



Assessing the potential of the rust fungus *Puccinia spegazzinii* as a classical biological control agent for the invasive weed *Mikania micrantha* in Papua New Guinea



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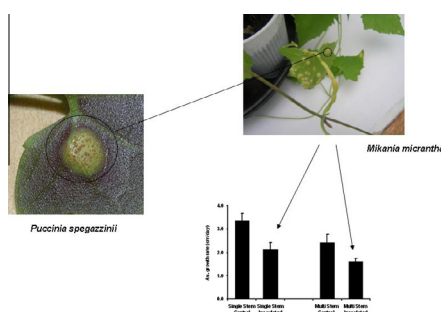
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HIGHLIGHTS

- *M. micrantha* in different countries has different susceptibilities to *P. spegazzinii*.
- The host specificity of *P. spegazzinii* is confirmed.
- *P. spegazzinii* has the potential to reduce populations of *M. micrantha*.

GRAPHICAL ABSTRACT



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ABSTRACT

The rust fungus *Puccinia spegazzinii* was introduced into Papua New Guinea (PNG) in 2008 as a classical biological control agent of the invasive weed *Mikania micrantha* (Asteraceae), following its earlier release in India, mainland China and Taiwan. Prior to implementing field releases in PNG, assessments were conducted to determine the most suitable rust pathotype for the country, potential for damage to non-target species, most efficient culturing method and potential impact to *M. micrantha*. The pathotype from eastern Ecuador was selected from the seven pathotypes tested, since all the plant populations evaluated from PNG were highly susceptible to it. None of the 11 plant species (representing eight families) tested to confirm host specificity showed symptoms of infection, supporting previous host range determination. A method of mass-producing inoculum of the rust fungus, using a simple technology which can be readily replicated in other countries, was developed. Comparative growth trials over one rust generation showed that *M. micrantha* plants infected with the rust generally had both lower growth rates and lower final dry weights, and produced fewer nodes than uninfected plants. There were significant correlations between the number of pustules and (a) the growth rate, (b) number of new nodes and (c) final total dry weight of single-stemmed plants placed in open sunlight and between the number of pustules and number of new nodes of multi-stemmed plants placed under cocoa trees. The trials suggest that field densities of *M. micrantha* could be reduced if the rust populations are sufficiently high.

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1. Introduction

Mikania micrantha Kunth (Asteraceae), or mile-a-minute, is a fast growing, perennial vine native to the Neotropics. It is a major invasive weed in many countries of Southeast Asia, where it was introduced as a cover crop (Wirjahardja, 1976; Waterhouse, 1994). *M. micrantha* is also present in the Pacific, where it is found in 18 countries and territories, including Papua New Guinea (PNG)¹ (Waterhouse and Norris, 1987; PIER – Pacific Island Ecosystems at Risk., 2012), affecting plantations and subsistence farms in areas up to an altitude of 1100 m above sea level (Waterhouse and Norris, 1987; Day et al., 2012). It flowers prolifically and the seeds are dispersed by wind or by people, becoming attached to clothing or possessions, and domestic animals (Holm et al., 1991; PIER – Pacific Island Ecosystems at Risk., 2012). In PNG, *M. micrantha* can grow about 4 cm/day and can form dense mats, smothering and killing crops such as banana, taro, papaw and young cocoa trees, thereby potentially reducing food security (Day et al., 2011).

Although some commercial plantations use herbicides, most control of *M. micrantha* in PNG is achieved through hand-pulling and slashing (Day et al., 2012). However, such methods are labour-intensive and unsustainable for small subsistence farmers, as plants quickly regrow from stem fragments (Holm et al., 1991; Day et al., 2012). Chemical control is not feasible for many subsistence farmers due to its high cost relative to the small scale of production and low income generated (Waterhouse and Norris, 1987; Orapa et al., 2001; Day et al., 2012). Therefore, biological control is seen as the only viable option to control this aggressive weed in most parts of PNG.

Biocontrol of *M. micrantha* first began in 1978, with exploration by CABI Europe-UK (CABI) in tropical America for potential agents, resulting in the introduction of *Liothrips mikaniae* (Priesner) (Thysanoptera: Phlaeothripidae) into the Solomon Islands in 1988 and Malaysia in 1990. However, this insect failed to establish in either country. It was also imported into PNG in 1989, but died out in quarantine before any field releases could occur (Cock et al., 2000).

In 1996, CABI surveyed tropical America for potential fungal pathogens for the control of *M. micrantha* in India, under a project funded by the UK-Department for International Development. Of several fungi found, the autoecious microcyclic rust fungus *Puccinia spegazzinii* de Toni (Pucciniales) was thought to be particularly damaging, as it attacks stems, petioles and leaves. Studies by CABI found that the strains of *P. spegazzinii* differed in their aggressiveness towards *M. micrantha* populations found in various invaded countries (Ellison et al., 2004). Host-specificity testing on 115 different plant species found the rust to be highly specific within the genus *Mikania* (Ellison et al., 2008). Different strains of the rust were subsequently released in India (2005), mainland China (2006) and Taiwan (2008), but the rust established only in Taiwan. However, in India and mainland China, the rust was released at very few sites, which may explain why it has apparently failed to establish (Ellison and Day, 2011).

In 2006, a new project on the biocontrol of *M. micrantha* commenced in PNG and Fiji, funded by the Australian Government and managed by the Queensland Government. The project aimed to introduce host-specific biocontrol agents that had been utilised elsewhere. The butterflies *Actinote antea*s (Doubleday and Hewitson) and *A. thalia pyrrrha* Fabr. (Lepidoptera: Nymphalidae), which were reported damaging *M. micrantha* in Indonesia (Desmier de Chenon et al., 2002) (R. Desmier de Chenon Indonesian Oil Palm Research Institute pers. comm., 2006), and the rust *P. spegazzinii*,

were prioritized. This paper reports the pathogenicity and host-specificity testing conducted by CABI on behalf of PNG and the culturing and potential impact of the rust on *M. micrantha* in PNG.

2. Materials and methods

2.1. Rust pathotype selection

Seven isolates of *P. spegazzinii*, from Argentina, Brazil, Costa Rica, eastern and western Ecuador, Peru and Trinidad and Tobago, were assessed in isolation under quarantine conditions at CABI in the United Kingdom for their pathogenicity towards 10 accessions of *M. micrantha* from PNG and Fiji. Since the isolates varied in their pathogenicity towards genotypes within the species *M. micrantha*, they are referred to as pathotypes *sensu lato* (Ellison et al., 2004). Full details of the collection sites of the rust pathotypes and the method of shipment to the UK on living plants, are given in Evans and Ellison (2005). This involved collecting 3–4 recently infected plantlets (rooted nodes of runners), originating from the same plant, with pre-erupting rust telia (5–10 per plantlet), at each site. A culture of each pathotype using all field material of that pathotype was then maintained separately to avoid cross-contamination with other pathotypes under quarantine (Ellison et al., 2008). Each pathotype was deposited as dried material in CABI Fungal herbarium (Herb. IMI).

M. micrantha plants were grown in a proprietary compost, maintained in an air-conditioned quarantine glasshouse, with supplementary lighting and a daily temperature range of 20–35 °C. The rust inoculation and assessment procedures were the same as those used in Ellison et al. (2008). Plant pieces (stems, petioles and leaves) containing the embedded teliospores of one of the pathotypes were suspended (by placing on a wire grid or attaching to a plant support frame with wire plant ties) over at least four young, healthy *M. micrantha* plants from each of the 10 accessions pruned to maximise the amount of meristematic tissue in a dew chamber (Mercia Scientific, Birmingham, UK) at 20 °C for 24 h to encourage basidiospore formation, release and plant infection. The test was conducted for each of the seven rust pathotypes, using four fresh plants of each accession each time. The complete test of all rust pathotypes was repeated at least once, using fresh plants of each accession each time.

Rust isolates were rated for aggressiveness and ability to fully attack the 10 accessions of *M. micrantha*. Test plants were examined daily for symptom development. After 21 days, which is the average time required for full symptom development on a susceptible host (Ellison et al., 2008), plants were given a score using the scale below:

1. No macroscopic symptoms.
2. Necrotic or chlorotic spots on inoculated leaves - no sporulation.
3. Abnormal infection site: chlorotic patches on leaves with very low teliospore production around edges of chlorosis. No infection of petioles and stems.
4. Abnormal infection site: pustules reduced in size (<4 mm diameter) or with low teliospore production in relation to compatible host-pathogen interaction; on leaves petioles and stems.
5. Fully compatible host-pathogen interaction. Normal pustule formation (4–7 mm diameter - often coalescing) on leaves, petioles and stems.

As well as a numerical value, a '–' or '+' after the score was used, to indicate that a result is 'just in' or 'better', respectively, than the allocated numerical value. This was recorded to help with the selection of the optimum pathotype for introduction into PNG.

¹ Abbreviations used: PNG, Papua New Guinea, SSI, single stem inoculated; SSC, single stem control; MSI, multi-stem inoculated, MSC, multi-stem control.

2.2. Host-specificity screening

The pathotype of the rust (IMI 393075, eastern Ecuador), which had been selected for release in PNG and Fiji on the basis of the pathogenicity results, was extensively screened against 115 plant species in 45 families before being released in Taiwan in 2008 (S.S. Tzean, National Taiwan University, pers. comm.). Therefore, only 11 additional plant species of importance to the South Pacific or within the same family as *M. micrantha*, representing eight families, were tested on behalf of countries in the South Pacific: *Bidens pilosa* L., *Zinnia* sp., *Hippobroma longiflora* (L.) G. Don f., *Scaevola sericea* Vahl., *Colocasia esculanta* (L.) Schott, *Anisomeles indica* (L.) Kuntze, *Hyptis pectinata* (L.) Poit., *Plectranthus scutellarioides* (L.) R. Br., *Psidium guajava* L., *Piper methysticum* Foster f. and *Morinda citrifolia* L. (full details given in [Table 2, Supplementary Material](#)).

Host specificity was determined using the same inoculation procedure summarized above under 'Rust pathotype selection', with four plants of each of the eleven species tested at the same time against the eastern Ecuador pathotype. The complete test of all 11 species was repeated once, using four fresh plants of each species. All inoculations included a known, fully-susceptible *M. micrantha* plant as a control. Inoculated plants were observed daily for the development of symptoms, and all test plants were monitored for six weeks, which is twice the time required for full symptom development on *M. micrantha* ([Ellison et al., 2008](#)).

Due to the inoculation method, involving release of basidiospores, it is difficult to accurately quantify the amount of inoculum that a plant will receive in each experiment. However, the technique used (checking inoculum viability and position above test plant meristem, replication, repeat runs and control plants) ensures the results are scientifically robust.

2.3. Culturing techniques and pathogen development in PNG

Following approval by authorities in PNG to allow the rust to be released in the field, four small, bare-rooted *M. micrantha* plants of Fijian origin, inoculated with *P. spegazzinii* (IMI 393075) seven days previously, were imported under permit from the quarantine containment at CABI-UK into an air-conditioned post-entry quarantine laboratory (26 ± 1 °C), with natural lighting at the National Agricultural Research Institute's (NARI) Islands Regional Centre (IRC) at Kerevat, East New Britain Province, PNG in November 2008. The plants were transplanted into four pots (200 mm diameter by 180 mm high), filled with steam-sterilized topsoil mixture and placed in a sealed inoculation chamber made from clear Perspex ($600 \times 300 \times 600$ mm high) to aid recovery and allow the development of the rust pustules.

When the first pustules on the imported plants showed signs of maturing (approximately seven days after receiving the plants), as evidenced by a change in colour from bright yellow to golden brown, the plants were placed on a wire mesh stand in the inoculation chamber. Three or four fresh clean *M. micrantha* plants sourced from PNG in pots (200 mm diameter by 180 mm high) were placed directly below the infected plants, to maximise the chance of basidiospores landing on the fresh plants.

The humidity in the chamber was raised to 100% by spraying the uninfected plants with a fine layer of distilled water. This spraying facilitated basidiospore production from the teliospores on the infected plants, and provided the necessary conditions for infection of the fresh plants (i.e. through the provision of free water on the leaves). The infected plants were kept in the chamber for seven days, as the teliospores in the pustules develop sequentially, and this allowed time for younger teliospores to mature and germinate. Each day, a new batch of 3 or 4 fresh clean plants of PNG origin was added to the chamber and the previous batch removed, thus providing a 24-h period for infection of each batch of plants.

Over the seven days the original infected plants were in the chamber, 23 new plants were inoculated. These plants, of PNG origin, were later released from quarantine.

Inoculated plants removed from the chamber each day were held on a bench in the quarantine laboratory under a light bank, with 10-h light/14-h dark cycle, supplementing natural light for two days. The plants were then transferred to the quarantine greenhouse, with ambient temperature (ranging 28–40 °C) and hand-watered when required. Once pustule development was evident on the new plants, the original imported plants were burnt. Further inoculations of fresh clean plants continued as above, as pustules on inoculated plants matured, to maintain a rust culture in PNG to facilitate field-releases.

Through daily observations of *M. micrantha* following inoculation, the life cycle and the duration of each stage of the developing pustules were determined. Descriptions and size of the pustules of each stage were recorded and compared with previous studies ([Ellison et al., 2008](#)).

To determine the optimum method for culturing *P. spegazzinii* under PNG conditions for field releases, seven culturing techniques were investigated (full details of each culturing method is presented as [Supplementary Material](#)).

2.4. Comparative growth studies

To determine the potential impact of the eastern Ecuador pathotype of *P. spegazzinii* on the growth of *M. micrantha*, a series of comparative growth experiments using a single inoculation were conducted under three different conditions: (a) in the quarantine greenhouse; (b) in open sunlight and (c) under cocoa trees. These three experiments were not conducted simultaneously.

For each experiment, 150 cuttings of mature *M. micrantha* stems of approximate equal length (ca 10 cm) and possessing at least one node, were collected from the field and propagated by planting single stem pieces, with the node exposed, in plastic drinking cups (70 mm diameter \times 85 mm high) filled with steam-sterilized topsoil. Plants were grown to the 4–5 leaf stage in the quarantine greenhouse, usually about 3–4 weeks after planting. All side shoots were removed, so only one actively growing stem remained on each plant.

For the quarantine 'screenhouse' experiment, 10 similar-sized plants were assigned randomly to each of four treatments: single stem inoculated (SSI), single stem control (SSC), multi-stem inoculated (MSI) and multi-stem control (MSC). An additional 10 plants were used to obtain initial dry weights to confirm uniformity of the plants. For the 'open-sunlight' and 'under-cocoa-trees' experiments, 20 plants were used in each of the four treatments and to obtain initial dry weights.

All test plants were inoculated by placing the plants under a raised wire mesh platform supporting 10–15 small, heavily rust-infected plants, containing mature pustules in the inoculation chamber in the quarantine laboratory. The fresh clean plants were positioned under the infected plants such that released spores could fall onto the young shoots of the clean plants below. Inoculated plants were removed from the inoculation chamber after 48 h and kept on a bench under a light bank in the quarantine laboratory for two days before being placed in the quarantine greenhouse, as already described. For the quarantine screenhouse experiment, the height of each plant was measured and the number of nodes on the main stem recorded. Inoculated and control pots were placed in their respective groups, about 1 m apart in the quarantine screenhouse to minimise the chance of the control plants becoming infected.

For the open-sunlight and under-cocoa-trees experiments, all plants were transplanted into larger pots (200 mm diameter \times 180 mm high), one week following inoculation. The height

and the number of nodes present on each plant were recorded prior to plants being placed out in the field. Plants in the open-sunlight experiment were placed out in the open, away from trees and buildings, while plants in the under-cocoa-trees experiment were placed in an area shaded by cocoa trees.

For both the open-sunlight and under-cocoa-trees experiments, wooden stakes 1 m long were placed in pots as supports to allow plants to grow upright. Inoculated pots were placed 3 m from the control plants to minimise the chance of the control plants becoming infected. An insecticide application of Karate® (Lambda-cyhalothrin) 0.5 mL:500 mL of water mixed in a hand-held sprayer was applied to the young developing leaves and growing tips to control thrips after weekly measurements were taken. A buffer zone around the study site was also sprayed with Karate® and maintained for the duration of the experiment.

For all three experiments, the height and number of nodes were recorded weekly. Developing side shoots on the single-stemmed plants were removed, while those on the multi-stemmed plants were allowed to develop (and counted each week) to determine whether rust infection influences the development of side shoots. The total number of infected leaves, petioles and stems, and the total number of pustules on each inoculated plant were recorded weekly.

The experiments continued for five weeks, allowing one generation of the rust to be completed on the plants, upon which all surviving plants were carefully uprooted. Roots were washed thoroughly under running tap water to remove all soil. Each plant was separated into leaves, stems and roots, which were placed in individual paper bags in an oven (Labec Equipment Pty Ltd, temperature controller) and dried at 60 °C for three days, after which they were weighed separately, using an electronic balance (Sartorius BP 2100 S, Biolab Scientific Ltd).

2.5. Data analysis

Growth rates, the number of new nodes and dry weights of inoculated and control plants were compared using a two-way ANOVA, with stem density (single vs multi-stem) and treatment (inoculated vs control) as factors in the screenhouse experiment and a three-way ANOVA (with stem density, treatment and site (open sunlight and under cocoa trees) in the open-sunlight and under-cocoa-trees experiments. Secondary stem data were analysed using a one-way ANOVA for the screenhouse experiment and analysed using a two-way ANOVA for the open-sunlight and under-cocoa-trees experiments. Differences among means were examined using Fisher's LSD test. Correlations between the number of pustules and (a) growth rate, (b) the number of new nodes and (c) final dry weight were examined. Simple linear regressions were conducted to determine the best model to describe the relationship between the number of pustules and (a) growth rate, (b) the number of new nodes and (c) final total dry weight.

3. Results

3.1. Rust pathotype selection

There was substantial variation in the aggressiveness of the different rust isolates towards the different *M. micrantha* accessions tested from Fiji and PNG. The rust pathotype from eastern Ecuador was the most aggressive, scoring 4+ for all but one of the plant accessions tested. The rust pathotypes from Trinidad and Costa Rica also rated highly and so can be considered suitable for PNG and Fiji. However, the pathotypes from Argentina, Peru, Brazil and western Ecuador were not considered for release in Fiji and

PNG, because at least one population of *M. micrantha* tested received a score of less than 4 (including 4–) (Table 1).

3.2. Host-specificity screening

None of the 11 plant species tested became visibly diseased with *P. spegazzinii*, or showed any reaction to the inoculation with the rust basidiospores. The *M. micrantha* control plants included in each test run always expressed a fully compatible reaction to the rust fungus (see Table 2, Supplementary Material).

3.3. Culturing techniques and pathogen development in PNG

Telia continued to develop on the original plants sent from CABI over the seven days the plants were held in the inoculation chamber. By day 7, all pustules on the original plants had matured and sporulated, infecting a total of 23 plants.

The first visual symptoms of infection are chlorotic spots (mean diameter: 1.3 ± 0.1 mm; $n = 60$) on the underside of leaves, usually 7–10 days after inoculation. The spots enlarge into telia (mean diameter: 2.6 ± 0.1 mm; $n = 60$), which are bright yellow and appear after 13–16 days. The teliospores begin to mature, as evidenced by dark brown spots on the raised sori (mean diameter: 3.8 ± 0.10 mm; $n = 60$) after 18–22 days, after which time they are ready to form basidiospores if conditions are suitable.

The most effective culturing method was when 3–4 week old cuttings were placed in the inoculation chamber with infected plants situated above. Using this method, all inoculated plants became infected (see Table 3 in Supplementary Material).

3.4. Comparative growth studies

Pustules developed normally on the inoculated plants and there was no cross-contamination of the control plants. However, the short duration of the experiment did not allow for the development of secondary infection on the test plants.

3.5. Quarantine screenhouse experiment

All inoculated plants developed pustules, with an average (\pm standard error) of 28.8 ± 9.0 pustules/plant for single-stemmed plants ($n = 10$) and 50.0 ± 18.0 for multi-stemmed plants ($n = 10$). Inoculated plants had significantly lower mean growth rates than the uninfected plants ($F_{1,37} = 13.57$, $p < 0.001$) (Fig. 1). There was no interaction between stem density and infection rate ($F_{1,36} = 0.58$, $p = 0.451$) and there was no correlation between the number of pustules and the growth rate of single- ($r = 0.415$, $p = 0.234$) and multi-stemmed plants ($r = -0.090$, $p = 0.804$).

Inoculated plants had significantly fewer new nodes than uninfected plants ($F_{1,37} = 8.39$, $p = 0.006$) (Fig. 2). There was no interaction between stem density and infection rate ($F_{1,36} = 0.15$, $p = 0.697$) and there was no correlation between the number of pustules and the number of new nodes of single- ($r = 0.462$, $p = 0.178$) and multi-stemmed plants ($r = 0.457$, $p = 0.184$).

Inoculated plants had a significantly smaller final total dry weight than uninfected plants ($F_{1,37} = 31.04$, $p < 0.001$) (Fig. 3), so separate analyses of leaf, stem and dry weights were not conducted. There was no interaction between stem density and treatment ($F_{1,36} = 0.01$, $p = 0.926$) and there was no correlation between the number of pustules and the final total dry weight of single- ($r = 0.266$, $p = 0.458$) and multi-stemmed plants ($r = 0.188$, $p = 0.604$) or the dry weights for leaves, stems and roots.

There was no difference in the number of secondary stems produced between inoculated and uninfected multi-stemmed plants ($F_{1,18} = 0.00$, $p = 1.00$).

Table 1

The degree of infection of *Puccinia spegazzinii* pathotypes collected from seven locations in the native range to accessions of *Mikania micrantha* from Viti Levu, Fiji and East New Britain Province (ENBP), West New Britain Province (WNBPN) and New Ireland Province (NIP), Papua New Guinea. Ratings: (0) no macroscopic symptoms; (1) necrotic or chlorotic spots visible but no sporulation; (2) chlorotic patches on leaves with very low teliospore production around edges of chlorosis; (3) pustules reduced in size (<4 mm diameter) or with low teliospore production in relation to compatible host-pathogen interaction; (4) fully compatible host-pathogen interaction (4–7 mm diameter – often coalescing) on leaves, petioles and stems. 'NT' not tested. As well as a numerical value, a '-' or '+' after the numerical score was used, to indicate that a result was 'just in' or 'better', respectively, than the allocated numerical value.

<i>M. micrantha</i> accession	Origin of <i>Puccinia spegazzinii</i> pathotype (IMI number)						
	Argentina (393078)	Peru (393076)	Brazil (393061)	Eastern Ecuador (393075)	Western Ecuador (393074)	Trinidad (393067)	Costa Rica (393069)
Naidascri, Fiji	4	4–	NT	4+	4	4	4+
Nausori, Fiji	4	4	4	4+	4+	4	4
Koronivia, Fiji	4–	3	1	4+	4	4	4+
Varoka, Fiji	4+	4	3–	4+	4–	4	4
Waibau, Fiji	4–	4–	4	4+	4	4	NT
Gazelle Peninsula, ENBP, PNG	4	4+	4	4	4	4	4
Kerevat, ENBP, PNG	4	4+	3	4+	4	4	4
Napapar 4 Village, ENBP, PNG	4	4	3	4+	4	4	4
Central, WNBPN, PNG	4	NT	NT	4+	NT	NT	NT
Kavieng, NIP, PNG	4	NT	NT	4+	NT	NT	NT

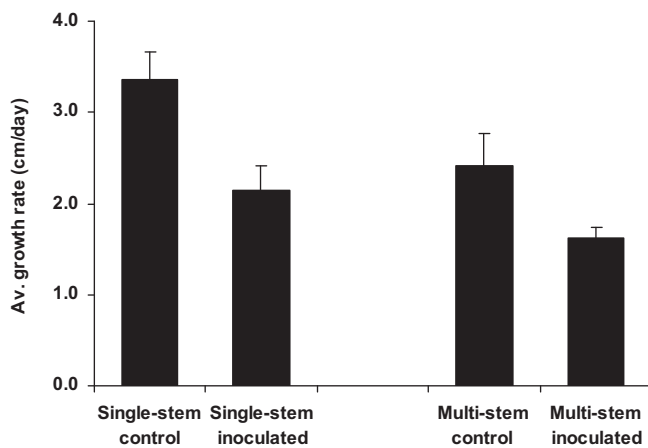


Fig. 1. The average growth rate (\pm s.e.) over five weeks of single- and multi-stemmed *M. micrantha* plants inoculated with *P. spegazzinii* and of single- and multi-stemmed non-inoculated plants grown in a quarantine screenhouse with ambient temperature (ranging 28–40 °C).

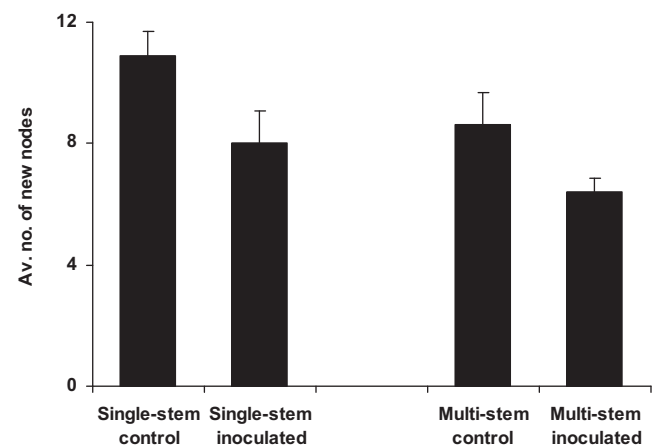


Fig. 2. The average number (\pm s.e.) of new nodes formed over five weeks on single- and multi-stemmed *M. micrantha* plants inoculated with *P. spegazzinii* and on single- and multi-stemmed non-inoculated plants grown in a quarantine screenhouse with ambient temperature (ranging 28–40 °C).

3.6. Open-sunlight and under-cocoa-trees experiments

All inoculated plants in the open sun trial developed pustules, with an average (\pm standard error) of 79.8 ± 14.4 pustules/plant for single-stemmed plants ($n = 20$) and 80.5 ± 10.0 for multi-stemmed plants ($n = 19$). However, one plant in the multi-stemmed trial died during the experiment.

All inoculated plants in the coca block trial developed pustules, with an average (\pm standard error) of 20.4 ± 4.3 pustules/plant for single-stemmed plants ($n = 18$) and 43.4 ± 16.8 for multi-stemmed plants ($n = 15$). However, two plants in the single-stemmed trial and five plants in the multi-stemmed trial died during the experiment.

There was a significant interaction between sites, stem number and inoculation status in the growth rate of plants ($F_{1,131} = 9.13$, $p = 0.003$) (Fig. 4). Single-stemmed inoculated plants placed in open sunlight had a significantly lower growth rate than uninoculated plants placed in open sunlight. In addition, multi-stemmed inoculated plants placed under cocoa trees had a significantly lower growth rate than uninoculated plants placed under cocoa trees. However, there was no difference in the growth rates of single-

stemmed inoculated plants and control plants placed under cocoa trees. Nor was there any difference in the growth rates of multi-stemmed inoculated plants and control plants placed in open sunlight.

There was a significant correlation between the number of pustules and growth rate of single-stemmed plants placed in open sunlight ($r = -0.517$, $p = 0.020$) but not between the number of pustules and growth rate of multi-stemmed plants placed in open sunlight ($r = -0.360$, $p = 0.143$). In addition, inoculated single-stemmed plants in open sunlight had a significantly lower growth rate than inoculated multi-stemmed plants ($F_{2,35} = 12.29$, $p < 0.001$) (Fig. 5).

There was no correlation between the number of pustules and growth rate with respect to single- ($r = -0.343$, $p = 0.163$) and multi-stemmed plants ($r = -0.480$, $p = 0.070$) placed under cocoa-trees. There was also no significant difference in the growth rate of inoculated single- and multi-stemmed plants placed under cocoa trees ($F_{2,30} = 2.09$, $p = 0.141$). However, there was a marginally significant regression when groups (single- and multi-stemmed) were ignored ($R^2 = 9.1$, $p = 0.049$) (Fig. 6).

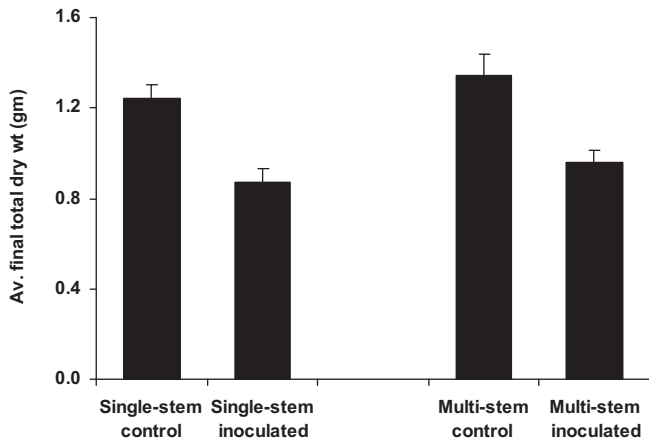


Fig. 3. The average final total dry weight (\pm s.e.) after five weeks of single- and multi-stemmed *M. micrantha* plants inoculated with *P. spegazzinii* and of single- and multi-stemmed non-inoculated plants grown in a quarantine screenhouse with ambient temperature (ranging 28–40 °C).

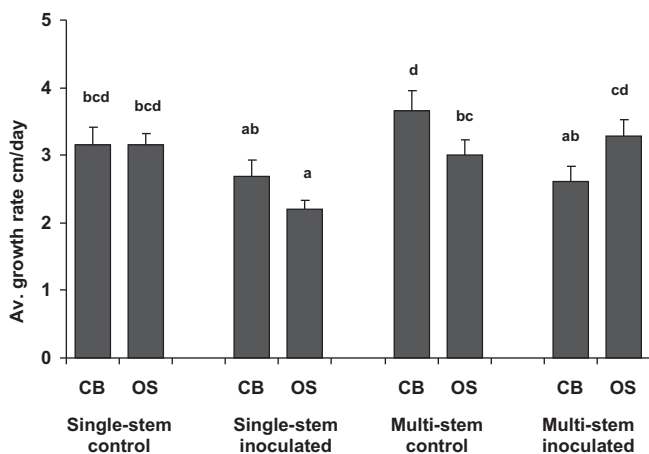


Fig. 4. The average growth rate (\pm s.e.) over five weeks of single- and multi-stemmed *M. micrantha* plants inoculated with *P. spegazzinii* and of single- and multi-stemmed non-inoculated plants when grown in open sunlight (OS) and under cocoa trees (CB). Growth rates with the same letter are not significantly different, using a Fisher's LSD test.

There was a significant interaction between sites, stem number and whether or not plants had been inoculated in the number of new nodes produced by plants ($F_{1,131} = 6.59$, $p = 0.011$) (Fig. 7). Single-stemmed inoculated plants placed in open sunlight produced significantly fewer new nodes than uninfected plants in open sunlight. In addition, multi-stemmed inoculated plants placed under cocoa trees produced significantly fewer new nodes than uninfected plants placed under cocoa trees. However, there was no difference in the number of new nodes of single-stemmed inoculated plants and control plants placed under cocoa trees. Nor was there any difference in the number of new nodes produced by multi-stemmed inoculated plants and control plants placed in open sunlight.

There was a significant correlation between the number of pustules and the number of new nodes produced by single-stemmed plants placed in open sunlight ($r = -0.496$, $p = 0.026$) but not between the number of pustules and the number of new nodes produced by multi-stemmed plants placed in open sunlight ($r = -0.415$, $p = 0.087$) (Fig. 8). In addition, inoculated single-stemmed plants in open sunlight had a significantly fewer new nodes than inoculated multi-stemmed plants ($F_{2,35} = 8.66$, $p < 0.001$).

There was a significant correlation between the number of pustules and the number of new nodes produced by multi-stemmed plants placed under cocoa trees ($r = -0.523$, $F_{1,31} = 0.045$) but not between single-stemmed plants placed under cocoa trees ($r = -0.219$, $p = 0.383$) (Fig. 9). There was also no significant difference in the number of new nodes of inoculated single- and multi-stemmed plants placed under cocoa trees ($F_{2,30} = 2.62$, $p = 0.089$). However, there was a significant regression when groups (single- and multi-stemmed) were ignored ($R^2 = 11.0$, $p = 0.034$) (Fig. 9).

There was a significant interaction between sites, stem number and whether or not plants had been inoculated in the final total dry weight of plants ($F_{1,130} = 12.93$, $p < 0.001$) (Fig. 10). Single-stemmed inoculated plants placed in open sunlight had a significantly smaller final total dry weight than uninfected plants in open sunlight. However, there was no difference in the final total dry weight of multi-stemmed inoculated plants and control plants placed under cocoa trees, single-stemmed inoculated plants and control plants grown under cocoa trees and multi-stemmed inoculated plants and control plants grown in open sunlight.

There was a significant correlation between the number of pustules and the final total dry weight of single-stemmed plants placed in open sunlight ($r = -0.531$, $p = 0.016$) but not between the number of pustules and final total dry weight for multi-stemmed plants placed in open sunlight ($r = -0.121$, $p = 0.632$). In addition, inoculated single-stemmed plants in open sunlight had a significantly smaller final dry weight than inoculated multi-stemmed plants ($F_{2,35} = 18.71$, $p < 0.001$) (Fig. 11). There was also a significant correlation between the number of pustules and the final dry weight of leaves ($r = -0.55$, $p = 0.012$) and roots ($r = -0.443$, $p = 0.050$) of single-stemmed plants placed in open sunlight when leaves, stems and roots were analysed separately.

There was no significant correlation between the number of pustules and final total dry weight with respect to single- ($r = -0.266$, $p = 0.286$) and multi-stemmed plants ($r = -0.330$, $p = 0.229$) placed under cocoa-trees. There was also no significant difference in the final total dry weight of inoculated single- and multi-stemmed plants placed under cocoa trees ($F_{2,30} = 1.77$, $p = 0.188$) or when the regression was analysed with groups (single- and multi-stemmed) ignored ($R^2 = 2.0$, $p = 0.207$).

There was no difference in the number of secondary stems produced between the inoculated and control multi-stemmed plants ($F_{1,63} = 1.65$, $p = 0.204$) and the interaction between stem density and treatment was not significant ($F_{1,62} = 2.32$, $p = 0.133$).

4. Discussion

P. spegazzinii from eastern Ecuador was the most effective pathotype against *M. micrantha* present in both PNG and Fiji and it is likely that, due to the movement of people in the Pacific and the way *M. micrantha* has spread in the region, this pathotype will also be the most effective for other countries in the Pacific. Pathogenicity testing showed that *M. micrantha* accessions from different countries have differing susceptibilities to different pathotypes of *P. spegazzinii* (Ellison et al., 2004). This suggests that *M. micrantha* was probably introduced to some countries or regions independently and probably from different areas within its native range. In India, *M. micrantha* was thought to have been introduced twice, once in the early 1900s (Choudhury, 1972) and again during World War II (Puzari et al., 2010). Pathogenicity testing showed that there were differences in the rust susceptibility of *M. micrantha* found in different parts of India, with *M. micrantha* in Assam being most susceptible to the pathotype from Peru and *M. micrantha* in Kerala being most susceptible to the pathotype from Trinidad (Ellison et al., 2004; Ellison et al., 2007).

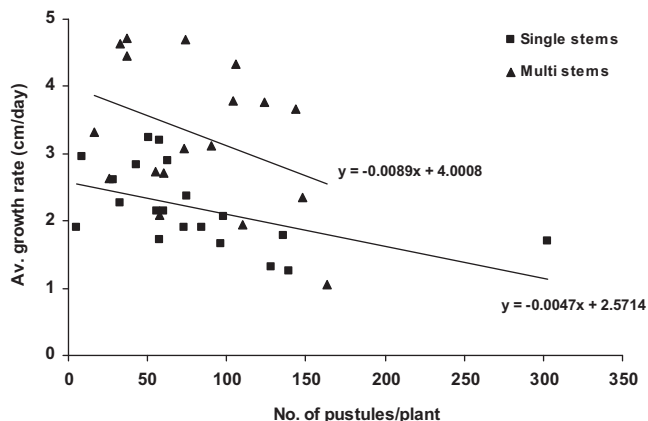


Fig. 5. The relationship between the number of pustules of *P. spegazzinii* present on *M. micrantha* and the growth rate over five weeks of single- and multi-stemmed plants grown in open sunlight.

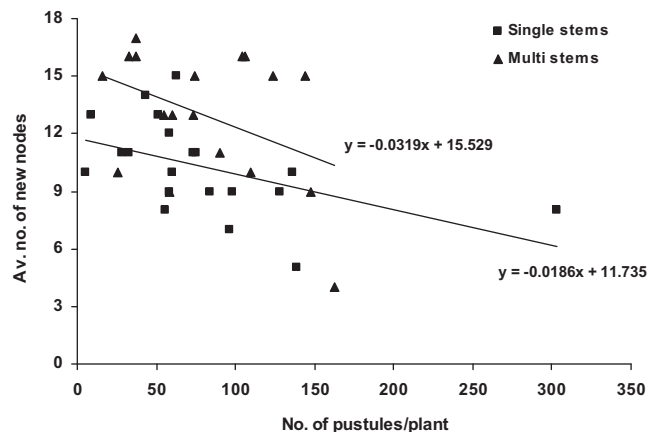


Fig. 8. The relationship between the number of pustules of *P. spegazzinii* present on *M. micrantha* and the number of new nodes produced after five weeks by single- and multi-stemmed plants grown in open sunlight.

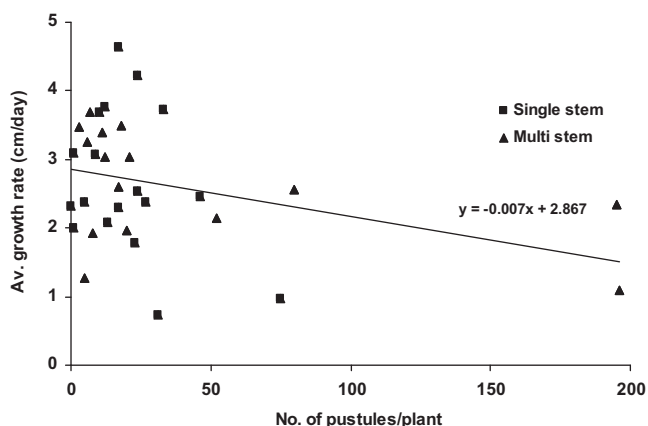


Fig. 6. The relationship between the number of pustules of *P. spegazzinii* present on *M. micrantha* and the growth rate over five weeks of single- and multi-stemmed plants grown under cocoa trees.

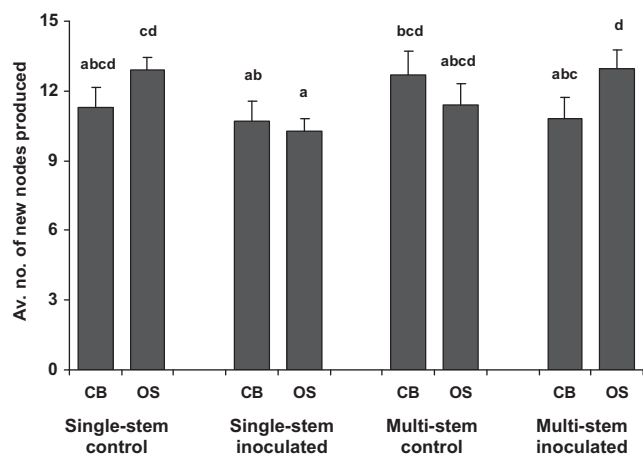


Fig. 7. The average number (\pm s.e.) of new nodes produced after five weeks on single- and multi-stemmed *M. micrantha* plants inoculated with *P. spegazzinii* and on single- and multi-stemmed non-inoculated plants when grown in open sunlight (OS) and under cocoa trees (CB). Numbers of new nodes with the same letter are not significantly different, using a Fisher's LSD test.

The east Ecuadorian pathotype of the rust was extensively screened before being released in Taiwan in 2008 and the host specificity results here confirmed the pathotype to be specific to

the genus *Mikania*, in common with all the pathotypes of this species that have been tested. The additional host specificity testing prior to release in the Pacific region focused on the interaction of the pathotype from eastern Ecuador with a selected number of important, or taxonomically-relevant plants (e.g. *Zinnia* and *B. pilosa*, which are in the same family as *M. micrantha*) from the Pacific.

Earlier testing confirmed the rust is able to infect *M. cordata*, which is native to, and found throughout Southeast Asia and parts of PNG (Holmes, 1982; Ellison et al., 2008). However, *M. cordata* is generally found in high altitude or cool areas while *M. micrantha* is found in lowland areas below 1100 m (Day et al., 2012). Following the submission of a dossier outlining the biology and host specificity testing of *P. spegazzinii* to the authorities in PNG, the rust was approved for field release. The rust was introduced into PNG in November 2008 and was cultured through one generation in quarantine prior to being field released.

Culturing methods trialled showed that plants suitable for inoculation can be easily grown in small plastic drinking cups which are readily available in PNG and many other countries. Infection was easily attained using young plants with actively growing tips, if humidity was sufficiently high to evoke the production and release of basidiospores and if there was free water on the inoculated plants for adequate time (>10 h) for infection. The success in using a simple, sealed inoculation chamber made from Perspex means that other countries, which do not have access to elaborate equipment, could easily culture the rust if required. In addition, achieving successful infection of plants in the shadehouse under natural conditions suggested that field inoculations and natural spread of the rust should be possible in PNG and other countries once the rust establishes in the field.

The comparative growth trials showed that *P. spegazzinii* can reduce the growth of *M. micrantha*, with growth rates of inoculated plants significantly lower than control plants. There were significant correlations between the number of pustules and (a) growth rate, (b) the number of new nodes and (c) final total dry weight of single-stemmed plants placed in open sunlight, as well as between the number of pustules and the number of new nodes of multi-stemmed plants placed under cocoa trees. However, in these experiments, fewer than 10 pustules were seen on some plants which suggest that even with low levels of infection, *P. spegazzinii* can have a negative impact on *M. micrantha*.

While these results are encouraging, these experiments looked at the impact of the number of pustules per plant resulting from only a single inoculation on the growth of 3–4 week old plants. Secondary infection of the inoculated plants was not observed in

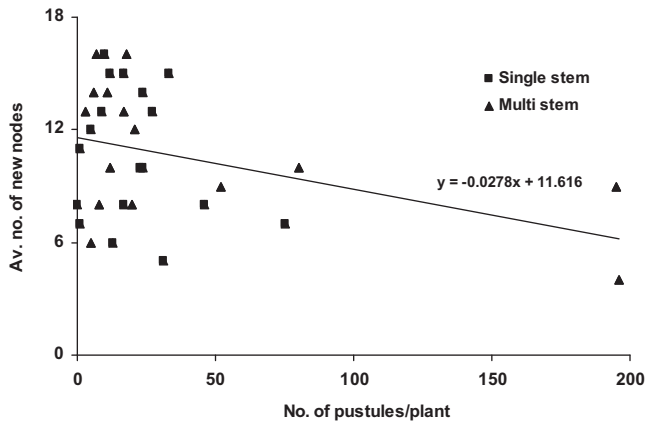


Fig. 9. The relationship between the number of pustules of *P. spegazzinii* present on *M. micrantha* and the number of new nodes produced after five weeks by single- and multi-stemmed plants grown under cocoa trees.

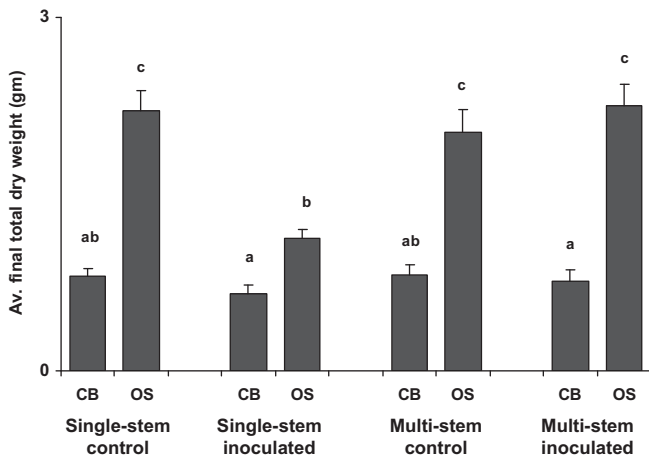


Fig. 10. The average final total dry weight (\pm s.e.) after five weeks of single- and multi-stemmed *M. micrantha* plants inoculated with *P. spegazzinii* and of single- and multi-stemmed non-inoculated plants when grown in open sunlight (OS) and under cocoa trees (CB). Dry weights with the same letter are not significantly different, using a Fisher's LSD test.

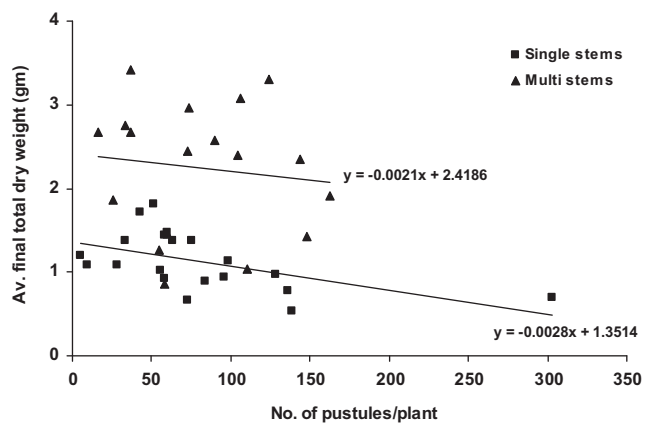


Fig. 11. The relationship between the number of pustules of *P. spegazzinii* present on *M. micrantha* and the total dry weight after five weeks of single- and multi-stemmed plants grown in open sunlight.

the present work and it is likely that prevailing conditions and the short (five weeks) duration of the experiments were contributing factors.

In the natural field situations, the age and size of *M. micrantha* plants are likely to be greater and secondary infection will occur, as established plants should receive repeated infections from surrounding infected plants over time. This will result in higher pustule density and longer infection periods than plants experienced in these experiments. Therefore, it is expected that damage to *M. micrantha* by *P. spegazzinii* in the field to be greater than seen here. Thus, *P. spegazzinii* has the potential to reduce the density of *M. micrantha*, benefitting farmers through reduced weed control and increasing food security through reduced crop losses (Day et al., 2012).

The variable results achieved in the experiments conducted in open sunlight and under cocoa trees may be due to a number of factors, including greater environmental variation under field conditions. One such factor was damage arising from attack by other organisms. For example, a large number of shoot tips were damaged, possibly due to feeding by insects despite the application of pesticides. Nevertheless, *P. spegazzinii* has been released and has established in numerous sites throughout PNG. Field releases are continuing, with regular monitoring being conducted at selected sites to determine the impact of *P. spegazzinii* on *M. micrantha*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biocontrol.2013.08.007>.

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