulata	+	-	+	-	-	+	+	+	+	NT	NT	NT	H	H
Sagittaria cf. sagittifolia	+	+	+	-	-	+	NT	NT	NT	NT	NT	NT	H	H
Alismataceae family														
Echinodorus cordifolius	-	-	-	-	-	-	NT	NT	NT	NT	NT	NT	L	L
Caldesia oligococca	NT	NT	NT	NT	NT	NT	-	-	-	NT	NT	NT	L	L
Hydrocleys nymphoides	+	+	+	-	-	-	+	+	-	NT	NT	NT	H	H
Damasonium minus	NT	NT	NT	+	+	-	+	+	-	-	-	-	M	M
Alisma plantago-aquatica	-	-	-	-	-	+	-	-	-	+	+	+	H	H
Non-Alismataceae species														
Cycnogeton procerum	-	-	-	-	-	-	NT	NT	NT	NT	NT	NT	L	L
Eleocharis dulcis	+	-	-	-	-	-	NT	NT	NT	NT	NT	NT	L	L
Oryza sativa	NT	NT	NT	-	-	-	NT	NT	NT	NT	NT	NT	L	L

H = High, M= Medium, L= Low

Listronotus sordidus: crown-boring weevil
Listronotus sordidus: Pre host-specificity studies

Abstract

Laboratory host testing trials found that *L. sordidus* could develop well on almost all *Sagittaria* species, all of which are exotic and some also invasive, and also on the native plant *Damasonium minus*. Species that are unlikely to be at risk of attack included the crop plants rice and water chestnut, native plant species *Caldesia oligococca*, *C. acanthocarpa*, and *Cycnogeton procerum*, as well as the ornamental *Echinodorus cordifolius*. Two plant species were suboptimal hosts but could support the entire life cycle of *L. sordidus*: the native plant *A. plantago-aquatica* and the ornamental and invasive plant *Hydrocleys nymphoides*.

Trials also reported here provided evidence that some of these species may not be utilised as hosts in the field at least under certain conditions. Larvae appear unable to develop on any plant species if the crown of the plant is inundated by water. All the species tested grow in water that inundates the crown for at least part of the growing season. During this time these plants will be protected from crown damage.

A no-choice oviposition test was designed to measure the insect's level of motivation to oviposit on each test plant species. The results of this timed oviposition trial suggested that *L. sordidus* was less motivated to oviposit on plant species outside the *Sagittaria* genus, except for *D. minus*.

Host testing and associated trials reported here did not provide evidence that populations of the native plant species *D. minus* and *A. plantago-aquatica* would not be significantly impacted by the release of this agent. An application for release of this agent will not be submitted until further studies that examine the host finding behaviour of *L. sordidus* are completed.

For further information on *Listronotus sordidus* host specificity testing results of this report contact DEJPR, Victoria.

Introduction

Listronotus sordidus is a crown-boring weevil that could destroy infestations of the aquatic weed *Sagittaria platyphylla* (family: Alismataceae). No-choice trials were used to assess which plant species present in Australia could be at risk of damage by *L. sordidus* if it was released as a biocontrol agent. The plants most at risk of attack are those that are most closely related to the target weed as they are most likely to share chemical and physical attributes that are suited to the insect's development. Plants that grow in similar environments may also be at risk of spillover attack if large populations of the agent overburden the target weed and disperse to the surrounding vegetation. For these reasons the plants included in the test list were native and ornamental plants from the Alismataceae family, as well as the aquatic crop plants rice and water chestnut, and the native aquatic plant *Cycnogeton procerum*. A sequence of host testing methods was used to provide certainty that if a plant species could support the development of *L. sordidus* then it would be detected in at least one trial.

Methods

Container trials

Adults were confined to containers set up with a bouquet of foliage for food and dried petioles (leaf stems) as oviposition substrate (Figure 103) from a single species as per Table 3.3.2.3.1. After five days the petioles were removed and sealed into petri dishes. The number of larvae that emerged from the petioles or the number of eggs dissected from the petioles was counted.

Petiole trial

Replicates were set up as per the container trials except that in each container the adults were provided with six petioles, each dried for a different number of days (0, 3, 5, 7, 10, or 12). After four days the number of eggs dissected from the petioles was counted.

Potted plant trials

In the single generation trial adults were confined to a potted plant (Figure 104) of a single species (Table 23) for six weeks, after which time the pots were destructively sampled and the number of progeny in each pot was counted. The continuation trial was set up as above, but adults were removed after one week. After eight weeks the plants were destructively sampled, and the progeny collected. Adult progeny were placed in containers set up as per the container trial with material from the plant species they emerged from. Immature larvae were collected from the petioles from the containers and applied to potted plants at the rate of ten per pot. Adults were counted as they emerged, and this method was continued for one further generation.

The single generation potted plant trials were set up in pairs at two water levels: half of which had their crowns submerged in water (high water level), the other half had their crowns exposed (low water level).

Figure 104. Setup of a container trial replicate

Project outcomes

Timed oviposition trial
Containers were set up as per the container trial. Petioles were removed and replaced after six hours, 24 hours and 4 days. The number of eggs laid into the petioles was counted per container at each time period. Plant species tested included all of those in Alismataceae listed in Table 23, except *Alisma lanceolatum* and *S. subulata*.

Larval starvation trial
Larvae were transferred to potted plants encased in mesh sleeves. After six weeks plants were destructively sampled, and the number of surviving individuals was counted per pot.

Water level trial
Adults were confined to potted *Sagittaria platyphylla* plants, half of which had their crowns submerged in water (high water level), the other half had their crowns exposed (low water level). After one week, adults were removed, and after 25 days the plants were destructively sampled to collect the larvae. The number and size of the larvae in each pot was recorded.

Detailed methods for all trials (except for the petiole trial) are in either Steel *et al*. 2019 available from DEJPR.

Results and discussion

Assessing host suitability of plant species for *L. sordidus* was complicated by several important aspects of the insect's biology. Initial trials were conducted to determine conditions under which the insects were most likely to accept the test plant as a host, to avoid false negative results.

Initial petiole trials revealed that *L. sordidus* lay eggs inside the stems of leaves (called petioles) and prefer to oviposit in dried plant material. In order to observe the number of eggs laid it was necessary to dissect the plant material. The first host testing trials involved counting larval emergence as a more time-efficient way to measure oviposition, whereas in later oviposition trials eggs were counted directly from dissection. The results of the first trials were not reproduced in later trials and this may be due to this difference in the assessment methods. Species which appeared to support significantly lower levels of oviposition in the container trials (*S. calycina*, *S. latifolia* and *Damasonium minus*) (Table 23) were found in the subsequent timed oviposition trials to have overall similar levels of oviposition compared to the target weed (Table 24). It is possible that counting larval emergence as a measure of oviposition was not accurate as there may have been egg or larval mortality from plant defence chemicals and/or cannibalism between larvae. It should be noted that the initial oviposition trials for *Echinodorus cordifolius* and *Oryza sativa* (rice) were assessed by dissecting petioles to count the number of eggs present so these results are more certain.

Figure 105. Setup of a potted plant trial replicate.

Table 23

Summary of container and potted plant trial results for effect of plant taxon on host utilization from Steel et al. (2019). The test plant genera are listed in approximate order of relatedness to Sagittaria based on molecular phylogenies.

Taxa	Container trial		Potted plant trials Oviposition and larval development	
	Larvae per container	Eggs per container	Single generation trial Total progeny per plant at 6 weeks	Multiple generation trial Adults per plant
L. appendicu-latus				
Sagittaria platyphylla[a]	44	17	27^	17^
Sagittaria calycina[a]	**1**	-	**1^**	-
Sagittaria latifolia[b]	**22**	-	**5^**	-
Sagittaria subulata[b]	**5**	-	**0**	-
Sagittaria sagittifolia[b]	41	-	**84^**	-
Alismataceae family				
Echinodorus cordifolius[b]	-	**0**	-	-
Caldesia oligococca[c]	**11**	-	**0**	**0**
Hydrocleys nymphoides[b]	**10**	-	**0**	-
Damasonium minus[c]	**19**	-	**0^**	**2^**
Alisma lanceolatum[b]	38	-	-	-
Alisma plantago-aquatica[c]	51	-	**0**	**0^**
Non-Alismataceae species				
Cycnogeton procerum[c]	**4**	-	**0**	-
Eleocharis dulcis[d]	**0**	-	-	-
Oryza sativa[d]	**-**	**0**	**0**	-

Plant species:[a] invasive in Australia;[b] ornamental species;[c] native to Australia;[d] food plant. ^Adult development was observed (note that values were derived from a statistical model so that a small number of progeny may be represented by a zero value); - test not conducted. Values in italics are associated with plants that died and may be underestimates. Values in bold were statistically different from the target weed results.

Project outcomes

The larvae of *L. sordidus* feed on the crown (starchy area that joins root and leaf parts of the plant) and then migrate into the soil to pupate. Initial trials found that fewer, smaller larvae were collected from *S. platyphylla* (target weed) plants that had their crowns submerged compared to those that were exposed (Table 25). In the potted plant trials, no adults were collected from plants with submerged crowns, even on the target weed. Consequently, water levels were kept below crown level in the potted plant trials to provide optimal aquatic conditions for larval development. Results from these trials suggested that several test species were unsuitable hosts due to low numbers of progeny emerging from these plants. This included almost all Alismataceae species tested, except for *S. sagittifolia*. However, all the other test plants in these trials were killed either by larval attack or desiccation, except for *S. platyphylla* and *A. plantago-aquatica*. Several of these species were nonetheless able to support development from oviposition through larval development with adults able to develop (albeit in low numbers) on all *Sagittaria* species (except *S. subulata*), as well as on *D. minus* and *A. plantago-aquatica*.

Table 24

Results of the timed oviposition trial and larval starvation trial (from Steel *et al.* in prep. a) Effect of genus on: the number of eggs laid in a container at each assessment point and for the overall total of the three assessments; and the proportion of larvae that survived to collection date. Values in bold were significantly different from the target weed results.

Genus	Timed oviposition trial				Larval starvation trial
	Number of eggs counted at assessment time:				Proportion larval survival
	6 hours	24 hours	4 days	Total	
S. platyphylla	1.2	3.4	40	47	0.43
D. minus	0.7	3.5	**62**	69	0.42
A. plantago-aquatica	**0.1**	1.6	**17**	**19**	0.21
H. nymphoides	**0.1**	**1**	**8**	**10**	0.48
C. oligococca	**0.1**	**0.1**	**1**	**2**	**0**
E. cordifoilus	**0**	**0**	**1**	**1**	**0.04**

Table 25

Effect of water level on number of larvae and average head width (from Steel et al. in prep. a)

Water level trial	Water level, i.e. crowns were:		P value
	Exposed	Submerged	
Number of larvae per pot	13.4	**1.9**	**0.0014**
Average head width (mm)	1.17	**0.77**	**0.0038**

Subsequent trials used a limited number of larvae transferred to potted plants to eliminate overburdening plants. These larval transfer trials revealed that all species tested in these trials were of similar suitability for larval development except for *Caldesia acanthocarpa* and *E. cordifolius*.

In summary, host suitability of the test plants could be divided into three groups:

1. Species that could not support both oviposition and development to adult in any trial. This included all tested species outside Alismataceae; the aquatic food crop species water chestnut (*Eleocharis dulcis*) and rice (*Oryza sativa*) on which oviposition was not observed, and the native plant *Cycnogeton procerum* on which very few eggs were laid and no larvae were able to develop to adult stage (Table 23). A very small level of oviposition was observed on three Alismataceae species, including *Echinodorus cordifolius*, *Caldesia acanthocarpa* and *C. oligococca*, but adults were not able to develop within the timeframe of these trials (although the C. *oligococca* trials were not conclusive as the plants died; Table 23). Oviposition on the four species in this group was either zero, or less than 5% of the number of eggs observed on the target weed (Table 24). These species are considered unsuitable host plants for *L. sordidus*.

2. Species that were able to support both oviposition and larval development to adult stage, but oviposition was observed at significantly lower amounts than the target weed. Oviposition on the native *A. plantago-aquatica* was 40% of that on the target weed, and on the exotic ornamental *Hydrocleys nymphoides* (also considered a weed species) was 20% (Table 24). Compared to the target weed there was no significant difference in the survival of larvae in at least one trial (Table 24), and both species were able to support some development to adult stage (Table 23). These species are considered physiologically suited to the development of *L. sordidus*, although suboptimal hosts compared to the target weed.

3. Species that could support oviposition and larval development at least as well as the target weed, with adult emergence observed. There was no significant difference between any of the *Sagittaria* species (all exotic) in the number of eggs laid, or number of larvae surviving in at least one trial (Table 24) (although the results for *S. subulata* were inconclusive (Table 23). Oviposition on the native plant *D. minus* was significantly higher than on the target weed, and survival of larvae was not significantly different (Table 24). These species are considered equally as well physiologically suited to the development of *L. sordidus* as the target weed.

Water level trials suggest that populations of plant species growing in inundated waterways will be protected from off-target damage by high water levels. In waterways with fluctuating water levels they may be protected from off-target damage at high water levels but become susceptible as water levels recede. For ephemeral species that are winter/dry-season deciduous and can also produce seed before water levels decline (*Caldesia* spp., A. plantago-aquatica, *H. nymphoides* and *D. minus*), population level impacts will be reduced when water levels are high.

A plant species that is able to physiologically sustain the development of an insect will not necessarily be utilized as a host in the field and the range of plant species considered suitable physiological hosts is usually larger than the range of plants utilized as hosts once an agent is released (Hinz *et al.* 2014). One explanation for this is that no-choice tests such as these do not allow the insects to exhibit host selection behaviours. However, choice trials in the laboratory may not provide the environment required to elicit normal host selection behaviours either. We used a no-choice timed oviposition trial to try to detect differences in the insect's response to the test plants at different time points during the trial, rather than just analysing total oviposition at the end of the four-day trial.

After six hours the average number of eggs laid on *Echinodorus*, was zero, and close to zero on *Caldesia Hydrocleys*, and *Alisma* (0.1) compared to an average of more than one egg (1.2) laid on *Sagittaria* (Table 24). These differences were significant for all species (2.1-2.4 SED) except for Alisma (1.9 SED). The average number of eggs laid on *Damasonium* was not significantly different from *Sagittaria* (Table 3.3.2.2). Within six hours of exposure to the test plants *L. sordidus* had commenced oviposition on the *Sagittaria* species but the insect was clearly less motivated to oviposit on *Caldesia*, *Echinodorus* or *Hydrocleys*, and similarly less motivated on *Alisma*. There appears to be a signal in the data within this time frame that *L. sordidus* may not consider these species to be suitable hosts. It is possible that if *L. sordidus* encountered any of these species in the field that this lack of motivation to oviposit would result in the insects leaving that plant in search of a more appropriate host.

It is interesting to note that *Damasonium* is one of the most distantly related species from *Sagittaria* and yet appears to be physiologically better suited to development with significantly more oviposition observed in at least one trial. It is possible that this plant species lacks physical and chemical defences (e.g. secondary plant compounds such as latex) that are present in the host and that operate against optimal development whilst remaining a suitable host. These secondary plant compounds can also be used by an insect to signal the location of a suitable host so if *Damasonium* lacks these signals then *L. sordidus* may not be attracted to it and unlikely to encounter it in the field. Similarly, compounds that stimulate oviposition in Sagittaria within six hours of exposure may be lacking in *Caldesia*, *Echinodorus*, *Hydrocleys* and perhaps *Alisma*. Behavioural studies that measure an insect's response to a plant's chemical may be used to explore these hypotheses.

Project outcomes

Whilst species outside the Alismataceae family, and some more closely-related species were not found to be at risk of damage from *L. sordidus*, traditional host testing procedures could not discount the possibility that the native species *D. minus* and *A. plantago-aquatica*, as well as the exotic ornamental *H. nymphoides*, may be utilized as alternate hosts in the field. Novel laboratory testing procedures explored as part of the PhD studies within this project may have detected behavioural responses to *Alisma* and *Hydrocleys* that suggest these two species may not be utilized as hosts in the field. However, behavioural studies are required to test this hypothesis. These trials have commenced in the laboratory using choice trials mediated by an olfactometer (Figure 105). An application for release of this insect will not be sought until these trials have occurred.

Further work (March -June 2020)

1. Olfactometer-mediated experiments to assess whether gravid *L. sordidus* females will orient towards *A. plantago-aquatica* and *D. minus* for oviposition.
1. Caged choice experiments to assess whether gravid *L. sordidus* females will orient towards A. *plantago-aquatica* and *D. minus* for oviposition.
2. Development of manuscript for publication.

Output 12(e) - Conduct thermal physiology studies and develop a degree-day model.

and Output 12(h) - Conduct bioclimatic models to predict the likely establishment of Sagittaria agent biotypes in different climatic regions of Australia.

A bioclimatic model was developed to identify regions of Australia that are most suited to the establishment of each of three *Listronotus* weevil species proposed as biocontrol agents for the aquatic weed, *Sagittaria platyphylla* (sagittaria). The model was developed using the results of laboratory temperature development trials to calculate the degree day requirements for each weevil species. Then, using a global dataset of average maximum and minimum temperatures, we could predict the optimum number of generations that each weevil species could undergo in different climatic regions of Australia. Although there are factors other than climate that influence the spatial distribution of species, climatic suitability provides an indication of the most suitable sites for establishment of biocontrol agents at a regional scale.

The model predicted that most of the recorded locations of *S. platyphylla* were within the most suitable climatic conditions for the establishment of *L. appendiculatus* (fruit-feeding weevil) and *L. frontalis* (tuber-feeding weevil). For the crown-boring weevil, *L. sordidus*, the model predicted that fewer generations were likely across most of the weed's current distribution in Australia. While it's likely that this candidate agent could establish in Australia, their populations may take longer to build-up and initial release strategies should take this into consideration.

The model outputs can be used to extract the arrowhead infestations within the optimal climatic envelope, and these can be ranked to list the suitable sites for establishment of an agent upon release. This information can be used to provide climatic data for a release strategy at a more localized scale.

Introduction

The rate at which an insect can complete development from egg to adult is known to be driven by temperature. For each insect species, there is a minimum temperature at which eggs can hatch and larvae complete development through to adulthood, known as the lower development threshold. As the temperature rises, so too does the development rate of the insect, until it reaches an upper development threshold, Hence, each species has an optimum temperature at which it develops most quickly, and either side of this temperature, the rate of development is slower. Generally, insects with high rates of development can complete multiple generations per year (i.e. multivoltine), enabling populations to build up at a more rapid rate compared to univoltine insects that have only one generation per year. For biocontrol agents, the likelihood of establishment and subsequent impact upon the target weed is likely to occur sooner if agent populations have a high rate of development and population increase.

We can estimate how many lifecycles an insect can potentially complete under climatic conditions at any location if we know the average temperatures at that location across the year, and how quickly the insect can develop at those temperatures. A bioclimatic model such as this can help us decide where a biocontrol agent from one part of the world should be released in its new range to maximise the chances of it establishing. Such a model was developed for estimating the climatic suitability of Australian climatic regions for the establishment of each of three *Listronotus* weevil species, candidate agents for the biocontrol of the aquatic weed *Sagittaria platyphylla* (Sagittaria).

Methods

A bioclimatic model was developed for each *Listronotus* weevil species using three main steps:

1. Degree Day Requirement. Development trials were conducted in the quarantine laboratory to calculate the rate of insect development from egg to adult under different constant temperatures. This data was then used to calculate the degree day requirement for each weevil species to complete its lifecycle.

2. Estimation of weevil generations across different climates. We used a global gridded dataset of average monthly maximum and minimum temperatures and the insect's degree day requirement to estimate the number of generations the insect could complete across the climatic conditions in its native range (USA) and in the new range (Australia).

3. Prediction of establishment success. We downloaded a database of known locations for each of the weevil species in their native range across North America. For each species record, we noted the estimated number of generations produced by the model at that location. We matched these estimated number of generations to locations in Australia with the same number of generations to identify which parts of Australia are most likely, less likely, or unlikely to support establishment of the agent.

Results and discussion

The results of the development trial revealed that *L. appendiculatus* had a shorter generation time compared to *L. frontalis* and *L. sordidus*, requiring fewer degree days to complete development (Table 26). Based on these results *L. appendiculatus* would be expected to complete more generations per year than the other two species at any given temperature. In comparison, *L. sordidus* took almost three times longer to develop than *L. appendiculatus*.

The average number of estimated generations recorded for each species (Table 26) was used to identify the most suitable climatic conditions likely to favour establishment for each weevil species. One standard deviation either side of the average was used to classify the range of estimated values that were most suitable for establishment of each weevil species across Australia (depicted in Figure 106). These areas represent climatic conditions under which, for example, the fruit-feeding weevil was estimated to complete between 3.85 and 10.33 generations. Locations for this insect were recorded outside these values: down to 1.62 and up to 17.62. For insects with life expectancies shorter than one year, the minimum number of life cycles for a population to persist is one. Locations with an estimated number of generations between one and 3.85 could be considered suitable climates for establishment of the fruit-feeding weevil but at below optimal temperatures these populations would take longer to increase. As such, we have classified these areas as "less suitable" for insect establishment.

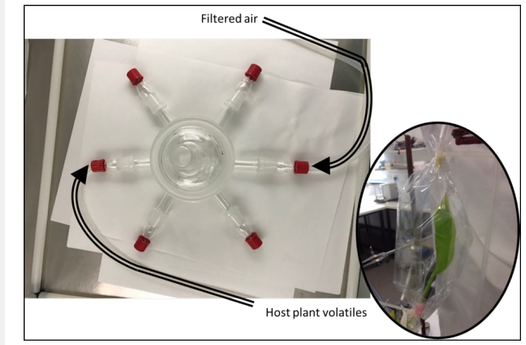


Figure 106. Setup for choice trials mediated by an olfactometer.

Table 26

Summary of degree day values calculated in growth chamber experiments and the estimated number of generations that could occur annually based on the climate at the US locations where the species is recorded.

Listronotus species	Degree day values	Number of generations estimated					
		Average*	Minimum^	L_1	L_2	U_1	U_2
L. appendiculatus	227	17.09 (3.24)	1	1.62	3.85	10.33	17.62
L. frontalis	417	4.85 (1.98)	1	0.20	2.87	6.83	8.27
L. sordidus	614	3.43 (0.89)	1	2.26	2.54	4.32	5.20

**Average number of generations at species' recorded locations (standard deviation (s.d.) in parentheses); ^Minimum number of generations required for establishment; L1: Lower value for suitable climate (lowest number of generations estimated at recorded locations); L2: Lower value for most suitable climate (one s.d. below average); U2: Upper value for most suitable climate (one s.d. above average); U1: Upper value for suitable climate (highest number of generations estimated at recorded locations).*

At the other extreme, in locations in the USA where the fruit-feeding weevil was predicted to have between 10.33 and 17.62 generations, the fruit-feeding weevil is not prevalent in these hotter climates. As such, these areas were also deemed "less suitable" because temperatures here were generally above the optimal temperature for insect development.

Parts of Australia that were most climatically suitable for establishment of each agent (between lower and upper values for most suitable climate in Table 26) are coloured green in the maps in Figure 106. Areas with less suitable climatic conditions are coloured blue for fewer estimated generations and purple for more estimated generations. Establishment is considered unlikely in areas coloured grey and white.

Almost all the recorded locations of Sagittaria in Australia are within the most suitable climatic conditions predicted for the fruit-feeding weevil (82%), most for the tuber-feeding weevil (69%) but less than 10% for the crown-boring weevil. Most of the Sagittaria infestations are within climatic conditions considered less suitable for the crown-boring weevil because average temperatures are below optimal (81%). This suggests that the fruit-feeding weevil and the tuber-feeding weevil are likely to establish well across most of the weed's range.

It is likely that the crown-boring weevil will also establish across the weed's range, but it may take more time for impacts on the weed to occur as populations will take longer to increase. The model predicts that almost all Sagittaria sites are likely to be suitable for release of the fruit-feeding weevil, but it would be prudent to choose optimal release sites for the tuber-feeding weevil, and especially for the crown-boring weevil to maximise the chance that they will establish. The model outputs can be used to extract the Sagittaria locations within the optimal climatic envelope for each weevil species, and these can be ranked to list (for example) the ten most suitable sites for establishment of each weevil agent upon release.

Limitations of the model

Whilst climate plays a key role in determining species distributions, other factors such as habitat suitability, day length, host availability and interspecies competition are also important. For example, *L. appendiculatus* requires its host plant to be fruiting for initiating oviposition and for larval development to occur. These bioclimatic models only consider temperature, not other factors that are important in dictating the life history of insects and their spatial distribution. As such, the estimated number of generations per year is considerably higher than would be expected in the field.

The model also identifies arid parts of Australia as suitable climatic regions despite a lack of aquatic environments necessary for the presence of the host plant. Nevertheless, the model is still useful in predicting which Sagittaria infestations in Australia that should be targeted for future releases of biocontrol agents. The presence of aquatic environments and locations of Sagittaria infestations can be included in a release strategy at a more localized scale.

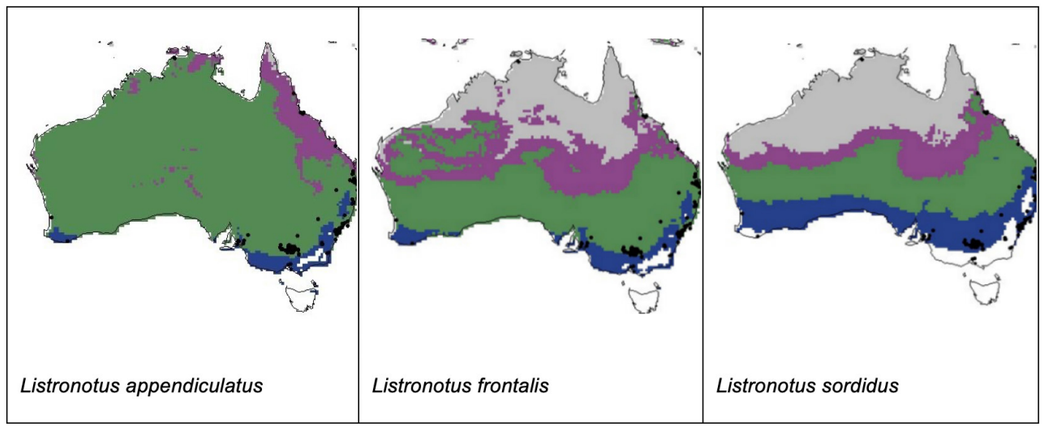


Figure 107. Climate suitability represented by colour across Australia for each proposed biocontrol agent based on estimated number of generations. Recorded locations for the weed, *Sagittaria platyphylla* are shown as black dots. Green = most suitable; blue = less suitable (colder); purple = less suitable (hotter); grey = least suitable (much hotter); white = unsuitable (less than one generation estimated).

Output 12(f) - Establish strategic nursery sites at a minimum of 15 sites. Monitor and release sites for establishment, dispersal and impact of suitable biocontrol agent{s}.

This component was not undertaken as no agent was approved prior to the completion of the project

Output 12(g) - Pending risks to non-target plants are acceptable, submit application to the Commonwealth regulators seeking approval to release at least one potential agent. Upon receiving approval, rear and release biocontrol agent(s).

An application to release *L appendiculatus* has been submitted for approval.

Significant productivity and profitability improvements for primary producers can be provided by the research and release of biocontrol agents to control weeds of production.

Output 12(i) - In consultation with waterways managers, identify strategic locations for release of approved biocontrol agent(s) and develop a biocontrol implementation plan.

Significant productivity and profitability improvements for primary producers can be provided by the research and release of biocontrol agents to control weeds of production. The silverleaf nightshade and Sagittaria subprojects contributed the following developments towards the release of biocontrol agents for agricultural weeds:

- identification of two new promising candidate biocontrol agents for silverleaf nightshade;
- early detection of unacceptable off-target damage in a third candidate agent prior to importation to Australian quarantine;
- an application for release of a biocontrol agent for Sagittaria (Appendix 25);
- development of methods to identify false positive results in the laboratory that might otherwise prevent the release of effective agents;
- development of methods for improving host-finding/ acceptance trials to add experimental rigour to the interpretation of host use data;
- bioclimatic models to inform successful establishment of biocontrol releases.

A detailed summary of these contributions is provided in Table 27.

Table 27

Expected compared to actual achievements of the Silverleaf Nightshade and Sagittaria sub-projects.

Expected	Actual
Characterisation of invasion pathways of the target weeds and identity of new agents through the application of advanced molecular techniques. **Relevant Outputs:** 11(a), 11(b), 11(c) 12(a)	**Silverleaf nightshade** Plants and insect speci-mens collected in Argentina, Paraguay and USA has been, or are in the process of being submitted for molecular analysis. In the case of insect and mite specimens, accurate identification of the two high priority agents is critical to separate them from closely related, and morphologically similar spe-cies (e.g. *Gargaphia arizonica* and *G. solani* in USA and *Aceria* sp. nov. and *A. bicornis* in Argentina) that have a different host-range. Concurrent stud-ies of variable ploidy in silverleaf nightshade popu-lations in Argentina may yield useful information of any correlation between ploidy level and ar-thropod fauna. **Sagittaria** Genetic analysis of *Sagittaria* across its native and invaded ranges detected three dis-tinct populations of the weed that had dispersed to Australia. Genetic barcoding did not find a genetic signal to link the weed populations to particular insect biotypes. Instead, we detected differences within *Listronotus* (Coleoptera: Curculionidae) species that were collected from different host plants within the Alismataceae family. These dif-ferences were observed across multiple collection sites, spanning a range of longitudes within the dis-tribution of the biocontrol target weed, *Sagittaria platyphylla* in its native range in southern USA. Molecular approaches to field surveys detected biotypes of at least one *Listronotus* species that have different host ranges. The barcoding approach allows researchers to ensure that they are testing a single biotype of the candidate agent, and that the biotype is the one associated with the target weed. In addition, molecular barcoding provid-ed evidence that the fundamental host range of two weevil species from laboratory host-specificity testing overestimated the ecological host range of these insects in their native range.
Geographic projections of potential distribution (in native and invaded ranges) and impacts (in invaded range) of new agents to control the target weeds through the use of big data-based bioclimatic/species distribution modelling techniques **Relevant Outputs:** 12(e) 12 (h)	A bioclimatic model was developed to identify regions of Australia that are most suited to the establishment of each of three *Listronotus* weevil species proposed as biocontrol agents for the aquatic weed, *Sagittaria platyphylla* (Sagittaria). The model was developed using the results of laboratory temperature development trials to calculate the degree day requirements for each weevil species. Then, using a global dataset of average maximum and minimum temperatures, we could predict the optimum number of generations that each weevil species could undergo in different climatic regions of Australia. Although there are factors other than climate that influence the spatial distribution of species, climatic suitability provides an indication of the most suitable sites for establishment of biocontrol agents at a regional scale.
Development of new biocontrol agents deemed safe for release into Australia, to facilitate adoption of biocontrol solutions for the target weeds **Relevant Outputs:** 11(d), 11 (e) 12(c), 12 (d), 12 (g)	**Silverleaf nightshade** Three new agents were identified from surveys conducted in Argentina, Paraguay and USA and developed through this pro-ject. Of these, the beetle *Gratiana lutescens* was rejected due to off-target feeding on potato in no-choice experiments in Buenos Aires, Argentina. The other two agents, *Aceria* sp. nov. and *Gargaphia arizonica* show considerable promise for biological control of silverleaf nightshade in Australia. Detailed experimental studies of *G. arizonica* have only just commenced, however *Aceria* sp. nov. was unable to form galls on eggplant in host-specificity tests in Argentina. Continued host-specificity testing and importation into Australian quarantine will be undertaken as part of the AgriFutures-led RRnD4P Round 4 project. **Sagittaria** Three weevil species were imported into quarantine from the southern USA for host specificity testing. Testing was successfully com-pleted for the fruit-feeding weevil enabling an application for its release to be submitted to the then Department of Agriculture. Host testing of the crown-boring and tuber-feeding weevils indicated that several ornamental *Sagittaria* species and two native Alismataceae were acceptable hosts. Further studies would be required to determine if the native species, *Alisma plantago-aquatica* and *Damasonium minus* would be at risk of attack under natural conditions.
Deployment of biocontrol agents (once approved for release) and explorations of suitable ways to integrate novel biocontrol solutions with other on-farm and off-farm weed management techniques, to facilitate integrated weed management in primary production of the target weeds **Relevant Outputs:** 12(f) 12 (i)	As explained above, no biocontrol agents were re-leased during this project. However, considerable research and planning has been conducted to en-sure the effective implementation of biocontrol once the Sagittaria fruit-feeding weevil is approved for release.
Ongoing stakeholder engagement through the following channels: • short RDC magazine articles • six-monthly updates to relevant RDCs and industry stakeholders, including project partners • field days • other extension opportunities that may arise during the course of the project. **Relevant Outputs:** 11(f)	Refer to Section 5 Extension and adoption for a complete list of all stakeholder engagement activities.

3.1.10 Prickly Acacia

Output 13(a) - Conduct native range surveys and catalogue insects, mites and pathogens associated with prickly acacia.

Native range studies for the prospective biocontrol agents for prickly acacia were conducted in Ethiopia, Senegal, Tanzania, Kenya, Nigeria, India and South Africa.

In Ethiopia, surveys were conducted in November 2016 and November 2017 across 24 sites. In Senegal, surveys were conducted in April 2017, October 2017, April 2018, October 2018 and June 2019 across 18 sites. In Kenya, surveys were conducted in May 2018 and November 2019 across four sites. In Tanzania, surveys were conducted in May 2018 and November 2019 across eight sites. In South Africa, surveys were conducted in December 2019 across 14 sites.

In each country, the subspecies status of the prickly acacia populations was recorded and insects and mites associated with various subspecies were catalogued. A greater emphasis was placed on gall-inducing agents, in view of their likely host specificity. When arthropod agents were collected from prickly acacia, related co-occurring Fabaceae species were also checked at the survey sites to ascertain field host range of the arthropods collected. The insects and mites collected were sent to relevant taxonomic experts in South Africa, Turkey and Australia for identification.

In Ethiopia, a gall thrips, *Acaciothrips ebneri* (Karny) (*Thysanoptera: Phlaeothripidae*) induced rosette galls in the axillary and terminal buds resulting in shoot-tip dieback (Appendix 27). On prickly acacia, the gall thrips were found only on subsp. tomentosa and subsp. indica and not on subsp. leiocarpa. There was no evidence of the gall thrips co-occurring on other Vachellia species like V. abyssinica and V. etbaica trees in Ethiopia highlighting its field host specificity to prickly acacia subspecies with moniliform fruits (see below). In Senegal, the gall thrips was seen on both subsp. tomentosa and subsp. adstringens, and not co-occurring on other Vachellia species (*Vachellia seyal*, *Vachellia tortilis* and *Senegalia senegal* trees).

In Ethiopia, a gall midge morphologically similar to *Lopesia niloticae* Gagné (*Diptera: Cecidomyiidae*), induced leaf rachis galls on all the three prickly acacia subspecies – subsp. indica, subsp. tomentosa, subsp. leiocarpa. A morphologically similar midge gall has also been seen on other subspecies of prickly acacia in Nigeria, Kenya, Tanzania and South Africa. There was no evidence of the gall midge on prickly acacia (on either subspecies) in Senegal.

In Ethiopia, three morphologically distinct Aceria mite galls were found on prickly acacia in Ethiopia (Appendix 28)- red spherical leaflet galls (type-1); creamy-white fluted leaflet galls (type-2); and hairy mushroom like galls on leaflets, rachides and shoot-tips (type-3;). Type-1 (Aceria sp. 1) leaflet galls were seen on all the three subspecies – subsp. *leiocarpa*, subs. t*omentosa* and subsp. *indica*. Type-2 (Aceria sp. 2) leaflet galls were seen only on subsp. leiocarpa and not on subsp. tomentosa or subsp. indica. Type-3 (*Aceria* sp. 3) galls on leaflets, rachides and shoot-tips were found only on subsp. *tomentosa* and subsp. *indica* and not on subsp. *leiocarpa*. Both type-1 and type-3 galls were often found on the same leaves. Galls morphologically similar to the three mite galls found on prickly acacia were not seen on co-occurring other *Vachellia* species in Ethiopia.

In Senegal, two morphologically distinct types of mite galls were found on two subspecies of prickly acacia. The type-2 creamy-white fluted leaflet galls were found only on subsp. *adstringens* in all survey sites, and the type-3 hairy mushroom like galls deforming leaflets and rachides were found on subsp. tomentosa and subsp. *adstringens*. There was no evidence of morphologically similar mite galls in co-occurring other *Vachellia* species (e.g. *V. seyal*, *V. tortilis* and *S. senegal* trees).

In Senegal, a stem gall-inducing fly *Notomma mutilum* (Bezzi) (Diptera: Tephritidae) has been identified as a prospective agent (Appendix 28). This is the first time a gall-inducing tephritid associated with prickly acacia has been collected. The gall fly was found in Senegal, on both subsp. tomentosa and subsp. adstringens, but not on other co-occurring *Vachellia*, *Acacia* and *Senegalia* species. The number of galls per shoot ranged from 1 to 12. There was no evidence of the gall fly in Ethiopia, Kenya, Tanzania, Nigeria and South Africa.

Output 13(b) - Prioritise prospective biocontrol agents based on field host range.

Based on field host range, geographic range and damage potential, a gall thrips (*Acaciothrips ebneri*), a gall mite (*Aceria* sp.), and a tephritid gall fly (*Notomma mutilum*) have been prioritised as prospective biological control agents for prickly acacia in Australia (Figure 108).

In Ethiopia, the gall thrips and gall mites were found only on prickly acacia subspecies with moniliform fruits (necklace-shaped fruit pods), similar to the fruit pods of Australian prickly acacia, and not on subspecies with non-moniliform fruit pods (not necklace shaped), with their margins straight or crenate or irregularly constricted. There was no evidence of the gall thrips on other co-occurring closely related V*achellia* and *Acacia* trees in Ethiopia.

In Senegal, the gall thrips were seen only on prickly acacia trees, and not on closely related co-occurring *Vachellia* and *Senegalia* trees.

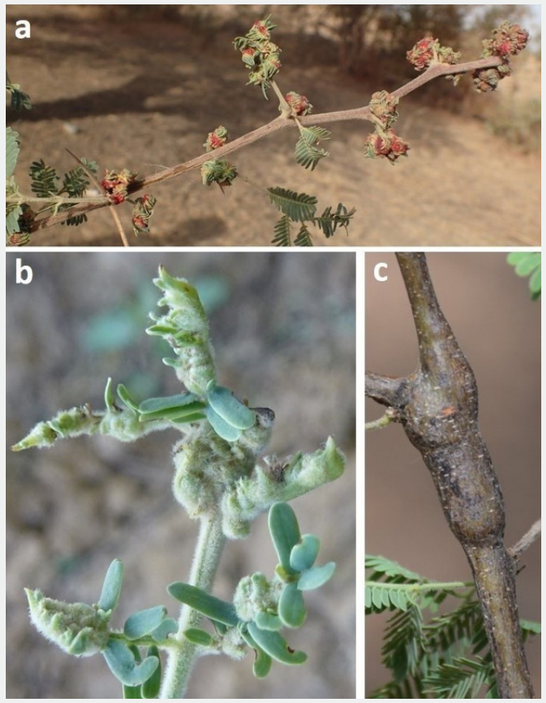


Figure 108. Prioritised prospective biocontrol agents: gall thrips (a) and gall mites from Ethiopia, (b) and gall fly (c) from Senegal.

Output 13(c) - Export prioritised agent(s) into quarantine (South Africa or Australia) for identification and colony establishment.

Three prioritised agents (Figure 108) were exported from the native range into quarantine facilities in Australia and South Africa for identification, colony establishment and host specificity testing.

The gall thrips (Figure 108A) from Shewa Robbit, Ethiopia was exported into a quarantine facility into a high security quarantine facility in Brisbane, Australia in December 2017.

A gall mite (Figure 108B) from Shewa Robbit, Ethiopia was exported into a quarantine facility in Pretoria, South Africa in December 2017. A trip to Ethiopia in February 2020 was planned to collect and export mite galls into quarantine in Pretoria, South Africa, to facilitate the host specificity testing of a large number of test-plant species at once. The trip to Ethiopia has been delayed due to the current coronavirus outbreak. A possible alternative being explored is to have collaborators collect the agent.

The prickly acacia gall fly (Figure 108C) was imported from Senegal, into quarantine in Brisbane, Australia, for colony establishment and host specificity testing. In October 2017, over 800 stem-cuttings with stem galls of the gall fly were imported, but no adults emerged from this material. In April 2018, about 600 stem-cuttings with stem galls of the gall fly were imported again from Senegal, and about 240 adults emerged from this second importation. In June 2019, about 900 stem galls from Senegal were imported into our quarantine facility in Brisbane and 680 adults emerged.

Output 13(d) - Conduct host specificity testing of prioritised agent(s) in quarantine (South Africa or Australia).

Host specificity testing

A gall thrips from Ethiopia was imported into a high security quarantine facility in Brisbane, Australia for colony establishment and host specificity tests. The colony of the gall thrips was established and maintained on Australian prickly acacia plants grown from prickly acacia seeds collected from north Queensland. Test plants (about 56 species) were sourced either as seeds and potted plants from nurseries or sourced as field collected seeds. No choice tests were conducted by inoculating various test plants with 20 newly emerged adult thrips (in an insect-proof cage each containing one potted test plant or control prickly acacia plant), with a minimum of five replications for each test plant species. The control and test plants were monitored for three months for gall development and any surviving adult thrips.

A gall mite from Shewa Robbit, Ethiopia was exported to a quarantine facility at Plant Health and Protection (PHP), Pretoria, South Africa in April and December 2017. A colony of the leaf-galling mite was established in the quarantine, on potted prickly acacia plants, grown from seeds sourced from prickly acacia populations in Australia. Seeds from 64 species of *Acacia*, *Vachellia* and other closely related test plants were also sourced from Australia, Ethiopia, Senegal and South Africa. The plants grown in the glasshouse from these seeds were used in the host specificity tests. No-choice host specificity tests were conducted by directly inoculating various test plants with the field collected gall mites sourced from Ethiopia, along with positive control plants (prickly acacia) with a minimum of five replications for each test plant species. Control and test plants were checked after six weeks for evidence of gall development.

Life cycle and host specificity testing of the gall thrips:

The test plant list for host specificity testing for prickly acacia biocontrol agents has been revised (Appendix 30). The *Acacia* genus has undergone great change over the past 20 years and these changes have ramifications for host specificity testing for weed biocontrol agents for prickly acacia (*Vachellia nilotica*).

Project outcomes

Once a member of *Acacia*, a large (>1000 spp.) and iconic group in Australia, prickly acacia is now part of the genus *Vachellia*. Current knowledge suggests that *Vachellia* is more closely related to the mimosoid genera than it is to *Acacia sensu stricta*. There has also been a recent reclassification of legume subfamilies with subfamily Mimosoideae (to which *Vachellia* and *Acacia* belong) now part of subfamily *Caesalpinioideae sensu lato*, and four new subfamilies established. Hence, a new host test list for quarantine testing has been developed. The newly developed test plant list is shorter than past lists, containing 46 species, including five *Vachellia* and six 'Mimoseae' species. Due to the importance of *Acacia* in Australia, it remains a significant component of the new list (Appendix 30).

In no-choice tests in quarantine facility in Pretoria, South Africa, the prickly acacia gall thrips from Ethiopia induced galls only on Australian prickly acacia (*Vachellia nilotica* subsp. *indica* – with moniliform fruits) and not on South African prickly acacia (*V. nilotica* subsp. *kraussiana* – with non-moniliform fruits), indicating high host plant specificity. Based on these results, a colony of the gall thrips from Ethiopia was established in a high security quarantine facility in Brisbane, Australia and host specificity tests are in progress (Appendix 29 and Appendix 30). So far, testing of 56 species has been completed for five replicates and there is no evidence of gall development on any of these non-target species. Only five plants of two species remain to be tested (two replicates for one test plant species and three replicates of the second test plant species; Figure 108). Tests for the remaining test plant species may be delayed by few months as we plan to import a new consignment of the gall thrips from Ethiopia, to reinvigorate the declining thrips colony in the high security quarantine in Brisbane, Australia.

Studies of the longevity and lifecycle of the gall thrips are in progress. This information is required by the Australian Department of Agriculture as part of an application to release a biocontrol agent. Three replicates of the longevity study have been completed; results suggested that adults live for up to 12 weeks. Females live longer than males, however more replicates are needed to complete the longevity study. As the gall thrips feed and breed within enclosed galls (Figure 109), it is difficult to conduct lifecycle studies, as the only way to monitor/follow the lifecycle is by destructive sampling of galls, which will result in the death of immatures in the galls. For this study, we introduce a newly emerged adult female on to a new prickly acacia plant and allow the female to induce galls and lay eggs for two weeks. Galls are then dissected every two weeks before the new adults emerge. An adult female is transferred to a new plant every two weeks. The results suggest that one female can produce approximately 200 progeny in a regular sized gall, however more replicates are required to validate the results.

Figure 109. No choice host specificity testing of gall thrips in high security quarantine facility in Brisbane, Australia.

The thrips colony in the high security quarantine has been declining since 2019. Several galls have been dissected out to identify the cause of the colony decline. Galls revealed both hatched and unhatched eggs; however, there were no larvae or pupae within the galls. Though the reasons behind the colony decline are not fully known, we suspect that this was possibly due to the malfunctioning of the automatic blinds in the high security section of the quarantine facility over the last two months, which may have exposed the thrips galls to direct sunlight, which would have resulted in extremely hot conditions. An additional importation is likely to be required to rebuild the thrips colony in the quarantine to complete the host specificity testing of the two remaining test plant species and the lifecycle studies. In the meantime, we are trying to resurrect the colony using the remaining galls to ensure that we have enough adult thrips for the host-specificity testing.

Host specificity testing of gall mites:
A colony of type-3 gall mite from Ethiopia was established on prickly acacia subsp. *indica* (sourced from Australia) in a quarantine facility in Pretoria, South Africa in December 2017.

Seeds from 64 species of *Acacia*, *Vachellia* and other closely related plants have already been sourced These include seeds both purchased or field collected in Australia, Ethiopia, Senegal and South Africa. In preparation of host-range testing, a subset of 39 species, encompassing seeds from each of these regions, has been sown and are currently in varying stages of growth. Sowing of additional batches of seeds is ongoing in anticipation of a collection trip to Ethiopia in order to have as many test plants available for testing as possible. During this trip, a large consignment of galls will be collected and returned to South Africa to facilitate the testing of a large number of test-plant species at once.

Figure 110. Gall development of thrips in high security quarantine facility in Brisbane, Australia.

To date, no-choice tests have been completed on *V. nilotica* subsp. *kraussiana*, *V. nilotica* subsp. *adstringens*, *V. nilotica* subsp. *tomentosa*, *V. sieberiana*, *V. hebeclada*, *Senegalia galpinii* and *Paraserianthes lopantha*. The type-3 gall mite has induced galls only on subsp. *indica* sourced from Australia (11 ± 5.4 galls per plant). Host specificity testing is ongoing for remaining test plant species (Figure 110).

Current focus is on boosting mite gall numbers in quarantine in South Africa. Unfortunately, new gall formation has slowed down with the onset of winter and by November 2019 no new gall development had been recorded in quarantine. As a result, further host-range testing was not possible due to low number of available mites for inoculation of test plants, as well as the negligible initiation of new galls on the culture plants.

A trip to Ethiopia in February 2020 was planned to collect a large consignment of mite galls for export to South Africa, to facilitate the host specificity testing of a large number of test-plant species at once. The trip to Ethiopia has been delayed due to the current coronavirus outbreak. Host specificity testing will continue when it is safe to travel to Ethiopia to collect and export gall mites to South Africa. Again, a possible alternative being explored is to have collaborators collect the agent.

Figure 111. No choice host specificity testing of gall mites in quarantine facility in Pretoria, South Africa.

Output 13(e) - Pending risks to non-target plants are acceptable, submit application to the Commonwealth regulators seeking approval to release at least one potential agent. Upon receiving approval, rear and release biocontrol agent(s).

As described above, testing of the gall thrips against 56 plant species has been completed for a minimum of five replications and there is no evidence of gall development on any of these non-target species. Only two more species remain to be tested. Tests for the remaining test plant species have been delayed for a few months due to sudden decline in the gall thrips colony in the high security quarantine in Brisbane, Australia in December 2019. The host specificity tests for the remaining plants will resume in April 2020 and likely to be completed in July 2020.

A release application for the gall thrips is currently being prepared for submission to the Department of Agriculture, Water and the Environment (DAWE) for approval. The draft application will be circulated among other biocontrol researchers for feedback, before submission to DAWE (likely date – October 2020).

3.2 References

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3.3 Contribution to program objectives

Generating knowledge, technologies, products or processes

This project has resulted in the development of new biocontrol options for seven weeds (provided the candidate agents are approved for release), including four that are Weeds of National Significance (African boxthorn, cabomba, Sagittaria and prickly acacia), one which is the most significant herbicide-resistant weed for grain production systems in Australia (fleabane), a serious environmental weed (ox-eyed daisy) and a major pasture weed (giant rat's tail grass).

This has been achieved through the

- Characterisation of the invasion pathways of the target weeds and confirmed the identity of candidate biocontrol agents through the application of advanced molecular techniques and detailed native range surveys
- Development of geographic projections of potential distribution of the weeds and the candidate biocontrol agents (in native and invaded ranges) using big data-based bioclimatic/species distribution modelling techniques;
- Identifying and undertaking underpinning research on new candidate biocontrol agents and applied to the relevant authorities for their consideration for release into Australia;
- Identified suitable ways to integrate novel biocontrol solutions with other on-farm and off-farm weed management techniques, to facilitate integrated weed management for a several of the weeds resulting in the upgrading of a number of Best Practice manuals.

These agents, when released and established in the environment, will benefit primary producers through the general landscape level through the reduction in weed pressures on rangelands, croplands and water assets, thereby enabling better integrated weed management outcomes.

No agents were found to be sufficiently host specific for the control of sowthistle (another herbicide resistant cropping weed) and mother-of-millions. A promising agent for silverleaf nightshade identified in RnD for Profit 1 was subsequently found to not be sufficiently host specific, but two other potential agents have been identified and are in the early stages of host specificity testing.

Other significant outcomes from the project were the development of a method to identify false positive results in the laboratory that might otherwise prevent the release of effective agents and the development of methods for improving host-finding/acceptance trials to add experimental rigour to the interpretation of host range data.

The project discovered that giant rat's tail leaf smut, a very damaging and effective pathogen, had naturalised in Queensland, rendering heavily infected tussocks sterile. It also resolved taxonomic identification errors often associated with the genus *Sporobolus*.

The project generated knowledge on various biocontrol agents for prickly acacia, which resulted in the importation and testing (technology) of two biocontrol agents that will benefit the grazing industry in Queensland. Specific knowledge and technology developed as part of the project are:

Strengthening pathways to extend the results of rural R&D

This project has been built on stakeholder engagement from the problem formulation stage (development of weed management goals). This engagement has been maintained throughout the project via effective communication of

(a) the status of the ongoing R&D,

(b) any setbacks or slippages in delivery and

(c) planning for outcomes of impending releases.

Collectively, this has enabled the development of a strong model for needs-based RD&E in the context of development of biocontrol solutions of Australian weeds.

Results from this project have been presented at review meetings, conferences and workshops and through scientific publications. For details of extension activities undertaken see the section Extension and adoption activities, below. At a grass roots level, a number of field days and forums were held that resulted in significant interest from stakeholders for continued research, monitoring and evaluation of biocontrol agents when packaged as part of an integrated control program.

The regular involvement of stakeholder through the progression of the project provided a strong basis for their collaboration building up to release of the agents (if approved.)

Establishing and fostering industry and research collaborations

Weed biological control research relies on international collaboration either through direct sourcing of potential biological control agents, swapping of research ideas or working together to tackle a species of mutual interest.

All the research undertaken in the project was done in close consultation with relevant industry partners. (see Collaboration section below).

The project involved the establishment of (and in some instances, continuing) collaboration with stakeholders, community groups and other research agencies in Australia and overseas. Where appropriate collaborations for the weeds continuing as part of the Round 4 RRD4 Profit program will continue. Through this project stronger linkages were formed which have the potential, in a number of instances, to extend into work on other weeds.

All collaboration will continue as part of the Round 4 RnD4 Profit program.

Impact on the productivity/profitability of businesses/primary industries.

All the weeds identified for this project have significant or potentially significant impacts on primary production and in some case, are environmental weeds as well. These weeds have also proven to be difficult or impractical to control by standard weed control methods and where these are employed, result in significant cost to asset managers.

As this project is yet to release agents into the field the future impact cannot be quantified. However, as an indication of the importance of this project, giant rat's tail grass. causes losses in production of about 80% as well as impacting directly on the health of cattle and horses, silverleaf nightshade costs farmers $70 million every year and Sagittaria can cause yield reductions of up to 75%, increased production costs and reductions in rice quality. Prickly acacia is estimated to cost primary producers around $9 million per year, while herbicide control of ox-eye daisy is not possible in environmentally sensitive areas.

Section 4 Collaboration

All KPIs related to the project outputs detailed below have been met. A summary of achievements related to each output is indicated below. Additional details are captured in Appendices (available on request from authors).

4.1 Research Collaborations

African Boxthorn

CSIRO: Jessica Bovill, Linda Broadhurst, Francisco Encinas-Viso, Gavin Hunter, Kylie Ireland, Mireille Jourdan, Darren Kriticos, Kumaran Nagalingam, Noboru Ota, Michelle Rafter, and Thierry Thomann

Rhodes University, South Africa: Evans Mauda, Lenin Chari, Grant Martin, Iain Paterson

University of Queensland: Graham McCulloch, Dean Brooks, Komal Gurdasani, James Hereward, Gimme Walter

Queensland Department of Agriculture and Fisheries: Tobias Bickel, Joseph Vitelli

Cabomba

Fleabane

Sow Thistle

SupAgro France: Manon Hervé, Vincent Lesieur, Maeva Miranda, Melodie Ollivier.

Agriculture Research Council – Plant Protection Research Institute South Africa: Alan Wood.

Universidade Regional De Blumenau Brazil: Davi Mesquita de Macedo, Eduardo Adenesky Filho, Marcelo Diniz Vitorino.

Colombia Universidad Nacional de Colombia Colombia: Mauricio Alberto Salazar Yepes, Laura Carolina Álvarez Morales, Juan Gonzalo Morales-Osario, Carlos Velasquez, Liseth Suarez, Sandra Uribe Soto.

FuEDEI Argentina: Guillermo Cabrera Walsh, Fernando McKay, Carolina Mengoni, Marina Oleiro.

Louisiana State University USA: Rodrigo Diaz, Carlos Wiggins

Mother-of-millions

Madagascar (University of Antananarivo). Tahina Rajaonera. PhD student.

Ox-eye daisy

CABI Switzerland

Giant rat's tail grass

QLD Department of Agriculture and Fisheries; Pathogen Laboratories

University of Queensland

NSW Department of Primary Industries

Queensland Herbarium

Rhodes University, South Africa

Silverleaf nightshade

FuEDEI, Argentina, Alejandro Sosa

NSW DPI Dr Hanwen Wu Dr David Gopurenko

South Australian Herbarium: Laurie Haegi

University of Melbourne, Cindy Hauser and Libby Rumpff (PhD Supervisors)

University of Texas (UT), Edinburg, Texas, USA Dr Alex Racelis

US Army Corps of Engineers, Lewisville, Texas Chetta Owens

USDA Texas, Edinburg, USA Dr John Goolsby

Sagittaria

Mississippi and Texas US Army Corps of Engineers: Nathan Harms and Julie Nachtrieb.

Tennessee Wildlife Department: Allan Trently.

University of West Alabama: Professor Brian Keener.

Rhodes University South Africa: Grant Martin.

Monash University (9) Deakin University (5), Trobe University (3), University of Melbourne (2): Students to assist with Laboratory research.

Prickly acacia

Agricultural Research Council- Plant Health and Protection, Pretoria, South Africa.

Forest Research Centre, Addis Ababa, Ethiopia.

Project partner: National Centre for Agronomic Research, Bambey, Senegal.

National Herbarium of Tanzania, Arusha, Tanzania.

National Museum of Kenya, East African Herbarium, Nairobi, Kenya.

Kogi State University, Anyigba, Nigeri.

Institute of Forest Genetics and Tree Breeding, Coimbatore, India.

Ondokuz Mayis University, Samsun, Turkey: Dr Sebahat Ozman Sullivan.

University of Pretoria, South Africa: .Prof Merv Mansel

4.2 Co-investors (cash and in-kind)

African Boxthorn

Shire of Ravensthorpe (Western Australia):- cash and inkind contributor

Cabomba

SEQwater:- cash and inkind contributor

Sow Thistle

Grains Research and Development Corporation: - cash and inkind contributor

Fleabane

Grains Research and Development Corporation: - cash and inkind contributor

Agrifutures Australia: - cash and inkind contributor

Primary Industries Research South Australia - cash and inkind contributor

US Department of Agriculture:- cash and inkind contributor;

CSIRO:- cash and inkind contributor.

Mother-of-millions

NSW Weed Biocontrol Taskforce:- cash contributor.

Northwest Local Land Services: inkind contributor

Local landholders (four pre-release site):- inkind contributor

NSW DPI: cash and inkind contributor.

Ox-eye Daisy

NSW Weed Biocontrol Taskforce:- cash contributor.

NSW DPI: cash and inkind contributor.

Giant rat's tail grass

Bundaberg Regional Council:-cash and inkind contributor.

Gladstone Regional Council:-cash and inkind contributor.

AgForce Queensland:-inkind contributor.

Gympie Regional Council:-inkind contributor.

HQPlantations Pty Ltd:-cash contributor.

NSW Weed Biocontrol Taskforce: cash contributor.

NSW DPI: inkind contributor.

Silverleaf nightshade

Bland Shire:- inkind contributor.

Primary Industries Research South Australia Dr John Heap - cash and inkind contributor

Lachlan Valley Weeds Committee - inkind contributor

Murrumbidgee Landcare - inkind contributor

DPJR (Victoria)- - inkind contributor

Glacial Ridge Potato Seed Co, Erskine, Minnesota, USA – test material

GRDC: - cash and inkind contributor

Sagittaria

Goulburn Broken Catchment Management Authority - inkind contributor

Central Murray County Council - inkind contributor

NQ Dry Tropics - inkind contributor

Murray Local Land Services - inkind contributor

Central Coast Council - inkind contributor

Murrumbidgee Irrigation Ltd - inkind contributor

Goulburn Murray Water - inkind contributor

Coleambally Irrigation - inkind contributor

NSW Office of Environment and Heritage - inkind contributor

Prickly acacia

Nil

Section 5 Extension and adoption activities

Stakeholder engagement was at the forefront of this project, including the general public, the scientific community, industry and governmental committees with biosecurity responsibilities. Regular face-to-face meetings with key stakeholders and co-investors, and regular updates of websites established for each of the weeds, enabled effective and direct communicate project progress.

During the course of the project, there were ten media releases, the maintenance of five web pages, five social media interactions, 12 newsletters, eight industry meetings, seven field days, a survey and 22 stakeholder meetings. This extensive communication was in the context of a biological research program which was in its early stage of the identification of potential agents.

The project also undertook extensive consultation with the nursery and garden industry and Plant Health Committee (PHC) in light of results that the African boxthorn rust fungus investigated could also cause disease symptoms on species of Goji berry grown in Australia (Ireland et al 2019c). Engaging early with these stakeholders has enabled identification os possible barriers to the release of biocontrol agents for African boxthorn and specific states where additional stakeholder engagement is needed. This approach has demonstrated a path that others may follow when faced with a similar situation.

Based on our stakeholder engagement in this project, and our past experience in this regard, we are confident that once biocontrol agents are approved for release by DAWE, there will be extensive interest from land managers and community groups to participate in large-scale release programs. This has been factored into our plans for the new Rural R&D for Profit project (AgriFutures Australia Project number: PRJ-12377; 2019-2022).

Media

CSIRO. Contribution to a Guardian (Australia) series on invasive species and biocontrol; specifically, spoke to the impacts of weeds and the ongoing work on weed biocontrol and integrated weed management (including of the weeds in this project) (January 2019)

CSIRO. ABC Focus Program interview on biocontrol (February 2019)

Buchanan, K. (2018). Natural assassin hunt uncovers new diseases that can be weaponised to fight invasive grasses. QLD Country Hour Posted 20 March 2018

McConnachie, A. National Geographic documentary, entitled 'Only in Australia' (17 December 2019)

http://agriculture.vic.gov.au/about-us/media-releases/promising-new-biocontrol-for-invasive-weed.

Post of the media release published on the Weed Society of Victoria Facebook Page on 22 November 2019

ABC Country Hour interview with Raelene Kwong and Angus Verley (host) conducted on 29 November 2019

Media release published on22 November 2019 highlighting the efforts to find new biocontrol agents for silverleaf nightshade, titled, 'Promising new biocontrol for invasive weed'. http://agriculture.vic.gov.au/about-us/media-releases/promising-new-biocontrol-for-invasive-weed

Vitelli, J.S. (2018). Natural assassin hunt uncovers new diseases that can be weaponised to fight invasive grasses. ABC Queensland Country Hour. 20th March 2018. Interview by Kallee Buchanan (http://www.abc.net.au/news/rural/2018-03-20/new-pathogens-found-in-fight-against-weed-grass/9566052)

Vitelli, J.S. (2018). Endemic pathogens of Giant Rat's Tail Grass. ABC Queensland Country Hour. 20th March 2018. Interview by Kallee Buchanan

Webpages

The following weed-specific webpage was maintained for the life of this project to communicate project delivery to all stakeholders and the general public.

https://research.csiro.au/african-boxthorn/

https://research.csiro.au/cabomba/

https://research.csiro.au/flaxleaf-fleabane/

https://research.csiro.au/sowthistle/https

Social media

CSIRO. GRDC twitter post 28/04/2017

CSIRO. GRDC Facebook post 27/04/2017

CSIRO. CottonInfo twitter post 13/04/2017

Facebook Group (NSW Weed Biocontrol Taskforce) administered.

Extension and adoption activities

Newsletters

Anon. Newsletter: Agrifutures Biocontrol of Weeds – Autumn 2018 update

Anon. Newsletter: Agrifutures Biocontrol of Weeds – Spring 2018 update

Anon. Newsletter: Agrifutures Biocontrol of Weeds – Autumn 2019 update

CSIRO. Article on African boxthorn biocontrol in "A Good Weed" Spring 2018 issue, the newsletter of the NSW Weeds Society

CSIRO. NORTHERN GRDC e-newsletter May 2017

DEDJTR . Flyer emailed to project collaborators and key Sagittaria contacts around Australia for dissemination through appropriate networks. The flyer was uploaded to three web sites as listed below: (https://www.gbcma.vic.gov.au/news_events/biological-control.html)

http://www.riverinaweeds.org.au/mdb-aquatic-weeds/sagittaria-portal-3/

http://www.riverinaweeds.org.au/about-us/latest-news/

Raelene Kwong and Greg Lefoe were featured in two articles highlighting the silverleaf nightshade research and biological control research in the Agriculture Victoria Research News, February 2020. Article titles: "Promising new biocontrol for invasive weed",

"At the forefront of Victoria's biological control" https://www.dropbox.com/s/l59i3odnb9r84q9/AVR_News_February%202020_Final%20.pdf?dl=0

Kwong, R. Biocontrol update leaflet published on the Riverina Weeds website and circulated by email to all project collaborators. http://www.riverinaweeds.org.au/wp-content/uploads/2015/03/Sagittaria-Biocontrol-project-update-June-17.pdf

Lefoe, G. "Assessing the risks of biological control to crop and ornamental plant cultivars". Presentation to the National Biocontrol Collaborators workshop, Canberra, 24 September 2019

McConnachie, A. NSW Weed Biocontrol Taskforce newsletters - Spring # 2 (December 2019)

Industry Meetings

GRDC Northern Grains Forum (August 2017 Wagga Wagga, NSW; October 2018 Toowoomba, QLD)

NSW DPI/NSW Weed Biocontrol Taskforce stand at the NSW Weeds Conference, Newcastle (26-30 August 2019)

NSW DPI/NSW Weed Biocontrol Taskforce stand at the Australian National Field days. (24-26 October 2019)

McConnachie, A. NSW Weed Biocontrol Taskforce presentation (November 2019)

Vitelli, J.S. (2019) Biological control investigations. Presented to the GRT Best Practice Technical Workshop. Ecosciences Precinct, Dutton Park, Brisbane. (12th February 2019).

Vitelli, J.S. (2019) Current GRT research and interim findings. Presented to the GRT Best Practice Technical Workshop. Ecosciences Precinct, Dutton Park, Brisbane. (13th February 2019).

Vitelli, J.S. (2019) Research update, looking back at 35 years. Presented to the Weed Society of Queensland, 44th AGM. Ecosciences Precinct, Dutton Park, Brisbane. (22nd February 2019).

Vitelli, J.S. (2019) *Sporobolus* research update. Presented to land maintenance staff of Powerlink Queensland. Powerlink Queensland, Virginia, Queensland. (13th March 2019).

Field Days

Kwong, R Biocontrol of Weeds – theory and current research'. Warby Ranges Landcare. Taminick, Victoria, 25 March 2019

Kwong, R Weed biocontrol display. Tallangatta Farm and Water Expo, Tallangatta Victoria, 11 April 2019

Kwong, R Biocontrol of Weeds – theory and current research', Warranbayne-Boho Landcare Group, Boho, Victoria, 17 May 2019

Kwong, R Biocontrol of Weeds – theory and current research', Molyullah-Tatong Landcare, Molyullah, Victoria, 12 October 2019

Lefoe G. "Is biocontrol an option for managing widespread weeds?" Seymour Alternative Farming Expo. Seymour, Victoria, February 2019

Vitelli, J.S. Update on the latest in biocontrol research for GRT. Bundaberg Regional Council GRT Field Day and Farm Walk. 150 Tableland Rd Tirroan. (Friday 16th March 2018). (220 attendees)

McConnachie A Mother of million field days at Walgett, Moree and North Star (April 2019)

Surveys

CSIRO. On-line surveys to better understand management goals and expectations of biocontrol (African boxthorn, fleabane, sowthistle);

Stakeholder meetings

CSIRO. SEQWater meetings (6-monthly)

DEDJTR Provided a project update to the Deputy Secretary, Agriculture Research which was published in "A message from the Deputy Secretary – 15 May 2017".

CSIRO. NSW Weed Biocontrol Taskforce meeting (6-monthly)

CSIRO. Annual General Meeting of Sydney Weeds in Sydney (November 2019)

CSIRO. Presentations on principles underpinning host-specificity testing at the national workshop of weed biocontrol practitioners with the regulators (Department of Agriculture and Department of Environment and Energy; October 2019)

CSIRO. Communication of survey results as a summary via email to participants in the survey on management goals for African boxthorn, fleabane and sowthistle (late 2017).

CSIRO. Presentations on aspects of the project given at South Australian Landcare Meeting (August 2017); Illawarra Landcare Forum (August 2017) and to the NSW State Weeds Committee (August 2017) and GRDC Northern Regions Weeds meeting (August 2017).

Dhileepan, K. Biological control prickly acacia: research updates. Prickly acacia Alliance (stakeholders) meeting. Ecosciences Precinct, Boggo Road, 18 June 2018.

Dhileepan, K. 2018. Weed Biocontrol Program. Queensland Invasive Plant and Animals Committee (QIPAC) Meeting (04/18), George Street, Brisbane, 30 November 2018.

Biocontrol workshop run for Central Tablelands LLS in Bathurst -64 people attended (23 September 2019)

Kwong, R., and Lefoe, G. Research progress on the biocontrol of sagittaria and silverleaf nightshade" Northern Victoria (Tatura) and southern NSW (Deniliquin, Griffith), 16-18 May 2018

Kwong, R., and Steele, J "Biological control of sagittaria". Murrmbidgee Irrigation office, Griffith NSW, 15 March 2017

Kwong, R., and Steele, J "Biological control of sagittaria" Murray Local Land Services office, Deniliquin NSW, 16 March 2017

Kwong, R., and Steele, J Central Coast Council 20 September 2017

Kwong, R., and Steele, J. Sagittaria biocontrol agent selection and risk. , NSW National Parks and Wildlife Service, Lane Cove National Park, NSW, 21 September 2017

Lefoe, G Silverleaf nightshade stakeholder meetings. Charles Sturt University, Wagga Wagga, and property, Gumly Gumly, NSW. 17 May 2017

Lock, C. (2018). Biological control of giant rat's tail grass using endemic fungal pathogens. School of Earth & Environmental Science, The University of Queensland, St Lucia, 18 May 2018.

Vitelli, J.S. (2017). Update on GRT research. Fraser Coast Regional Council Giant Rats Tail Grass - An Integrated Approach Information Day. Woocoo Hall, Oakhurst. (Saturday 29th April 2017). (85 attendees)

Vitelli, J.S. (2018). Herbicide treatments and current GRT research. Gympie Regional Council Weedy Sporobolus Forum Day. Prospectors Hall, Gympie Civic Centre, Gympie. (Tuesday 27th February 2018). (250 attendees)

Vitelli, J.S. (2018). GRT research update and its management. Gladstone Regional Council Community forum on Giant Rat's Tail Grass, Miriam Vale Community Hall, Miriam Vale. (Thursday 17th May 2018) (80 attendees).

Vitelli, J.S. (2019). Current research into GRT management with herbicides and promising GRT pathogens. Presented to producers of AgForce and Coochin Creek Fruit Growers Co-operative at the Giant Rat's Tail Grass Field Day. Conondale Hall, Conondale. (19th June 2019). (over 120 attendees).

Vitelli, J.S. (2019). GRT paddock walk looking at the importance of integrated control, involving leaf smut, crush grazing, pasture sowing and wick wiping as tools to reduce the dominance of GRT. Giant Rat's Tail Grass Field Walk. Elgin Station, Conondale. (19th June 2019). (over 100 attendees)

As the GRT project was based at Rhodes University, South Africa, where researchers were conducting surveys to find potential agents and conduct host specificity testing, there were limited extension and adoption activities undertaken in this project. Project progress was regularly reported to Queensland local governments through a number of stakeholder committees, the annual publication 'Technical Highlights' (distributed to local governments and other stakeholders) and at field days for other projects on management of giant rat's tail grasses. Two papers were presented at conferences.

Conference presentations

Sutton, G, Day, M.D., Canavan, K. & Paterson, I. 2018. Prospects for the biological control of invasive giant rat's tail grasses (*Sporobolus* spp.) in Australia. International Symposium on the *Biological Control of Weeds*. Engelberg, Switzerland. August 2018.

Taylor, D.J.B., Dhileepan, K., Day, M. & Pople, T. 2019. Biological control of Queensland weeds: achievements and progress. *Australasian Entomological Society Conference*. Brisbane, Australia. December 2019.

Section 6 Lessons learnt

There were a number of learnings and issues that arose during the conduct of the project.

Contracting/timing

In general, timeframes to develop projects were far too short. It takes time to develop a project that is well planned and suitably funded, as well as engage with suitable collaborators. This is especially so, if almost all research is going to be conducted off-shore. In some cases, there may need to be preliminary visits to determine if facilities and capacity (including staff) are sufficient to complete the work. For this project, it was fortunate that there was existing collaboration between the organizations and so some of these concerns were not relevant. However, if working on weeds in countries with no prior linkages, it becomes more challenging or not feasible. This means that some important weeds may not be studied, as there is no prior knowledge of who could do the work.

Apart from the time to identify the collaborators, there was also insufficient time to seek out cash support. This was critical, especially since the Australian government was providing 2:1 for all cash pledged.

The size of the project meant that the process of going through the checks and balances of all participating institutions took significantly longer for subcontracts to be executed.

This delayed the start of some components. Based on significant goodwill, between DAWE and AgriFutures and certain agencies, meant work was able to be commenced with confidence, in advance of the subcontracts being signed. However, streamlining of the contracting process would eliminate delays in the commissioning of future projects.

International collaborations and permitting

Despite the complexity inherent to international collaborations, the project progressed well through productive collaborations between the project teams and the network of international collaborators spanning Australia, Europe, Africa, South America and North America and Asia.. Some issues were associated with the granting of export permits for some of the agents but familiarity with international permitting process across multiple jurisdictions, and under new and merging conventions (e.g. Nagoya Protocols) will hold in good stead for future international collaborative projects on weeds RD&E and beyond.

KPI expectations

The project was optimistic in believing that, for particular weeds, some of which had never been a target for biocontrol anywhere in the world, it was possible, over the course of a 4-year project, to identify candidate agents, complete risk assessments, and obtain approval from the Australian authorities to release them in the field. Some deliverables were adjusted towards the end of the project. However, the project has demonstrated that it is possible to get at least to the stage of submitting a release application in a more rapid timeframe than in the past through an appropriate level of investment to enable optimizations of projects in weed biocontrol.

Facilities and Capacity

Currently, Australian quarantine facilities are fully committed. NSW DPI has committed in a significant way to expanding its facilities, albeit it not in time to make a difference for the projects undertaken in this round funding. Enhanced containment facilities are needed to ensure that more work can be conducted in Australia, and more rapidly.

Technical Issues

Adverse conditions in quarantine conditions – on one occasion, malfunctioning equipment in quarantine and an unexpected power failure resulted in erratic temperature fluctuations within the quarantine, adversely affecting the biocontrol agent colony, thereby delaying the host specificity testing.

Some of the test plants for host specificity testing were difficult to procure from commercial nurseries. Hence, in one situation specialist plant taxonomists had to be engaged for the field collection of seeds of difficult to source test plants for inclusion in host specificity testing.

There were difficulties in raising some of the agents in quarantine in Australia. Close cooperation with overseas facilities enabled some of the testing to be carried out in those locations prior to introducing agents to Australia.

Research cooperation

Establishment of a PhD student and employing an early career researcher associated with the Sagittaria sub-project encouraged innovative thinking to explore and progress the science of host testing biocontrol candidates. Conversely, collaboration with undergraduate interns from Melbourne's top universities revealed that students have little to no exposure to biocontrol as a successful method of weed management.

Project Continuity

Sub-projects such as silverleaf nightshade have benefited enormously from the continuity provided by multiple rounds of RRnD4P. Even though *Leptinotarsa texana* was rejected as a prospective agent in Round 1, the project nevertheless addressed important knowledge gaps, and provided a foundation for further research. The AgriFutures-led Round 2 project then built on this foundation to reject unsuitable agents and focus international exploration and research efforts on the most promising and apparently host-specific candidates. Two new agents are now in culture in Argentina and USA and will be shipped to Australian quarantine under the AgriFutures Round 4 project

Liaison

The convening of an annual Biocontrol workshop in Canberra involving research and project leaders, Department of Agriculture, Department of Environment and Agrifutures was an important forum to address strategic and structural issues associated with the project. Key issues raised were the likely pipeline of agent release applications, opportunities to streamline the approval process through a more interactive process with reviewers and proponents and the presentation of a tool for selecting crop cultivars for host-specificity testing.

Partner contributions

It is important to account for possible changes in both the level of in-kind contribution, and which agencies are required to provide contributions. Activities associated with the release of biocontrol agents for silverleaf nightshade and Sagittaria did not proceed as currently no agents are available for release. Consequently, project collaborators who committed to biocontrol agent releases were unable to meet their full in-kind contribution. Despite these constraints, considerable in-kind contributions were received from a range of stakeholders involved in other aspects of the project.

These learnings will hold us in good stead in the new Rural R&D for Profit project (AgriFutures Australia Project number: PRJ-12377; 2019-2022), and for future biocontrol projects.

Section 7

Appendix - additional project information

7.1 Project, media and communications material and intellectual property

A summary of extension and stakeholder activities is provided in Section 5.

Conference Presentations

CSIRO. Results and research plans for the sowthistle project were presented as two posters at the 5th International Symposium on Weeds and Invasive Plants (10 –14 October 2017, Chios, Greece)

Oral and poster presentations relating to this project were delivered at the 21st Australasian Weeds Conference, 9-12 September 2018, Sydney https://www.21awc.org.au/

An oral presentation showcasing GRTG project at the Plant Biosecurity Research Symposium in Brisbane (August 2019).

Dhileepan, K., Shi, B., Callander, J., Teshome, M., Neser, S., Diagne N. and King, A. 2017. Biological control of prickly acacia in Australia: prospective agents from Ethiopia and Senegal. 26th Asian Pacific Weed Science Society Conference, Kyoto, Japan, 19-22 September 2017.

Johnson, S. Wu, H. Weston, L (Eds.), Proceedings of the 21st Australasian Weeds Conference (2018), pp. 204-10. http://caws.org.nz/old-site/awc/2018/awc201812041.pdf

Kwong, R 'Impacts of a pre-dispersal seed predator on achene production in the aquatic macrophyte, *Sagittaria platyphylla*'. Proceedings of the 21st Australasian Weeds Conference (2018) http://caws.org.nz/old-site/awc/2018/awc201812041.pdf

Kwong, R "Evaluation of a pre-dispersal seed predator for the biological control of an aquatic weed Australian Entomological Society Conference Terrigal NSW 17-20 September 2017

Kwong, R. Do host races exist in the Sagittaria fruit-feeding weevil? XV International Symposium, on Biological Control of Weeds, (26-31 August 2018, Engelberg, Switzerland)

Kwong, R. and Steel, J. 'Biological control of Sagittaria platyphylla – agent selection and risk assessment'. 4th Combined Australian and New Zealand Entomological Societies Conference (27 20 November, 2106)'

Kwong, R. "Does enemy release explain the invasion success of *Sagittaria platyphylla* in Australia and South Africa?". 15th International Symposium of Aquatic Plants, Queenstown, New Zealand. 20 February 2018

Kwong, R. Biocontrol of weeds in Victoria – an overview of R&D projects. Victorian Weeds Conference 7-8 May 2019 Echuca, Victoria, 7-8 May 2019. The conference program is available at: https://www.wsvic.org.au/wp-content/uploads/2019/04/Program-for-Victorian-Weeds-Conference-7-8-May-2019_v10.pdf

Lefoe, G. Prospects for biological control of silverleaf nightshade in Australia. Victorian Weeds Conference, Echuca, Victoria, 7-8 May 2019

The conference program is available at: https://www.wsvic.org.au/wp-content/uploads/2019/04/Program-for-Victorian-Weeds-Conference-7-8-May-2019_v10.pdf

Lefoe, G. Risks and decisions: is Leptinotarsa texana suitable for biological control of silverleaf nightshade in Australia? (poster). Victorian Weeds Conference . Echuca, Victoria, 7-8 May 2019

The conference program is available at: https://www.wsvic.org.au/wp-content/uploads/2019/04/Program-for-Victorian-Weeds-Conference-7-8-May-2019_v10.pdf

McConnachie, A. Use of drones in assessing the impact of weed biocontrol. Presented at the International Symposium of the Biological Control of Weeds (26-31 August 2018, Engelberg, Switzerland).

Steel, J. Unexplored risks in biocontrol. Victorian Weeds Conference Echuca, Victoria, 7-8 May 2019.

The conference program is available at: https://www.wsvic.org.au/wp-content/uploads/2019/04/Program-for-Victorian-Weeds-Conference-7-8-May-2019_v10.pdf

Lefoe, G. "Kangaroo apples, bush tomatoes and spuds: the challenges of assessing risk", Victorian Biodiversity Conference. Melbourne University, February 2019.

Steel, J. "Host specificity testing process for Sagittaria - a case study". AgriBio Science Conference, 26-27 October 2017

Steel, J. "Predicting the realised host range of a biocontrol agent imported from USA to control Sagittaria platyphylla in south-eastern Australian aquatic environments". 15th International Symposium of Aquatic Plants. Queenstown, New Zealand. 20 February 2018

Steel, J. "The Physiological, ecological and climatic limits of a crown-boring weevil (Listronotus sordidus) for assessing its suitability as a biological control agent for the aquatic weed Sagittaria platyphylla (Delta arrowhead) in South Eastern Australia. PhD Confirmation seminar. AgriBio, Melbourne, 6 March 2018

Steel, J. One level up…and Quadrant across: biocontrol research is forging ties across AgriBio'. AgriBio Science Conference, Melbourne, 7 November 2018

Steel, J. Application of DNA barcoding to compare the fundamental and ecological host ranges of a proposed biocontrol agent, Listronotus sordidus, the crown-boring weevil, for the aquatic weed delta arrowhead Sagittaria platyphylla. XV International Symposium, on Biological Control of Weeds. (26-31 August 2018, Engelberg, Switzerland)

Lefoe, G 'Risks and decisions: is Leptinotarsa texana suitable for biological control of silverleaf nightshade in Australia?' XV International Symposium, on Biological Control of Weeds. (26-31 August 2018, Engelberg, Switzerland)

Lefoe, G., Kwong, R., Heap, J., Wu, H, Gopurenko D and Haegi, L. '*Biological control of silverleaf nightshade* Solanum elaeagnifolium *in Australia: a new hope*. The 4th Combined Australian and New Zealand Entomological Societies Conference (27 20 November, 2106)

Lefoe, G. et al. "Biological control of silverleaf nightshade in Australia". USDA Biological Control Laboratory, Edinburg, USA, .26 April 2017

March, N., Vogler, W. and Dhileepan, K. 2017. Advancing prickly acacia management through War on Western Weeds initiative, pp. 39-42. In: T. Sydes (ed.), *Proceedings of the 14th Queensland Weed Symposium*. The Weed Society of Queensland, Port Douglas, 4-7 December 2017.

McConnachie, A. Results of Ox-eye Daisy research presented at the International Symposium of the Biological Control of Weeds (26-31 August 2018, Engelberg, Switzerland)

McConnachie, A. Plenary talk on biocontrol of weeds. New South Wales Weeds Conference, Newcastle (26-30 August 2019)

McConnachie, A. and Harvey, K.Australian weed biocontrol: A look at the past, present and future (p. 203) Australasian Weeds Conference, Manly 2018:

Simmons, L., Vitelli, J.S., and Csurhes, S. (2019). New technologies for weed eradication - invasive plants have no place to hide when DNA is involved. Presented to the International Tropical Agriculture Conference (TROPAG), Shaping The Science of Tomorrow. Brisbane Convention & Exhibition Centre, Brisbane. 11-13 November 2019. (12th November 2019).

Simmons, L. (2019). New technologies for weed eradication - invasive plants have no place to hide when DNA is involved. Presented to the International Tropical Agriculture Conference (TROPAG), Shaping The Science of Tomorrow. Brisbane Convention & Exhibition Centre, Brisbane. 11-13 November 2019. (12th November 2019).

Snow, E, Jones, P., Riding, N., and Day, M. pp. 226-229)Promising new biological control agents for Queensland (Australasian Weeds Conference, Manly 2018:

Steel J, Butler KL. Blacket MJ, Kwong RM (2019) The fundamental and ecological host ranges of the crown-boring weevil, Listronotus sordidus - a proposed biocontrol agent for the aquatic weed delta arrowhead, Sagittaria platyphylla. Proceedings of the XV International Symposium of Biological Control of Weeds, Engelberg, Switzerland, 26-31 August, 2018. The proceedings were published on-line https://www.ibiocontrol.org/proceedings/

Appendix - additional project information

Sutton, G, Day, M.D., Canavan, K. & Paterson, I. 2018. Prospects for the biological control of invasive giant rat's tail grasses (*Sporobolus* spp.) in Australia. International Symposium on the biological control of weeds. Engelberg, Switzerland. August 2018.

Taylor, D. J. B., Dhileepan, K., Day, M. and Pople, T. 2019. Biological control of Queensland weeds: achievements and progress. *Australasian Entomological Society Conference*, Brisbane, Australia, 1-4 December 2019.

Vitelli, J.S. (2019). Will Australian endemic pathogens weaken the might of Giant Rat's Tail (GRT) grass? Proceedings of Pest Animal & Weed Symposium. The Weed Society of Queensland, Gold Coast. 21 May, 2019

Vitelli, J.S., Holdom, D.G., Shivas, R.G., Lock, B.C., Tan, Y.P., Bransgrove, K., Chamberlain, A., Riding, N. and Hosking, J. (2019). Will Australian endemic pathogens weaken the might of Giant Rat's Tail (GRT) grass? Proceedings of Pest Animal & Weed Symposium. The Weed Society of Queensland, Gold Coast. 20-23 May, 2019.

Scientific reports and publications

Bickel, T., Vitelli, J. and Raghu, S. (in preparation). Integrated management of Cabomba caroliniana: recommendations.

Chari et al. (in press) Insect herbivores associated with Lycium ferocissimum (Solanaceae) in South Africa and their potential as biological control agents in Australia. African Entomology (in press)

DAF 2017. Biocontrol of giant rat's tail grass. Technical Highlights: Invasive Plants and Animals. pg. 13. https://www.publications.qld.gov.au/dataset/technical-highlights/resource/89ee7722-6db7-4ff2-99ce-5a02e896f4f3

DAF 2018. Biocontrol of giant rat's tail grass. Technical Highlights: Invasive Plants and Animals. pg. 12. https://www.publications.qld.gov.au/dataset/technical-highlights/resource/d81d5f65-7921-434a-8b63-a4455333cc51

DAF 2019. Biocontrol of giant rat's tail grass. Technical Highlights: Invasive Plants and Animals. pg. 11. https://www.publications.qld.gov.au/dataset/technical-highlights/resource/e783ac13-320b-48e2-aba2-8a70a72d0d11

Dhileepan, K., Shi, B., Callander, J., Teshome, M., Neser, S. and Senaratne, K.A.D.W. 2018. Gall thrips *Acaciothrips ebneri* (Thysanoptera: Phlaeothripidae) from Ethiopia, a promising biological control agent for prickly acacia in Australia. *African Entomology* 26:237-241.

Dhileepan, K., Shi, B., Callander, J., Taylor, D., Teshome, M., Neser, S., Diagne N. and King, A. 2019. Biological control of prickly acacia (*Vachellia nilotica* subsp. *indica*): New gall-inducing agents from Africa. In: H.I. Hinz et al. (eds.), *XV International Symposium on Biological Control of Weeds*, Engelberg, Switzerland, pp. 13-19, 26-31 August 2018.

Dell Q, Vance T, Kumaran N and Raghu, S. (in preparation) Notes on Methodology to Inform Mass Rearing of the cabomba weevil, *Hydrotimetes natans*

Encinas-Viso F, Bovill J, Morin L, Raghu S, Knerr N, Roux C and Broadhurst L (in preparation) The origins of sowthistle (*Sonchus oleraceus*) invasion in Australia.

Gurdasani K, Hereward JP, McCulloch GA, Morin L, S. Raghu S, Walter GH. (in preparation) Precision provenance testing for biological control? Using population genomics to trace the invasion history of African boxthorn (*Lycium ferocissimum*) in Australia.

Ireland KB, Delaisse C, Hunter GC, Morin L (in preparation) Information package to support the application to release the rust fungus *Puccinia rapipes* for the biological control of African boxthorn (*Lycium ferocissimum*) in Australia.

Ireland KB, Hunter GC, Wood A, Delaisse C, Morin L (2019a) Evaluation of the rust fungus *Puccinia rapipes* for biological control of *Lycium ferocissimum* (African boxthorn) in Australia: life cycle, taxonomy and pathogenicity. Fungal Biol. 123:811–23

Ireland KB, Rafter M, Kumaran N, Raghu S, Morin L (2019b) Stakeholder survey reveals priorities for African boxthorn biocontrol research in Australia. Biocontrol Sci. Technol. 29: 1123–28

Kriticos DJ, Ireland KB, Morin L, Kumaran N, Rafter M, Ota N, Raghu S (in preparation) Integrating ecoclimatic niche modelling methods into classical biological control programmes

Kwong, RM, Sagliocco J-L, Harms NE, and Nachtrieb JG. Impacts of a pre-dispersal seed predator on achene production in the aquatic macrophyte, Sagittaria platyphylla.

Kwong, RM, Sagliocco, JL, Harms, NE, Butler, KL, Martin, GD, Green, PT (2019) Could enemy release explain invasion success of Sagittaria platyphylla in Australia and South Africa? Aquatic Botany 153, 67-72. The article is available on-line at https://www.sciencedirect.com/science/article/pii/S0304377018301505

Lefoe, G., Hauser, C. E., Steel, J., Slater, A. T., Kwong, R. M., Lubanga, U. K., & Rumpff, L. (2020). Systematic cultivar selection for weed biological control risk assessment. In prep.

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Lesieur V, Jourdan M, Thomann T, Hervé M, Ollivier M, Malik C, Martin J-F, Maëva M, Tavoillot J, Tixier M-S, Vaast P, Morin L, Raghu S (in preparation) Could classical biological control of *Sonchus oleraceus* be a solution for management in Australia?

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Lock, B.C. (2018). Investigation of fungal pathogens for the biological control of giant rat's tail grass (*Sporobolus natalensis*) in Australia. Honours Research Thesis. Bachelor of Environmental Management The University of Queensland, Brisbane.

McConnachie, A. (2019). Nomination of a target weed for biological control: *Leucanthemum vulgare* (Asteraceae). Prepared by NSW DPI

Martin, G D., Coetzee, JA., Lloyd, MA. Nombewu, SE., Ndlovu MS., Kwong RM(2018) Invaded habitat incompatibility affects the suitability of the potential biological control agent Listronotus sordidus for *Sagittaria platyphylla* in South Africa, Biocontrol Science and Technology, DOI: 10.1080/09583157.2018.1460314.

Mauda, EV (2020) Investigations into biological control options for *Lycium ferocissimum* Miers, African Boxthorn (*Solanaceae*) for Australia. PhD thesis, Rhodes University, Grahamstown (submitted January 2020).

McCulloch GA, Mauda EV, Chari LD, Martin GD, Gurdasani K, Morin L, Walter GH, Raghu S (2020) Genetic diversity and morphological variation in African boxthorn (Lycium ferocissimum) – Characterising the target weed for biological control. Biological Control 143:104206

Morin L, Ireland KB, Delaisse C, Zeil-Rolfe I, Hunter GC (in preparation) Information package to support the application to release the rust fungus *Puccinia cnici-oleracei* (ex. *Conyza*) for the biological control of flaxleaf fleabane (*Conyza bonariensis*) in Australia.

Noble MR, Adair RJ, Ireland KB (in preparation) The Biology of Invasive Plants. 1. *Lycium ferocissimum* Miers.

Ollivier M, Kazakou E, Corbin M, Sartori K, Gooden B, Lesieur V, Thomann T, Martin J-F, Tixier M-S (in press) Trait differentiation between native and introduced populations of the invasive plant *Sonchus oleraceus* L. (Asteraceae). Neobiota

Ollivier M, Labouyrie M, Raghu S, Tavoillot J, Tixier M-S, Martin J-F, Lesieur V (in preparation) What can we learn for biological control by sampling the invasive range of a weed? The case study of *Sonchus oleraceus* (Asteraceae) in Australia

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Appendix - additional project information

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Hunter GC and Morin L (2018) Proposed plant host test list for assessing risk of candidate biological control agents for *Sonchus oleraceus*. Prepared by CSIRO.

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- *Neopestalotiopsis nebuloides* C. Lock, Vitelli, Holdom, Y.P. Tan & R.G. Shivas, sp. nov.
- *Pestalotiopsis etonensis* C. Lock, Vitelli, Holdom, Y.P. Tan & R.G. Shivas, sp. nov. were submitted to *Persoonia* are due for publication in Fungal Planet XXX – 2020.

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7.2 Equipment and assets

NSW DPI 5 x Steridium MODEL pledt-rh-500 growth cabinets.

7.3 Monitoring and evaluation

MER framework was developed and reported as part of Milestone 2.

The purpose of the Plan was to:

- Ensure the objectives of the funded project and the process for achieving them are clear for applicants, partners and the Australian Government
- Identify data and information that can be reasonably collected as evidence of project delivery and outcomes
- Provide input into the evaluation of the overarching Rural Research and Development for Profit Programme.

The components of the MER were as follows:

- The development of a governance structure (Steering Committee) to monitor strategic aspects of the project. Members of this group consisted of the research managers from each of the institutions involved in the project. This committee met at six monthly intervals, after the submission of Milestone reports. As well as addressing strategic issues, it was responsible for overseeing the Risk and Issues registers for the project.
- The establishment of an operational committee structure involving the project researchers which met six monthly prior to the milestone deadlines. A key function of these meetings was to identify any issues which may have resulted in the failure to meet KPIs.
- Review of subproject Milestones with clarification sought if required and a consolidated report forwarded to the Federal Government funding body.

An independent mid-project evaluation of the MER plan was undertaken by A Ball and R Pattinson and submitted as a component of Milestone 4. Their report made a number of recommendations which were considered by Agrifutures management and the Project Steering Committee and where appropriate, actioned.

Project achievements

The Steering committee functioned well. As a result of the review, the importance of the issues and risks registers was reinforced and reviewed at scheduled meetings. The Committee was also expanded to include a representative of one of the major external funding bodies.

A number of KPIs in the original contracts were dependent on the release and establishment of biocontrol agents the field. While it was likely clear that some agents would be nominated by the end of the funding period, approval by the relevant authorities to release was not expected to occur. Relevant Milestones were modified in 2019 to allow more flexibility in the delivery of outcomes.

All KPIs have been met.

Consideration was given to using the CSIRO ADOPT® program to provide some evaluation of the economic benefits of the program. However, in review, it is not an appropriate tool as it is driven by a farmer's motivation to adopt certain practices. The essence of biological control is that once established, the agents by and large, are beyond the control of the landowner. The initial justification for the inclusion of the chosen weeds was based on their economic impact.

The extent to which this benefit will be realised will be dependent of the successful establishment of the agents, which in most cases will not be evident for several years after release.

7.4 Budget

The audited income and expenditure statement will be provided as a separate document in June 2020.

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