Journal of Nematology 33(4):239–247. 2001.
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Fergusobia/Fergusonina-induced Shoot Bud Gall Development on *Melaleuca quinquenervia*[1]

R. M. Giblin-Davis,[2] J. Makinson,[3] B. J. Center,[2] K. A. Davies,[4] M. Purcell,[3] G. S. Taylor,[4] S. J. Scheffer,[5] J. Goolsby,[3] and T. D. Center[6]

Abstract: *Fergusobia* nematodes and *Fergusonina* flies are mutualists that cause a variety of gall types on myrtaceous plant buds and young leaves. The biology of an isolate of the gall complex was studied in its native range in Australia for possible use in southern Florida as a biological control agent against the invasive broad-leaved paperbark tree, *Melaleuca quinquenervia.* Timed studies with caged *Fergusonina* flies on young branches of *M. quinquenervia* revealed that females are synovigenic with lifetime fecundities of 183 ± 42 (standard error; SE) eggs and longevities of 17 ± 2 days. None of the male flies but all dissected female flies contained parasitic female nematodes (range = 3–15), nematode eggs (12–112), and nematode juveniles (78–1,750). Female flies deposited eggs (34 ± 6; 8–77 per bud) and nematode juveniles (114 ± 15; 44–207 per bud) into bud apices within 15 days. Histological sections of shoot buds suggested that nematodes induce the formation of hypertrophied, uninucleate plant cells prior to fly larval eclosion. Enlarged size, granular cytoplasm, and enlarged nucleus and nucleolus characterized these cells, which appeared similar to those of other species galled by nematodes in the Anguinidae. Observations of ovipositional behavior revealed that female *Fergusonina* sp. create diagnostic oviposition scars. The presence of these scars may facilitate recognition of host use during specificity screening.
Key words: Australia, biocontrol, Diptera, *Fergusobia, Fergusonina,* Fergusoninidae, fly, gall development, life history, mutualism, Myrtaceae, Nemata, nematode, oviposition, Tylenchida.

Fergusobia (Currie) (Tylenchida: Neotylenchidae) nematodes are involved in an apparently mutualistic association with *Fergusonina* Malloch (Diptera: Fergusoninidae) flies (Fig. 1) that induces a variety of gall types in young meristematic/apical tissues of myrtaceous hosts in Australasia (Giblin-Davis et al., 2000, 2001a). This unique gall-forming interaction was first studied by Currie (1937) on species of *Eucalyptus.* Currently, approximately 20 *Fergusonina* fly species have been described in association with *Fergusobia* nematodes and myrtaceous plant species (Tonnoir, 1937), but many more remain underscribed (Giblin-Davis et al., 2001a). Recently, *Fergusobia/Fergusonina* galls have been found for the first time on several broad-leaved *Melaleuca* species in Australasia including *M. quinquenervia* (Cav.) S. T. Blake, a highly invasive weed in southern Florida and the Florida Everglades (Balciunas et al., 1995).

The *Fergusobia/Fergusonina* interaction involves an extremely close association between the nematodes and the flies. The nematode appears to be responsible for gall induction, and the fly for gall maintenance and for dispersal and sustenance of the nematode (Currie, 1937). The female fly deposits its eggs along with juveniles of *Fergusobia* nematodes in plant tissue (Currie, 1937). As these nematodes feed, a gall initiates and the nematodes develop into parthenogenetic females. These lay eggs giving rise to amphimictic male and female nematodes. Inseminated pre-parasitic females are infective and invade fully-grown fly larvae (third instar). They develop inside the fly into parasitic female nematodes that undergo an additional separation (apolysis) and shedding of the cuticle (ecdysis) without the development of a new cuticle. The epidermis becomes hypertrophied and the corresponding surface area is increased with large numbers of epidermal microvilli, presumably for more efficient nutrient acquisition (Giblin-Davis et al., 2001b). The nematode parasite deposits eggs into the fly's hemolymph. The juvenile nematodes that hatch from these eggs move to the oviducts of the adult fly and are deposited with her eggs into appropriate plant tissue, thus beginning the next generation. All female flies contain nematodes; males never do (Currie, 1937).

Sequence comparisons within *Fergusonina* flies (mtDNA) and *Fergusobia* nematodes (rRNA) from a variety of gall types, hosts, and geographical isolates have shown a high degree of host specificity within the Myrtaceae (Giblin-Davis et al., 2000; Scheffer et al., unpubl.). This suggests that one or more members of this complex might be useful for control of *M. quinquenervia.* Very little is known about the basic biology of the *Fergusonina/Fergusobia* complex (Davies et al., 2001; Giblin-Davis, 1993, 1999; Giblin-Davis et al., 2000, 2001a; Goolsby et al., 2000; Scheffer et al., unpubl.). We report herein results from a study of the association between *Fergusobia* sp. and *Fergusonina* sp. from *M. quinquenervia* in Australia.

Materials and Methods

Multilocule (multichamber), *Fergusonina/Fergusobia*-induced shoot bud galls with pupal windows were col-

Received for publication 19 December 2000.
[1] Contribution of the Florida Agricultural Experiment Station, Journal Series R-07914.
[2] Fort Lauderdale Research and Education Center, University of Florida/IFAS, 3205 College Ave., Davie, FL 33314.
[3] USDA Australian Biological Control Laboratory, PMB 3, 120 Meiers Rd., Indooroopilly, Queensland, AUSTRALIA 4068.
[4] Department of Applied and Molecular Ecology, Waite Campus, Adelaide University, PMB 1 Glen Osmond, South Australia, AUSTRALIA 5064.
[5] USDA-Agricultural Research Center, Systematic Entomology Laboratory, Bldg. 005, Rm. 137, BARC-W, 10300 Baltimore Ave., Beltsville, MD 20705.
[6] USDA-Agricultural Research Service, Invasive Plant Research Lab, 3205 College Ave., Davie, Florida 33314.
The authors thank Jim Plaskowitz, Systematic Botany and Mycology Laboratory, USDA, ARS, PSI, Beltsville, MD for assistance with scanning electron microscopic imaging and Susan Winewriter, University of Florida, for the photomicrograph of the ovipositing *Fergusonina* sp. fly. This project was funded, in part, by USDA-ARS Specific Cooperative Agreement No. 58-6629-9-004 from the USDA Invasive Plants Research laboratory.
E-mail: giblin@ufl.edu
This paper was edited by Parwinder Grewal.

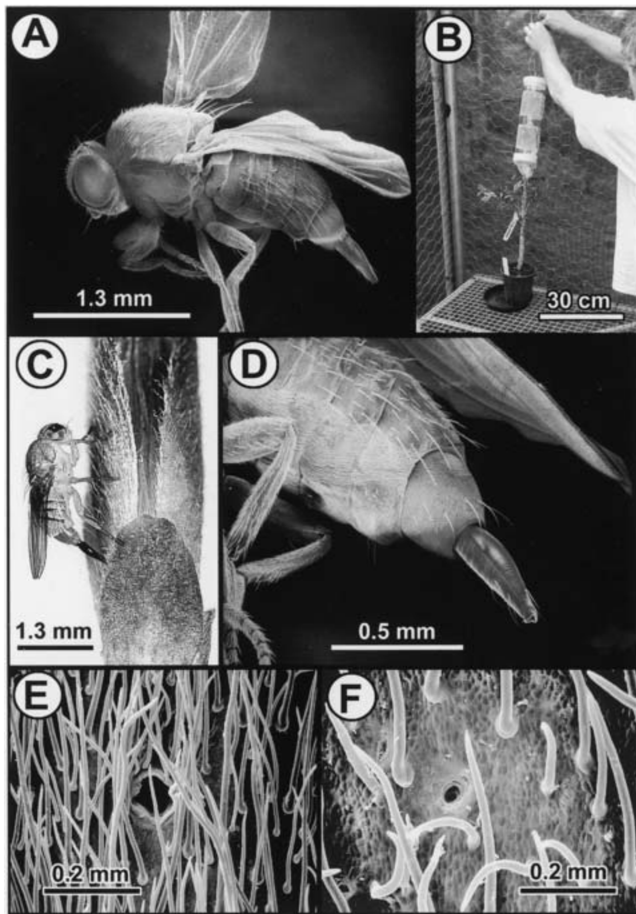

FIG. 1. Scanning electron micrographs (SEM) and light photomicrographs of study cage and adult females of *Fergusonina* sp. from *Melaleuca quinquenervia* showing ovipositor, oviposition, and oviposition evidence on a bud. A) SEM of lateral aspect of female fly with ovipositor retracted. B) Cage for confining a male and female fly onto a branch of a potted *M. quinquenervia* sapling. C) Lateral aspect of female fly ovipositing into a stage I bud. D) SEM close-up of lateral aspect of fly abdomen with retracted ovipositor. E) SEM of oviposition scar from female fly in outer leaf of stage I bud. F) SEM of oviposition scar from female fly in first inner leaf of stage I bud.

lected from *M. quinquenervia* at Stradbroke Island, Queensland (27° 29′45″S, 153° 30′53″E), during May to August 1999. Each gall was set up at room temperature in a capped plastic shell vial (3-cm diam. × 7 cm high) and monitored twice daily for adult *Fergusonina* sp. fly emergence. Newly emerged male and female flies were aspirated from the emergence vial and then placed in pairs in a plastic shell vial with a drop of honey:water solution (1:10). About 12 hours later, they were released into screened confinement cages that contained young shoots of potted *M. quinquenervia.* The cages with flies and plants were held under screenhouse conditions. In some cases, time-to-fly egg maturation was measured by dissecting newly emerged female flies kept with males at 12 to 24-hour intervals after emergence until 100 hours post-emergence. Male flies were dissected to determine the presence of nematodes.

The 4-m^3 screen house consisted of a fine-screened (185-µm opening) mesh to prevent the introduction of exogenous flies, parasites, or pests. Plants were 1 to 2-year-old saplings that had been established and maintained in 14-cm-diam. × 13-cm-high pots. Pots were watered daily and fertilized as needed. Branches were pruned from plants several weeks before the initiation of an experiment to induce new shoot bud development. Branches to be enclosed and exposed to a *Fergusonina* pair were photographed with a digital camera, and shoot buds were numbered by position, measured with calipers, and staged. Buds were staged as follows: 0 to 5 mm were classified as 00, 5 to 10 mm were classified as 0, greater than 10 mm with leaf bracts were classified as I, and buds greater than 10 mm without bracts were classified as II. Small, cylindrical (9-cm-diam. × 30 cm long) screened confinement cages constructed from 5-mil acetate sheeting with four 7 × 8-cm nylon mesh-covered windows (185-µm hole opening) and wax-coated cardboard container lids were used to retain flies over the chosen branch (Fig. 1B). A radius cut was made in one of the container lids, and a hole was bored in the center. This cardboard lid was gently placed around the base of the branch to be confined, the radius cut covered with tape, and the hole around the branch sealed with cotton. The lid at the distal end had a 6-cm-diam. nylon mesh window. The confinement cage was then assembled around the rest of the branch and suspended from an armature in the screen house using cotton guidelines to prevent it from breaking the branch (Fig. 1B). Daily maximum-minimum temperature readings were taken each morning. Flies were observed twice daily for morbidity and fed a (1:10) honey/water solution as needed in a drop through the mesh windows.

Random observations were made to determine general ovipositional behavior using a video camera for documentation. The screened enclosure was removed after both adults had died, and the plant was monitored for subsequent gall development until harvest time. Flies were transferred to a new plant for continual observation through the duration of their lives if the plant was harvested prior to their demise. Data collected included fly longevity, fecundity, oviposition behavior, starting and ending bud stage, number of ovipositional scars, fly eggs or other stages and nematodes (and stage) in each bud, and gall development. Gall development was assessed by destructive harvests of complete plants at timed intervals (15, 20–21, 25, 30–31, 35, 44, and 65–66 days after initiation of the experiment). Five or more cages were established three times between May and August 1999.

Lifetime fecundity estimates for the flies were made by counting the total number of eggs recovered from dissected buds from each cage. Only buds that were caged for fewer than 35 days were used in these estimates because eggs were more difficult to recover as the

gall developed and because of the possibility of larval cannibalism after eggs hatched. An interpolated average was used in cases where buds were not dissected (i.e., buds for sectioning).

The number, location, and stage of flies and nematodes were recorded from measured, staged, and dissected buds, and several buds/galls were arbitrarily fixed in FAA (formalin, acetic acid, ethanol; 5:5:90) retained for scanning electron microscopy (SEM) or dehydrated in a tertiary butyl alcohol series and embedded in paraffin as per the method of Johansen (1940). Embedded buds/galls were sectioned 11 µm thick, mounted on slides treated with Mayer's albumin (50 ml fresh egg albumin, 50 ml glycerin, 1 g sodium salicylate), stained with 1% aqueous safranin and 0.5% fast green in clove oil and 100% ethanol (1:1), and then examined and photographed with a compound photomicroscope. For SEM, two adult female flies that had been killed and held in 95% ethanol and several *M. quinquenervia* buds that had been exposed to flies for 15 days were taken from the FAA fixative, rinsed twice in distilled water, post-fixed in 4% aqueous OsO_4, and run through an ethanol series to 100%. The specimens were dried in a critical-point drying apparatus using CO_2 as the transitional fluid, mounted on stubs using double sticky tape, coated with gold-palladium, and viewed with a JEOL T300 scanning electron microscope at 15 kV.

Oviposition scars and fly eggs were measured with a camera lucida. In most cases, female flies were dissected after they died to count the remaining fly eggs and the number and stages of nematodes retained.

Field observations were made on 223 arbitrarily collected buds from Stradbroke Island, Queensland, on 16 July 1999. Ovipositional scars were counted (if present), and buds/galls were dissected and observed for fly and (or) nematode development.

Four saplings of *M. quinquenervia* were used to see if injected nematodes would establish galls and develop without flies in various stages of buds. Plants were grown as described above. Immediately prior to treatment, the plants were photographed and shoot buds were numbered and measured. Buds were paired according to stage and arbitrarily assigned a treatment (with or without injected *Fergusobia* sp. juveniles). Injections were done with a Becton-Dickinson and Co. (Rutherford, NJ) 1-ml Tuberculin syringe with a 26G ½ non-toxic, pyrogen-free needle. Recently emerged (<48 hours old) female *Fergusonina* sp. flies from Stradbroke Island, Queensland, were dissected without rupturing the ovaries into phosphate buffered saline (PBS) (pH 7.4) and distilled H_2O in a 1:1 ratio. The fly, her ovaries, and eggs were removed, leaving nematode juveniles and eggs, which were quantified and taken up into a single drop (4–6 µl total volume with 20 to 75 nematode juveniles) into a primed syringe and injected into the apical region of one of the pair of similar-sized buds. The other bud received an injection of an equivalent volume of the carrier fluid without nematodes. About half of the inoculations were executed by penetrating the outer leaves about one third of the way up from the bud base into the apical region; the other half of the inoculations were done by pushing the tips of the leaves apart with the needle and penetrating to the apical region. Paired treatments were injected in the same manner. Plants were maintained in the screen-house for 28 to 35 days post-inoculation, and buds were measured, staged, dissected, and examined for nematode development and gall formation. A total of 34 stage I and four stage 0 buds were injected.

Results and Discussion

Based on field observations, May to August is the optimum time for gall development on *M. quinquenervia* (Goolsby et al., 2000). All of the research reported from this study was accomplished from May to August during the austral winter. The mean daily maximum and minimum temperature readings in the screen-house during the course of the experiments were 25.8 ° (33.0–17.5 °C) and 11.2 ° (18.0–4.5 °C), respectively. The mean daily maximum and minimum temperature readings from a nearby weather station were 21.0 ° (26.5–17.0 °C) and 9.6 ° (16.5–2.0 °C), respectively.

Fergusonina sp. association with Fergusobia sp.: Dissections of newly emerged *Fergusonina* sp. from galls on *M. quinquenervia* revealed that none of 17 male flies (<96 hours after emergence) had *Fergusobia* sp. present within the hemocoel. This is consistent with Currie's (1937) observations for *Fergusobia* spp. associated with *Eucalyptus* spp. and Davies et al.'s (2001) observation of no parasitic nematodes associated with male *Fergusonina* sp. from *M. quinquenervia* larvae or pupae. None of 12 female flies that were <48 hours old had mature fly eggs present in the ovarioles, but they did have a mean of 153 ± 11 (standard error; SE) (range = 93–219) immature ova. Female flies (n = 6) 72 to 96 hours after emergence had 97 ± 26 (27–205) eggs present, suggesting a 72-hour egg maturation period. The average mature parasitic female *Fergusobia* sp. burden from the abdomen of female flies <96 hours after emergence was 9 ± 1 (4–15) (n = 17), similar to numbers observed by Davies et al. (2001). In all cases, including two paraffin-sectioned female flies, hundreds to thousands of infective juveniles and many eggs were present in the hemocoel of the abdomen (Fig. 2) and juveniles were observed in the fly ovaries (Fig. 2A). No nematodes were observed in the head or thorax. In several cases, it appeared as if there were two juvenile size classes, suggesting a molt. The fly reproductive system and organs appeared healthy but, because none of the females were without nematodes, it was difficult to assess the impact

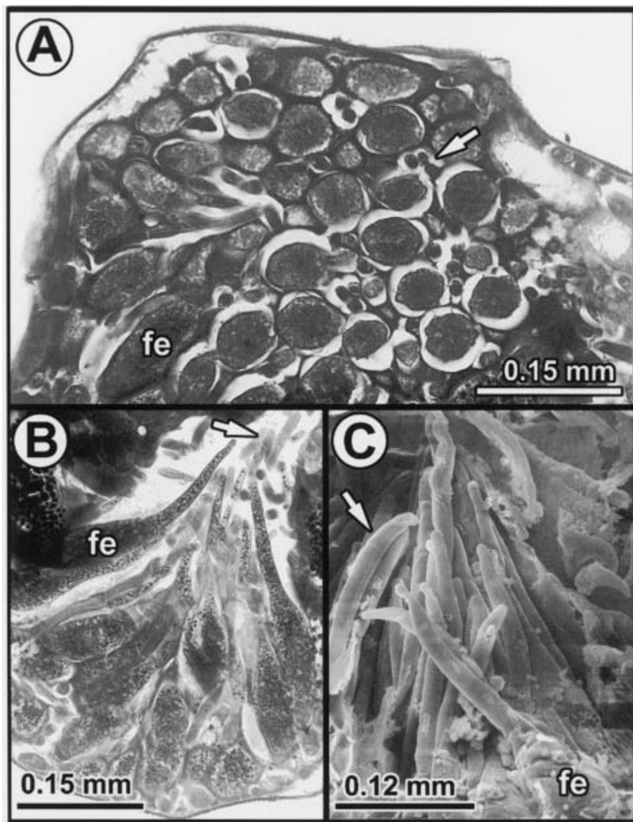

FIG. 2. Scanning electron micrographs (SEM) and light photomicrographs of the abdominal body cavity of female *Fergusonina* sp. showing fly ovary, eggs, and *Fergusobia* sp. nematode juveniles. A) Transverse histological section showing nematode juveniles (arrow) in the hemocoel and ovary of the fly. B) Longitudinal section of fly abdomen showing fly eggs (fe) and nematode juveniles. C) SEM of fly abdomen that was critical-point dried and cracked open to expose fly eggs and nematode juveniles.

of the parasitic phase of *Fergusobia* sp. Recent dissections of a cohort of flies in quarantine in Florida have suggested that stressing factors may change the balance of the relationship favoring the nematodes and disfavoring the flies. Nematodes were present in the thorax, head, and abdomen of several unproductive female flies that were dissected and examined, and the ovaries and organs appeared severely depleted (Wineriter and Boucias, unpub.).

Fergusonina sp. ovipositional behavior: The ovipositional behavior of 10 female *Fergusonina* sp. flies from *M. quinquenervia* was observed to look for signs or symptoms that might prove useful in future host-range studies evaluating the *Fergusonina/Fergusobia* complex for release into Florida. Oviposition occurred between 9:30 am and 5:00 pm, starting about 48 hours after caging. Although ovipositional activity seemed most intense during the first week or two after caging, it was observed throughout the life of some females. The basic behavior was for the female to alight near the base of a stage I bud and begin walking up and down the basal third of the outer leaf (a distance of about 6 mm). The walking was usually accompanied with dorsal-ventral movements of the head. After moving up and down several times (usually about 5 runs each direction), she would position herself with her head facing upward (distally) on the bud about one third of the way up from the base of the bud. She would straddle the leaf, extend her ovipositor (Fig. 1A,C,D), and insert it into the bud, presumably in the act of oviposition (Fig. 1C). This could last from 30 seconds to more than 10 minutes, and the ovipositor was often reinserted 1 to 4 times before final retraction. The female sometimes flew off at this point but usually resumed, with the up-and-down walking often accompanied with leg or face-cleaning behaviors on the same bud. Examination of the ovipositional site revealed one or more puncture holes (newly formed ovipositional scars) (Fig. 1D,E). These would darken over the next few days and, as outer leaves unfurled, would eventually form diagnostic small holes. Measurement of the fly egg diam. (61.3 ± 1.3 µm; CV = 9.8; range = 55.3–78.9 µm; [n = 20]) and the diam. of recent ovipositional scars (80.7 ± 3.9 µm; CV = 25.4; range = 36.8–142.1 µm [n = 28]) confirm that these marks are the result of ovipositor damage. On average, eggs are 4.3 times as long as wide and have a characteristic pointed, elliptical appearance (Fig. 2B,C). Females would usually string the above-described behaviors together for several times on the same bud, often alternating oviposition on opposite sides of the bud. A single series of ovipositing events could last more than 30 minutes on a single bud. Sometimes a female would then move to another bud and start again. We observed that the same female could oviposit onto the same bud on subsequent days, suggesting that inhibitory marking secretions may be absent. This needs to be tested with different female specimens and confirmed with egg-marking methods. This behavior also could be an artifact of the confinement cages. We never observed the males interfering with the females, and mating was not observed in the cages.

Fergusonina sp. longevity, fecundity, and association with Fergusobia sp. at death: The longevity of *Fergusonina* sp. from *M. quinquenervia* in the caged experiment was 17 ± 2 days (5–36 days) for females (n = 21) and 17 ± 2 days (0.25–34 days) for males (n = 20). Females that were dissected at death had 142 ± 27 unspent mature eggs in their ovaries. In addition, there were 5 ± 1 (3–9) live parasitic female nematodes (n = 12), 54 ± 9 (12–112) nematode eggs (n = 13), and 566 ± 145 (78–1,750) live nematode juveniles (n = 13). Mean fly age at death (for those flies that were dissected) was 19.5 days (n = 13). Different nematode juvenile size classes occurred inside the fly, suggesting a molt and perhaps parasitism. This needs to be confirmed by further study. The fly egg and nematode burdens at death approximated those of flies dissected at <96 hours old, suggesting sustained fly egg production (synovigenic vs. proovigenic) and nematode deposition throughout the life of the fly. Lifetime fecundity was 183 ± 42 eggs (65–344) (n = 7).

Association of Fergusonina/Fergusobia complex with buds of Melaleuca quinquenervia: The quantified association of *Fergusonina* sp. and *Fergusobia* sp. stages with branches and buds of *Melaleuca quinquenervia* after timed exposures to a confined pair of newly emerged *Fergusonina* sp. adults and after destructive harvesting is presented in Table 1. In addition, the percentages of different staged buds of *M. quinquenervia* with oviposition scars, fly eggs, or nematode juveniles from these destructive harvests are presented in Figures 3 and 4. The percentage of *Fergusobia* sp.-infested original buds (34–54%) and mean number of ovipositional scars per bud (9–37%) were similar regardless of the timed exposure to flies (Table 1). The mean number of fly eggs (≥24) and nematode juveniles per bud (≥85) appeared higher in the first 25 days of exposure to flies than in subsequent harvests (≤11 and 22, respectively) suggesting survivorship or recovery problems (Table 1). In general, there was proportionately more or equal ovipositional scarring, fly egg deposition, and nematode juvenile presence in stage I, 0 and unpresented 00 buds than in 00 buds (Figs. 4; 5).

Dissections and paraffin cross-sections at 15 days after caging showed that nematode juveniles and multiple fly eggs are deposited into the area directly above the terminal or axial shoot tip (the youngest part of the shoot including apical meristematic cells associated with leaf or inflorescence primordia) (Figs. 5; 6A,B,D,E). After 15 days (which could represent 0–15 days after oviposition), the tissues and cells near the point of nematode and fly deposition looked like non-infested shoots. At 20 to 21 days, results were similar with no nematode development or fly egg eclosion, but there was evidence of some fusion of primordial leaves around the shoot tip where nematodes were present (Fig. 6C,F). At 25 days, there was still no nematode development or fly egg eclosion but there was some fusion of primordial leaves around the shoot tip with nematodes (Fig. 7A,D). At 30 to 31 days, there was still no nematode development or fly egg eclosion. There was some fusion of primordial leaves around the shoot tip and granulated cytoplasm of cells near nematodes. In some cases, bud necrosis was observed associated with dead fly eggs and nematode juveniles (Fig. 8A,D). Some of the *Fergusonina*-infested buds at 30 to 31 days had an outward appearance of fusion when inspected with the dissecting scope. The first evidence of bud swelling with primordial leaf fusion occurred at 35 days. This was accompanied with proliferation of ground parenchymal cells with irregular-shaped pockets lined with one to two layers of hypertrophied cells (enlarged nucleus and nucleolus and granular cytoplasm) (Fig. 7B,C,E,F). These pockets of cells roughly corresponded to unfused areas of primordial or young leaves in the region of the bud apex and were often filled with many unhatched fly eggs and nematodes (Fig. 7B,C,E,F).

At 44 days after caging, nematodes were present in all

TABLE 1. Percentage association of *Fergusonina* sp. and *Fergusobia* sp. stages with caged *Melaleuca quinquenervia* plants and buds after different timed exposures to a confined pair of newly emerged flies.

Days after exposure to flies	Number of plants		% infested buds (n)		Mean no. per infested bud ± S.E. (range) with:					
	Tested	Infested	Original[a]	Total[b]	Ovipositional scars	Fly eggs	Fly larvae	Nematode juveniles	Parthenogenetic female nematodes	Nematode eggs
15	3	2	47 (32)	45 (33)	14 ± 3 (2–46)	34 ± 6 (8–77)	0	114 ± 15 (44–207)	0	0
20–21	2	1	53 (17)	54 (24)	15 ± 4 (1–37)	26 ± 7 (8–69)	0	85 ± 13 (21–148)	0	0
25	1	1	50 (20)	48 (23)	32 ± 5 (1–55)	24 ± 5 (5–44)	0	90 ± 20 (11–187)	0	0
30–31	2	2	52 (23)	50 (26)	24 ± 5 (5–50)	11 ± 2 (1–21)	0	17 ± 2 (5–23)	0	0
35	1	1	55 (11)	64 (14)	37 ± 10 (3–87)	11 ± 4 (3–32)	0	22 ± 9 (5–66)	0	0
44	1	1	54 (13)	28 (25)	9 ± 2 (4–22)	3 ± 1 (1–4)	0	5 (3–7)	4 (1–7)	0
66	6	5	34 (59)	24 (82)	11 ± 4 (1–38)	2 ± 1 (1–2)	1 ± 0 (1)	7 ± 3 (1–15)	9 ± 3 (1–20)	11 ± 6 (1–37)

[a] Buds that were originally observed and measured at start of experiment.
[b] Buds that were observed and measured at destructive harvest.

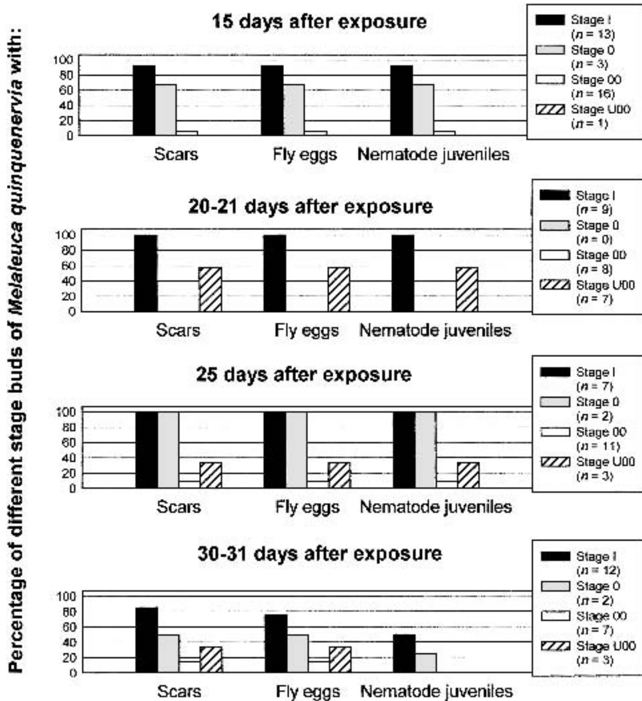

FIG. 3. The percentages of *Melaleuca quinquenervia* buds at different developmental stages with oviposition scars, fly eggs, or nematode juveniles from destructive harvests after 15, 20 to 21, 25, or 30 to 31 days of confinement with a female and male of *Fergusonina* sp. Stage I = buds >10 mm with leaf bracts; Stage 0 = 5 to 10 mm; Stage 00 = 0 to 5 mm; Stage U00 = 0 to 5 mm that were not visible at the onset of the experiment but were visible at harvest.

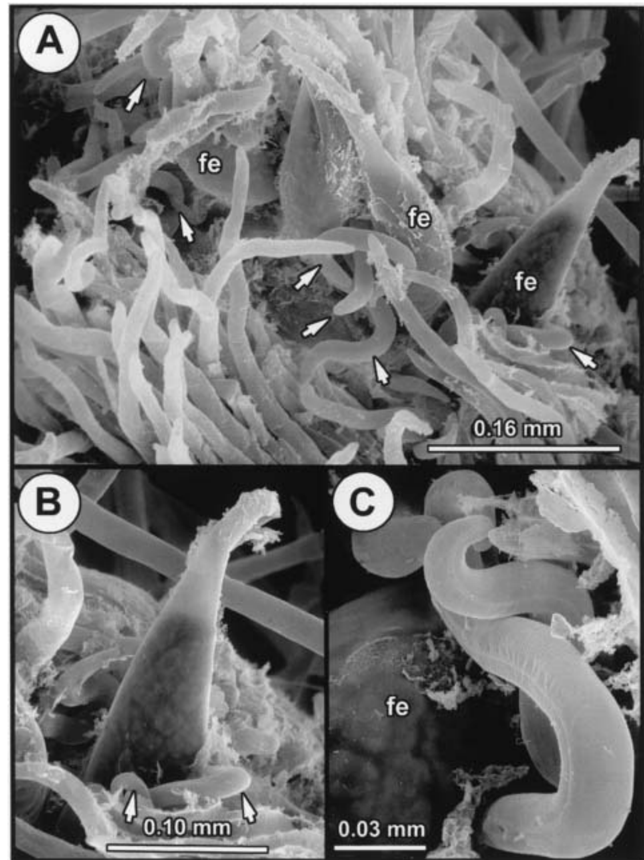

FIG. 5. Scanning electron micrographs (SEM) of a stage I axial shoot bud of *Melaleuca quinquenervia* that was confined for 15 days with a female and male *Fergusonina* sp. that was critical-point dried and cracked open to expose the apical region with newly deposited fly eggs and nematode juveniles. A) Fly eggs (fe) and nematode juveniles (arrows) among inner leaf hairs. B) Close-up of fly egg with nematodes at base in panel A. C) Close-up of nematode juveniles at base of fly egg.

of the infested and swelling buds as parthenogenetic females and juveniles (Table 1). Swollen buds were more robust than those observed at 35 days and had primordial leaf fusion and proliferation of ground parenchymal cells with irregular-shaped pockets, which were lined with one to three layers of hypertrophied cells (Fig. 8B,E). These pockets of cells usually surrounded one to a few unhatched fly eggs and the parthenogenetic female and juvenile nematodes. At 66 days, observed galls had primordial leaf fusion and proliferation of ground parenchymal cells with elliptical-shaped locules (chambers) lined with one to five layers of hypertrophied cells (Fig. 8C,F). These pockets of cells appeared as friable white callus in dissections and stained darkly in paraffin sections. The more regular locule shape and an increase in granulation of hypertrophied cells was roughly coincident with the hatch of the first-stage fly larva (Table 1), suggesting that the fly may contribute at this point to the maintenance or modification of the locule. At 66 days, only one fly larva and parthenogenetic female(s) and juvenile nematodes was present in each locule (Table 1), suggesting cannibalism or some other mechanism for isolating individual flies. These data are consistent with observations made by Davies et al. (2001), where parthenogenetic females (2 ± 1; 1–3) and juveniles were associated with

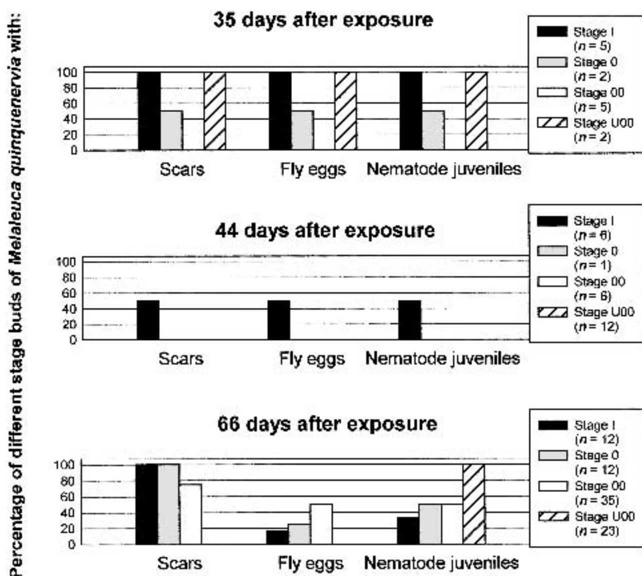

FIG. 4. The percentages of *Melaleuca quinquenervia* buds at different developmental stages with oviposition scars, fly eggs, or nematode juveniles from destructive harvests after 35, 44, or 66 days of confinement with a female and male of *Fergusonina* sp. (see Fig. 3 for legend).

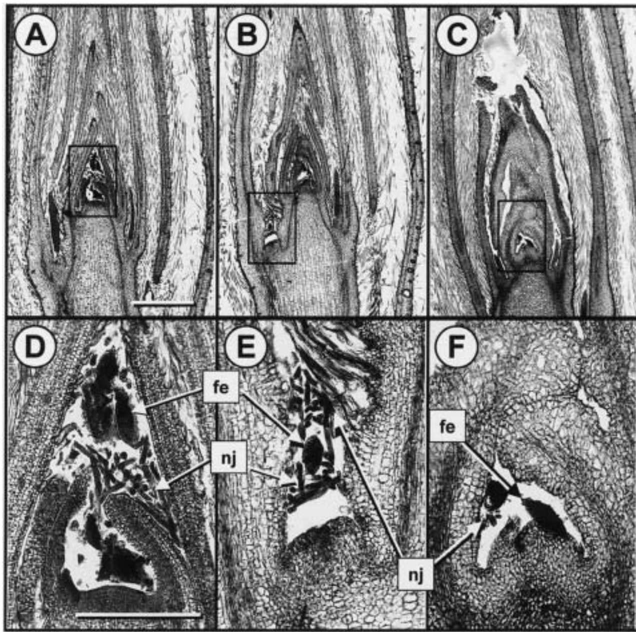

FIG. 6. Histological sections of the terminal apical region of *Melaleuca quinquenervia* stage I buds after exposure to a caged pair of *Fergusonina* sp. for pre-determined time periods. A) Terminal apical region infested with *Fergusobia* sp. nematode juveniles and fly eggs 15 days after caging. B) Same bud as in panel A with unpresented axial bud infested with nematode juveniles (nj) and fly eggs (fe). C) Terminal apical region infested with nematode juveniles and fly eggs 20 days after caging. D) Close-up of boxed portion of panel A. E) Close-up of boxed portion of panel B. F) Close-up of boxed portion of panel C. A, B, and C bar = 500 µm; D, E, and F bar = 200 µm.

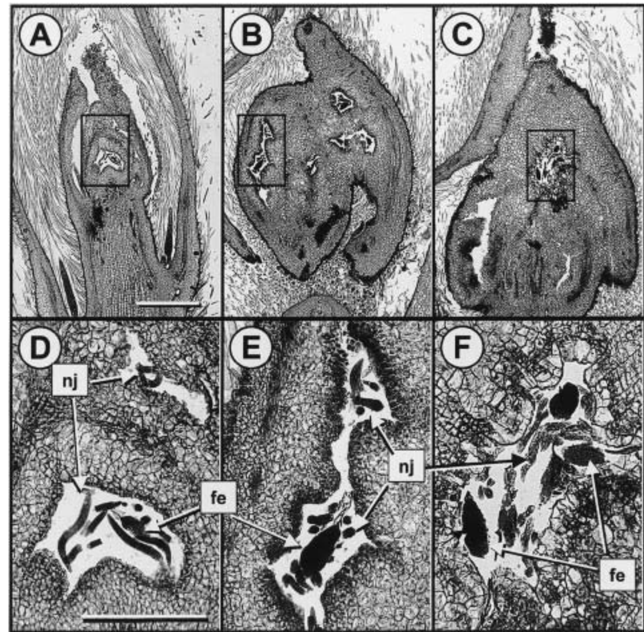

FIG. 7. Histological sections of the terminal apical region of *Melaleuca quinquenervia* stage I buds after exposure to a caged pair of *Fergusonina* sp. for pre-determined time periods. A) Terminal apical region infested with *Fergusobia* sp. nematode juveniles and fly eggs 25 days after caging. B) Terminal apical region infested with nematode juveniles (nj) and fly eggs (fe) 35 days after caging. C) Same bud but different section as in panel B with bud infested with nematode juveniles and fly eggs. D) Close-up of boxed portion of panel A. E) Close-up of boxed portion of panel B. F) Close-up of boxed portion of panel C. A, B, and C bar = 500 µm; D, E, and F bar = 200 µm.

first instar fly larvae, and male nematodes were not observed until the development of the second instar fly larva (neither observed within 66 days in this study).

In summary, the shoot bud gall created by the *Fergusonina/Fergusobia* complex from *M. quinquenervia* seemed to occur after an introduction of nematode juveniles and fly eggs into the spaces around the primordial buds during oviposition. Nematode juveniles apparently induced hypertrophied cells between young leaves that created a pocket around groups of fly eggs and nematode juveniles as the rest of the leaf surfaces fused. It is not clear if each pocket was due to a terminal or unpresented axillary bud or if multiple pockets were created from a single primordial bud.

Currie (1937) suggested that newly eclosed *Fergusonina* sp. fly larvae from *Eucalyptus macrorhyncha* F. Muell. Ex Benth. flower bud galls cut out small crypts between apposed masses of hypertrophied anther cells, and nematodes then aggregate around the fly as the locule becomes fused around them. A similar scenario is possible with *M. quinquenervia* shoot bud galls. Each first instar larva could be a focal point for individual locule development around itself and a cohort of nematodes. Cecidogenic secretions or the physical act of movement and feeding by the newly eclosed fly larva may stimulate the nematode-induced hypertrophied cells lining the pockets around nematodes and fly eggs. Stimuli from this event could be critical to downstream development of the amphimictic generation of nematodes that lead to inseminated infective female nematodes that penetrate the third instar fly larva. Buds where flies fail to develop often become leaf curls and not multilocule galls, suggesting that the fly larva is necessary for the complete manifestation of a single or multilocule gall (Davies et al., 2001). It is also possible that cecidogenic compounds are deposited at the time of oviposition or the physical act of oviposition by the female fly serves as the sole or partial inducer of hypertrophied cells prior to fly egg eclosion. However, the morphology of the hypertrophied cells in pockets around fly eggs in infested buds (Fig. 8E) is very similar to that described for tylenchid nematodes that induce seed galls in the Anguinidae (e.g., *Anguina agrostis* [Steinbuch] Filipjev), suggesting that nematodes are responsible for gall initiation (Stynes and Bird, 1982).

In a recent field study, gall density of *Fergusonina* sp. from *M. quinquenervia* increased in abundance in the winter (August/September) about 2 months after seasonal flushes of growing buds. For this reason, a 2-month interval was used as the estimated length of the life cycle (Goolsby et al., 2000). Balciunas et al. (1995) estimated the time from egg to adult for *Fergusonina* sp. from *M. quinquenervia* to be about 6 weeks. These times are similar to the time observed from cag-

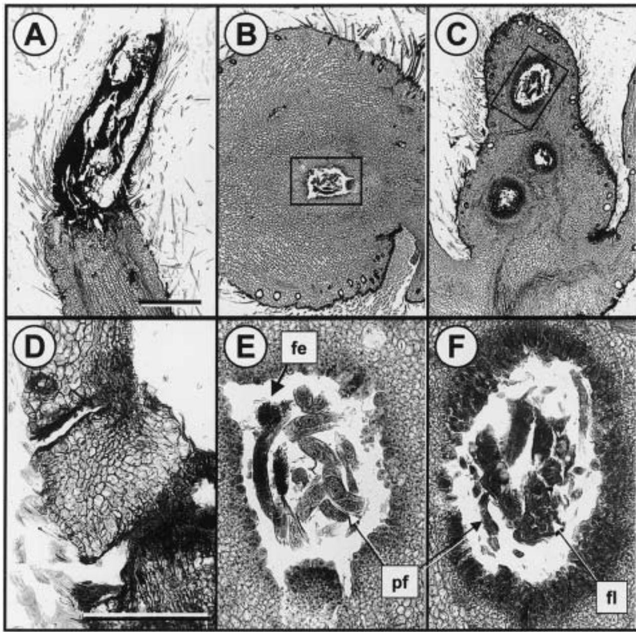

FIG. 8. Histological sections of the terminal apical region of *Melaleuca quinquenervia* stage I buds after exposure to a caged pair of *Fergusonina* sp. for pre-determined time periods. A) Terminal apical region infested with *Fergusobia* sp. juveniles and fly eggs 30 days after caging showing necrosis. B) Terminal apical region infested with nematode juveniles and fly eggs 44 days after caging. C) Terminal apical region infested with nematode juveniles and fly eggs 66 days after caging. D) Close-up of same bud as in panel A showing oviposition holes. E) Close-up of parthenogenetic female nematodes (pf) and fly egg (fe) from panel B. F) Close-up of locule with parthenogenetic and juvenile nematodes and first instar fly larva (fl) in C. A, B, and C bar = 500 μm; D, E, and F bar = 200 μm.

ing to the early stage of gall formation (presence of first instar fly larvae) in this study. Unfortunately, the time to adult fly eclosion was not observed because destructive harvesting was done prior to completion of the life cycle. Time to adult emergence might be another 2 to 6 weeks (total time 10 to 14 weeks), suggesting that the time from egg to adult may be closer to 90 days. If so, the flies attack buds at about a month or so prior to growth in the field.

Fergusonina sp. from *M. quinquenervia* populations can be hindered by heavy parasitism from at least eight species of wasp parasites (Davies et al., 2001; Goolsby et al., 2000). Our study demonstrated that confined females of *Fergusonina* sp. that were released from heavy parasitism were quite successful at ovipositing in growing stage I buds (92% infestation rate [$n = 52$]). However, only 25% of stage I buds with ovipositional scars produced young galls by 65 to 66 days. Some of the attrition can be explained by bud mortality due to overexploitation, an artifact of the confining cage. *Fergusonina* sp. also attacked stage 0 and 00 buds that were unseen at the onset of the experiment (Figs. 4; 5). The infrequent attack of stage 00 buds may be due to the fact that these buds were often in a dormant state and did not grow during the experiment (Figs. 4; 5). This is similar to field observations that showed that the small reddish, unexpanded and dormant buds were not galled (Goolsby et al., 2000). Also, *Fergusonina* sp. from *M. quinquenervia* did not attack individual flower buds on inflorescences. Thus, for culture or no-choice host testing of this complex, it appears that expanding shoot or inflorescence buds in stages 00, 0, and I are required with a preference for stage I buds (Figs. 4; 5).

There was no difference between the number of ovipositional scars or length of bud at the start of the experiment for terminal vs. axial buds when stratified by stage for the five fly-positive, 65 to 66-day caged plants ($P > 0.1$). There was also no difference between the numbers of ovipositional scars, lengths of buds at the start of the experiment, numbers of fly eggs, or numbers of nematode juveniles per bud for terminal vs. axial buds when stratified by stage for the seven fly-positive, 15 to 35-day caged plants ($P > 0.1$). There was a positive correlation between the numbers of fly eggs per bud and the numbers of nematode juveniles per bud in stage I buds from 15 to 35-day harvests ($P = 0.0001$; $n = 30$) but not for other bud stages or between these variables and bud lengths at start or numbers of ovipositional scars per bud. This significant positive correlation is consistent with the hypothesis that nematode deposition into the shoot tips is linked to fly oviposition. It is assumed that nematodes are passively deposited with eggs during oviposition based on sections and dissections of the fly ovaries (Currie, 1937).

Twelve percent (28/233) of dissected buds from recently expanded shoots of *M. quinquenervia* collected from Blue Lake Beach on Stradbroke Island, Queensland, in July 2000 (stages 0-I) had 3 ± 1 (1–10) ovipositional scars, and 64% of these scarred buds ($n = 18$) had galls and *Fergusobia* sp. and (or) *Fergusonina* sp. present. In some cases, there were ovipositional scars on outer leaves but no evidence of the *Fergusonina*/*Fergusobia* complex and the bud had been aborted. These data are consistent with the observations in the screen-house, suggesting that ovipositional scars can be used to assess host selection by *Fergusonina.* As with the screen-house data, there was no correlation between the number of scars and the number of progeny present in successful galls. In one example from the field, there was one ovipositional scar associated with 17 *Fergusonina* sp. larvae in a multilocule (separate-chambered) gall. The ratio of ovipositional scars to number of progeny may be related to a variety of factors, including ovipositional experience of the female, multiple oviposition events by the same or more than one female, fecundity, and mating status of females, among others.

The results from injections of *Fergusobia* sp. into *M. quinquenervia* shoot buds were inconclusive. Nematodes were not recovered from any of the buds except for one after 28 to 35 days, and there was little evidence of galling or bud fusion. In the one stage I bud with a *Fergusobia* sp. inside, it was a recently dead male in bud

tissue that appeared slightly deformed. Unfortunately, control injections without nematodes were sometimes associated with deformation because the needle apparently injured the apical area. The presence of the male suggests that at least one parthenogenetic female had successfully developed from injected juvenile nematodes. In addition, a few injections were tried with nematodes and *Fergusonina* sp. eggs or *Fergusonina* sp. eggs alone without success (Giblin-Davis, unpub.) Earlier attempts by other researchers to inject *Fergusobia* from *E. macrorhyncha* into its flower buds also failed (Currie, 1937). Thus, it remains unclear whether the fly or the nematode is the cecidogenic agent in gall formation by the *Fergusonina/Fergusobia* complex.

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