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## Assessing the Risk to *Neptunia oleracea* Lour. by the Moth, *Neurostrotta gunniella* (Busck), a Biological Control Agent for *Mimosa pigra* L.

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### Abstract

*Mimosa pigra* L. is native to tropical America and is an aggressive, invasive weed on the flood plains of the Northern Territory of Australia and in several countries in Southeast Asia. *Neurostrotta gunniella* (Busck) (Gracillariidae) was introduced into Australia from Mexico in 1986 for biological control of mimosa. It was released in 1989 following completion of extensive host range studies which determined that the moth bred readily on *M. pigra* and to a much lesser extent on *Neptunia dimorphantha* Domin, *N. gracilis* Benth., *N. major* (Benth.) Windler, *N. monosperma* F. Muell. and *M. pudica* L. Damage to these non-target species was assessed as insignificant. Subsequently, this moth was introduced to Thailand where quarantine studies showed substantial attack on an important vegetable, *N. oleracea* Lour., which is a perennial, aquatic herb which either grows prostrate near the water's edge or floats by forming spongy aerenchyma around the stems. *N. gunniella* was not released in Southeast Asia.

Further studies showed that *N. gunniella* oviposits and breeds similarly on potted *M. pigra* and the terrestrial form of *N. oleracea* but fewer eggs are laid and larval mortality is much greater on *N. oleracea*, when it grows as single stems over water. However, when tests were conducted on the aquatic form of *N. oleracea* growing more naturally as sparse and thick mats, there were no significant differences in the number of progeny produced from the terrestrial or aquatic forms of this species. This confirmed that *N. oleracea* could be heavily damaged by *N. gunniella* if this moth was released against *M. pigra* in countries or regions where *N. oleracea* is an important plant. These studies highlight the importance of a country reviewing plant genera at risk and testing local species in these genera before releasing a biological control agent that has been used and tested against different species elsewhere.

### Introduction

*Mimosa pigra* L., commonly called mimosa or giant sensitive plant, is native to tropical America and is an aggressive, invasive weed outside its native range. It is a very serious weed on the flood plains of the Northern Territory of Australia and in several countries in Southeast Asia (Lonsdale *et al.* 1995). Mature plants are multi-stemmed and grow to a height of 4-6m. In Australia, this weed has invaded about 80,000 ha of floodplains over a 700 km arc from the Arafura Swamp in central Arnhem Land to the Fitzmaurice River near the border with Western Australia (Anon, 1997). It has transformed grass- and sedgeland to near monospecific tall shrublands and has invaded billabongs and swamp-

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lands of *Melaleuca* spp. (Lonsdale *et al.* 1995).

A program to find safe and effective biological control agents for *M. pigra* in Australia commenced in 1979 with surveys in the native range of this weed to find suitable insects and pathogenic fungi. During surveys in Mexico in the mid-1980's, early instar larvae of the moth, *Neurostrota gunniella* (Busck) (Gracillariidae), were frequently observed mining the pinnae of young leaves of mimosa, and older larvae were observed tunneling in the tips of mimosa stems. In preliminary host range testing by CSIRO Entomology in Mexico, adults were reared from *M. pigra* and *Neptunia plena* (L.) Benth., which is also native to Mexico, but were not reared from any other related species (Davis *et al.* 1991). *N. gunniella* was introduced into CSIRO quarantine facilities in Brisbane, Australia in 1986 and the biology and host range of this moth was studied over the following 2 years. These studies determined that the moth bred readily on *M. pigra* and to a much lesser extent on *N. dimorphantha* Domin, *N. gracilis* Benth., *N. major* (Benth.) Windler, *N. monosperma* F. Muell. and *M. pudica* L. Adults oviposited on these non-target species in no-choice tests but larval mortality was very high, 70-96% compared to 25% on *M. pigra*. Damage to these non-target species was rated as insignificant (Davis *et al.* 1991). The moth was approved for release against mimosa in the Northern Territory of Australia in February 1989 following acceptance of the results of extensive host range studies by the regulatory authorities, the Australian Quarantine Inspection Service (AQIS) and Environment Australia (EA).

The moth spread rapidly following its release and became abundant, particularly towards the end of the wet season. Lonsdale and Farrell (1998) measured the impact of *N. gunniella* on mimosa over five years and tentatively concluded that the moth was having a negative impact on seed production with seed output being 60% lower than normal at high insect densities.

In 1992 (Wilson pers. com.) confirmed that *N. gunniella* was attacking *N. major* in the field near infestations of *M. pigra*. This observation was not alarming, given that host range studies had shown that the moth would attack *Neptunia* spp. in Australia, but it highlighted the need to measure the impact of this moth on non-target species, post release.

Species of *Neptunia* grow throughout the tropical and warm temperate regions of the world, usually occurring as terrestrial plants in open areas (Windler 1966). Loureiro described the genus *Neptunia* in 1790, based on a floating species which he named *Neptunia oleracea* Lour. *N. oleracea* has a pan-tropical distribution and inhabits warm, slow-moving, and frequently stagnant waters in Asia, Africa, and Central and South America. It is a perennial, aquatic herb that either floats on water or is prostrate near the water's edge (Windler 1966). Stems are rarely branched and may reach 1.5m in length. When the plant grows in water, a spongy-fibrous indument of aerenchyma is formed around the stem between the nodes and this helps to keep the plant afloat. Windler (1966) in his revision of this genus, commented that it had a greater affinity with species in the Mimoseae, which includes *M. pigra*, than the tribe where it was placed, Adenanthereae, and it has since been moved to the Mimoseae.

As part of a collaborative project between Australia and Thailand, supported by the Australian Center for International Agricultural Research (ACIAR), *N. gunniella* was sent from Australia to quarantine facilities in Thailand in 1990 with a strong recommendation from the senior author of this paper that *N. oleracea*, which occurs in Southeast Asia but does not occur in Australia, should be tested before the moth was considered for release.

*N. oleracea* is an economically important species in Thailand and Vietnam, where it is farmed in ponds and young shoots are harvested as a vegetable. In these countries, any substantial damage to this food source would be unacceptable.

The National Biological Control Research Center (NBCRC) of Thailand carried out host range testing on potted test plants and showed that *N. gunniella* bred readily on *N. oleracea*. As a result, the moth was not released in Thailand (Banpot Napompeth, pers. com. 1990). Subsequently, the senior author recommended that this moth should not be introduced into any Asian country regardless of the status of *N. oleracea*. Forno and Day (1994) expanded on the Thai studies by testing *N. oleracea* as an aquatic plant, with spongy aerenchyma surrounding the stems and as a terrestrial plant in pots without this tissue. Their results were partially inconclusive though they did confirm that the terrestrial form of *N. oleracea* was at risk from *N. gunniella*. Their results also indicated that the aquatic form was much less susceptible to attack by *N. gunniella* but further studies were required to understand the behaviour of the moth on the different forms of *N. oleracea*.

The studies reported in this paper compare the oviposition, larval development and adult emergence of *N. gunniella* on *N. oleracea*, growing as a terrestrial and an aquatic plant, and *M. pigra*. Two species, *N. major* and *N. monosperma*, which are native to Australia and had been previously tested, were also included in some of these trials.

## Methods

### Larval Development (Experiments 1 and 2)

In these experiments, a known number of fertile eggs were artificially placed on to the test plants.

In Experiment 1, 50 eggs of *N. gunniella* were placed on each of the following species: *M. pigra*, *N. oleracea* as a terrestrial (T) and an aquatic (A) plant (replicated 5 times), *N. major* (replicated twice) and *N. monosperma* (replicated three times). Plants were grown in 15 cm diameter pots and were ca. 50 cm high when the trial commenced, except *N. oleracea* (A), which was propagated from tip cuttings placed in nutrient enriched water where they grew and developed spongy aerenchyma around the stems. *N. oleracea* (A) plants had approximately the same number of leaves as *N. oleracea* (T) to standardise these treatments.

Eggs were obtained by placing four to five pairs of moths in clear plastic food containers, 260mm by 90mm by 100mm deep. Two sprigs of mimosa, each with three pairs of pinnae taken from the youngest fully expanded leaves were placed in each container. The sprigs were placed in water in small vials within the container and held in position with plasticine. The containers were covered with fine nylon mesh and then covered with plastic cling wrap, pierced to allow air to pass through. The sprigs were replaced daily and the sprig from the previous day was held in a plastic Petri dish for egg development.

Fertile eggs were identified on day 3, approximately 24 hours before they hatched, by the presence of a developing larva inside. The pinnae and rachi to which fertile eggs were attached were removed from plant sprigs using a binocular microscope. Fifty eggs were glued on to each plant with aqueous Aquadhere<sup>R</sup> by placing eggs singly on to the first five pairs of pinnae starting at the base of young fully expanded leaves of mimosa and one egg per pinna per fully expanded leaf on the *Neptunia* species.

Each plant was then placed into a separate cage, 460 mm by 460 mm by 900 mm high, constructed of extruded aluminium with a metal floor until adults emerged. The walls and top of the cage were covered with fine nylon organza. Development of larvae was moni-

tored by recording the number of sites where first instar larvae had entered the pinnae four days after egg hatch, the number of days to the emergence of the first adult and the number of adults that emerged. Moths were removed on the day that they emerged. Plants were held for at least one week after the last adult had emerged.

In Experiment 2, eggs were collected from moths emerging in experiment 1. Fifty eggs were glued on to the same species from which the adult moths had emerged. There were 3 replicates of each *Neptunia* species and four replicates of *M. pigra*. Numbers of adults emerging from these G1 eggs on each of five plant species were compared. The procedure for mating moths and obtaining eggs was the same as that for experiment 1 except that sprigs of the same plant treatment from which adults had emerged were placed in the oviposition boxes. The procedure for counting larval mines and recording adult emergence from each plant species was as described above.

### **Non-choice oviposition and larval development (Experiment 3)**

In experiments 1 and 2, the oviposition selection process was bridged so that the development of larvae from a cohort of 50 eggs could be followed. In this trial, moths were obtained from pupae collected from *M. pigra* in the field. Newly emerged moths were paired and three pairs were caged with each test plant. Moths were allowed to oviposit for 3 days on *M. pigra*, *N. oleracea* (T) and *N. oleracea* (A) in a non-choice situation. As most eggs/day are laid on days 2 and 3 and hatch after 4 days (Davis *et al.* 1991), adults were removed after 3 days so that eggs were of a similar age. Test plants were grown in pots except *N. oleracea* (A) which floated on water. Mines on the pinnae were counted after 4 days as an indicator that fertile eggs had been deposited on the plant. Plants were held separately in cages as for experiments 1 and 2 until adults emerged. Moths were sexed and the number of adults emerging from each test plant was recorded.

### **Development of *N. gunniella* on a mat of *Neptunia oleracea* (Experiment 4)**

The previous experiments gave information on the oviposition and the suitability of *M. pigra*, *N. oleracea* (T) and (A) as hosts for *N. gunniella* when growing as single plants. In nature *N. oleracea* grows more as a mat of stems on water with adventitious roots forming at the nodes. In sparse mats, the stems are covered by spongy-fibrous aerenchyma between the nodes but as plant density increases, the stems above the water grow more vertically and lose the spongy tissue. In this experiment, the suitability of a mat of *N. oleracea* (T), and *N. oleracea* (A) as hosts for *N. gunniella* were compared.

Rooted cuttings of *N. oleracea* were grown in a mixture of sand, peat and vermiculite and slow release fertilizer in 150mm diameter pots. Plants were pruned to promote lateral growth. When lateral shoots were ca. 30cm long, plants were transferred to tubs, 88cm by 65cm by 35cm deep, covered by an aluminium frame (95cm by 70cm by 60cm high) with removable panels on the top and sides. Panels were covered with fine nylon organza. Two potted plants were placed in each of 15 tubs. Water was added to 10 tubs until it was ca. 10cm above the pots. This promoted the formation of the spongy fibrous indument around the stems of plants in these tubs.

There were three treatments, each replicated five times. Treatments were arranged randomly in a glasshouse with daily temperatures ranging from 20 to 40°C over a 3 month period. Treatments 1 and 2 contained plants of *N. oleracea* (T) and *N. oleracea* (A) respectively, each with a total of 15 branches about 30cm long. Treatment 3 also had 15 branches but these were 90cm long to increase the density of plant material on the water

surface. Treatment 1 [*N. oleracea* (T)] had no water surrounding the pot, treatment 2 [*N. oleracea* (A)] had free water between the stems and treatment 3 [*N. oleracea* (A)] had a dense cover of stems with little or no free water between them. Three pairs of *N. gunniella* were added to each cage. Larval mines were counted 10-14 days and 20-21 days after moths were added. Each day thereafter, cages were checked for the emergence of adults and treatments were terminated after no adults emerged on three consecutive days after the first emergence. Adults were sexed as they emerged and numbers recorded.

## Results

### Larval Development (Experiments 1 and 2)

Mines were counted 4 days after eggs were glued onto the plants to give an indication that eggs had not been damaged during the delicate process of gluing these to the plants and that larvae had emerged and were feeding normally. In three trials, two on *M. pigra* and one on *N. oleracea* (A) there was no feeding on day 4 and these trials were therefore not included in the analyses.

An analysis of variance on the valid data showed no significant difference in the number of larval mines on day 4 indicating larvae had started to mine the pinnae on all species. There was no correlation between number of mines and eggs placed on the plant as larvae sometimes mined more than one pinna. There was no significant difference in development time from egg to adult between species although when the sum of squares was partitioned, the contrast between development time on *N. oleracea* (T) and *N. oleracea* (A) was significant at  $p < 0.05$ . There were more adults emerging on *M. pigra*, *N. major* and *N. oleracea* (T) than on *N. monosperma* and *N. oleracea* (A) ( $p < .05$ ) (Table 1).

**Table 1.**

**Mean number of larval mines, moths emerging and mean development time from egg to adult of *Neurostrota gunniella* on *Mimosa pigra* and 3 species of *Neptunia*. (Experiment 1).**

Plant species	Mines on day 4 Mean (SE)	Adults emerging Mean (SE)	Egg to adult Days (SE)
<i>M. pigra</i>	46.6 (6.6) a	23.3 (3.5) a	23.0 (1.2) a
<i>N. oleracea</i> (T)	40.2 (5.1) a	23.4 (3.3) a	23.3 (1.0) a
<i>N. oleracea</i> (A)	32.0 (5.7) a	10.9 (3.5) b	27.0 (1.0) a
<i>N. major</i>	51.5 (8.1) a	21.0 (4.2) a	24.1 (1.5) a
<i>N. monosperma</i>	30.7 (6.6) a	12.7 (4.1) b	25.7 (1.2) a

Values in the same column followed by the same letter are not significantly different

Similar results were obtained when larvae from eggs of G1 females in experiment 1 developed on the same species from which adults emerged. The number of mines on day 4 indicated that larvae were feeding on all species. Development from egg to adult took a similar number of days and significantly more adults emerged from *M. pigra* and *N. oleracea* (T) than the other *Neptunia* species ( $p < 0.001$ ). We assumed this difference was mainly due to larval mortality and results from *N. monosperma* and *N. major* were similar to those cited in Davis *et al.* 1991 where larval mortality was estimated at greater than 70% and 96% respectively.

**Table 2.**

**Mean number of larval mines, moths emerging and mean development time from egg to adult of *Neurostrotta gunniella* on *Mimosa pigra* and 3 species of *Neptunia* when eggs from G1 adults were placed on the same species from which adults emerged. (Experiment 2)**

Plant species	Mines on day 4 Mean (SE)	Adults emerging Mean (SE)	Egg to adult Days (SE)
<i>M. pigra</i>	58.0 (11.6) a	23.3 (1.8) a	26.5 (1.0) a
<i>N. oleracea</i> (T)	52.7 (13.4) a	29.3 (2.1) a	23.3 (1.1) a
<i>N. oleracea</i> (A)	37.3 (13.4) a	10.0 (2.1) b	26.7 (1.1) a
<i>N. major</i>	56.3 (13.4) a	10.0 (2.1) b	24.7 (1.1) a
<i>N. monosperma</i>	41.0 (13.4) a	13.7 (2.1) b	25.3 (1.1) a

Values in the same column followed by the same letter are not significantly different

### Non-choice oviposition and larval development

When adult moths were allowed to oviposit on the 5 test plants in a non-choice situation, the variation in the number of mines after 4 days and the number of adults emerging from each species and between replicates was large and required a square root transformation for analysis. Analyses of variance showed that although the mean number of mines on *N. oleracea* (T) and *M. pigra* was greater than the number on other species, the differences were not significant. We concluded that moths accepted all species for oviposition, laying more eggs on *N. oleracea* (T) and *M. pigra*. Significantly fewer adults emerged from *N. oleracea* (A) and *N. major* ( $p < 0.001$ ) again confirming that larval mortality on these species was greater than on the other species with consistently more adults emerging from *M. pigra* and *N. oleracea* (T). The mean development time was significantly less on *M. pigra* when the contrast between *M. pigra* and other species was analysed (Table 3).

**Table 3.**

**Mean number of larval mines, moths emerging and mean development time when moths were allowed to oviposit on test plants in a non-choice situation (Experiment 3).**

Plant species	Mines on day 4 Mean	Adults emerging Mean *(SE) [ ]	Egg to adult Days (SE)
<i>M. pigra</i>	15.5 a	6.8 (0.49) [48] a	35.8 (1.2) a
<i>N. oleracea</i> (T)	10.2 a	6.6 (0.44) [45] a	38.6 (1.1) a
<i>N. oleracea</i> (A)	2.0 a	2.3 (0.57) [5] b	40.0 (1.4) a
<i>N. major</i>	3.3 a	2.3 (0.57) [6] b	40.7 (1.4) a
<i>N. monosperma</i>	6.0 a	5.6 (0.98) [31] a	39.0 (2.4) a

Values in the same column followed by the same letter are not significantly different

\* Transformed estimates with (SE) after a square root transformation. Untransformed data in [ ]

**Development of *N. gunniella* on a mat of *N. oleracea***

In the *N. oleracea* (A) treatments, moths seemed to prefer the more aerial parts of the plant away from the water for oviposition. The number of mines on day 10 in all treatments was analysed using a square root transformation. Treatment differences were not significant though there was a difference in the contrast between the terrestrial and the aquatic forms with more mines on the former ( $p < 0.05$ ). By day 20 there were no significant differences. These data confirmed that moths would lay on both forms of *N. oleracea* and there was no evidence of significant larval mortality in any treatment. It was therefore not surprising that there were no significant differences between the number of adults emerging from each treatment. Development time was slightly longer on the terrestrial form of *N. oleracea*. The ratio of female to male moths was approximately 1:1 (Table 4).

**Table 4.**  
**Mean number of larval mines, moths emerging and development time from egg to first adult, when moths were allowed to oviposit on plants growing as a mat in a non-choice situation.**

Plant species	Mines on day 10 Mean *(SE)	Mines on day 20 Mean (SE)	Egg to first adult Mean (SE)*	Adults emerging Mean (SE)*	Number of Males: females
<i>N. oleracea</i> (T)	53.2 (8.8) a	63.0 (13.6) a	26.5 (0.62) a	124.0 (32.4) a	59.8 : 64.3
<i>N. oleracea</i> (A) sparse	23.8 (7.8) a	42.5 (13.6) a	21.6 (0.56) b	104.2 (29.0) a	51.6 : 52.6
<i>N. oleracea</i> (A) dense	31.2 (8.0) a	44.6 (12.2) a	22.2 (0.56) b	90.8 (29.0) a	43.4 : 47.4

Values in the same column followed by the same letter are not significantly different

**Discussion**

Studies to determine the host range and specificity of potential biological control agents for tropical weeds are usually carried out on potted plants in a quarantine facility where it is almost impossible to simulate natural conditions for growing each plant species to be tested. Whilst this may be relatively unimportant for most plants it had important consequences for determining the suitability of *N. oleracea* as a host for *N. gunniella*.

When the suitability of Australian native *Neptunia* spp. as hosts for *N. gunniella* was determined in 1986-88, it was acceptable to conduct tests on plants growing in pots as these *Neptunia* spp. are terrestrial and are sparsely distributed in the field. The initial studies to determine the suitability of *N. oleracea* in Thailand as a host for *N. gunniella* were also carried out on potted plants (Banpot Napompeth pers. com. and Forno and Day (1994)). Their studies concluded that *M. pigra* and the terrestrial form of *N. oleracea* were excellent hosts for *N. gunniella*. However in potted trials, it seemed that the aquatic form of *N. oleracea* may not be as suitable, possibly because moths avoid ovipositing or larvae may not be able to complete development, on stems floating on water and covered with

spongy aerenchyma. In these studies we tested the suitability of aquatic and terrestrial forms of *N. oleracea* as hosts for *N. gunniella* by comparing development on single plants in pots and plants with a mat of stems either floating on water or on the bottom of a cage. Our results conclusively show that *M. pigra* and both the terrestrial and aquatic forms of *N. oleracea* are excellent hosts for *N. gunniella*. They also support the findings of Davis *et al.* 1991 that *Neptunia* spp. in Australia are inferior hosts.

These studies demonstrate the importance of observations in the native range and host specificity studies carried out in other countries when evaluating the suitability of an agent for introduction elsewhere in the introduced range of the weed. For example, the observed attack by *N. gunniella* on *N. plena* in Mexico ensured that the host test list in Australia was widened to include all native species of *Neptunia* in Australia. *N. amplexicaulis* Domin could not be found and was not tested. Quarantine studies demonstrated that *N. dimorphantha*, *N. gracilis*, *N. major* and *N. monosperma* were inferior hosts compared to *M. pigra* and the risk to Australian *Neptunia* spp. was accepted, resulting in the release of *N. gunniella* (Davis *et al.* 1991). Other countries eg. Thailand was made aware of the need to test any local *Neptunia* spp. before releasing *N. gunniella* against *M. pigra*. Thailand conducted studies on potted *N. oleracea*(T) and concluded that *N. oleracea* was an excellent host for *N. gunniella*. The unnatural phenotypic appearance of *N. oleracea* in pots indicated the need to conduct more detailed studies on terrestrial and aquatic forms of the plant and subsequent studies highlighted the need to conduct studies on a floating mat of stems rather than single plants in pots. The outcome was that although *N. gunniella* may lay fewer eggs and larval mortality may be higher on *N. oleracea*(A) in pots, in reality, when *N. oleracea* grows as a mat, there is a mixture of floating “aquatic” stems covered with aerenchyma and aerial “terrestrial” stems allowing the moth to select the best material for reproduction. We concluded that this moth should not be released in any country with *N. oleracea*.

This is not the first instance where a biological control agent has been approved for release in one country but not recommended in others. For example, the mirid, *Eccritotarsus catarinensis* (Carvalho) was not recommended for release in Australia against water hyacinth because it could develop and sustain populations on native *Monochoria* species in the Pontederiaceae, (Stanley and Julien 1999). However, *E. catarinensis*, was released in South Africa following host range tests that showed that species in the Pontederiaceae, native to South Africa, cannot support significant populations of this mirid (Hill *et al.* 1999). Similarly the moth *Xubida infusella* (Walker) was released in Australia and South Africa but not in the USA as it attacked *Pontederia cordata* L., a native of southern USA (Julien and Stanley in press). These examples and this study illustrate the complexity of determining the extent of the complete host range and relative host specificity of an insect under laboratory conditions in different countries. Post-release assessment of the host range, specificity and impact of introduced biological control agents are not only important for testing the predictions made under less natural conditions but may also assist the methods used to measure risk assessment before agents are released.

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