Root and Stem Rot of Parrotfeather (*Myriophyllum brasiliense*) Caused by *Pythium carolinianum*

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ABSTRACT

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A disease of the emersed aquatic weed parrotfeather was observed during 1980 and 1981 in a drainage ditch in northern California. Diseased plants showed a severe rot of underwater roots and stems and frequently wilted and collapsed. An isolate of *Pythium carolinianum* from diseased plants was pathogenic to parrotfeather cuttings. Mycelium inserted into parrotfeather stems girdled the stems and caused collapse of the shoots. Zoospore inoculations of plants in solution culture resulted in root rot and suppressed growth. Parrotfeather grown in basins in the field at Davis, CA, and inoculated with the pathogenic isolate produced significantly less biomass and fewer shoots than did uninoculated controls.

The emersed aquatic weed parrotfeather (*Myriophyllum brasiliense* Camb.) is an increasing problem in California irrigation systems. Originally introduced from South America as an aquarium plant, parrotfeather has escaped to waters in much of the southern United States (21) and in other countries (5,19). Parrotfeather is rooted in the hydrosoil and produces leafy shoots that extend above the water (18). It can grow in water as deep as 2 m where its submerged stems and roots impede water flow and hinder navigation (8,21).

During a survey in northern California during 1980 and 1981 for pathogens of aquatic weeds (1), we observed a drainage ditch with large areas where parrotfeather plants were wilted and dying. Underwater stems of severely affected plants were chlorotic and devoid of rootlets. Frequently, the stem was girdled by a black canker several internodes long, often associated with a wound. Phase-contrast microscopy of cross sections from living stem tissue adjacent to the canker revealed fungal hyphae but no bacteria in cells of the cortex and aerenchyma. The presence of hyphae in stem cankers led us to examine the possibility that a fungus caused the disease. A preliminary report has been presented (2).

MATERIALS AND METHODS

Isolation and culture of fungi. The disease of parrotfeather was observed in both 1980 and 1981 in a drainage ditch crossing under State Highway 99 about 16 km north of Yuba City, CA. Stem cankers from diseased plants were surface-sterilized in 0.5% sodium hypochlorite for 0.5–2 min and serially sectioned, then transferred into petri dishes containing potato-dextrose agar (PDA) acidified with 25% lactic acid (200:1, v/v), a medium selective for pythiaceous fungi (22), and water agar. Plates were incubated at 22–24 C for up to 10 days. Mycelium was transferred from the edge of actively growing colonies onto either Difco cornmeal agar (CMA) or PDA.

For some experiments, an isolate of *Pythium carolinianum* Matt., Ag 23-81-12, was grown on V-8 juice and vermiculite (15). Vermiculite (500 ml) in 1-L jars was moistened with 250 ml of a broth containing 200 ml of V-8 juice, 800 ml of water, and 2.5 g of $CaCO_3$. The lids were fitted with foam plugs for ventilation, and jars containing medium were autoclaved for 1 hr. Mycelium of Ag 23-81-12 from agar plates was then introduced and the jars were covered with plastic bags. Vermiculite cultures were used after 3–4 wk of incubation at 22–24 C.

Growth of *P. carolinianum*. The effect of temperature on mycelial growth was determined in petri dishes containing mycelial disks 6 mm in diameter on 20 ml of CMA. Dishes were incubated for 24 hr at temperatures ranging from 6 to 42 C at intervals of 3 C, and colony diameters were measured. Optimum temperature for sporangium formation by Ag 23-81-12 was determined by incubating 6-mm disks from 5-day-old cultures on V-8 juice agar in 2% soil extract (10) at 6–42 C. After 18 hr, the sporangia around the edges of three replicate disks from each temperature were counted. Optimum temperature for zoospore release was determined by incubating 0.5 g of vermiculite culture of Ag 23-81-12 in 20 ml of tap water for 12, 24, and 36 hr at various temperatures. Zoospore concentrations were determined with a hemacytometer. Attempts were made to produce oospores by placing mycelium on autoclaved carrot disks, grass blades, hemp seeds in water (12), and two sterol-containing media (14,24). Cultures were incubated in darkness at 25 C for up to 1 mo.

Inoculation of parrotfeather in containers. Fungi isolated from diseased parrotfeather were tested for pathogenicity to parrotfeather cuttings in a greenhouse. Healthy cuttings 8 cm long were planted in pairs in 1-L crocks filled to within 3 cm of the top with sterilized Yolo fine sandy loam and flooded to 2 cm above the soil surface with water. Seven to 10 days after planting, longitudinal slits about 1 cm long were made in stems in the first internode above the water surface. A small weft of mycelium from a 4-day-old culture on PDA was inserted into the slit. The wound was then wrapped with a moist piece of cotton and plastic film. Alternatively, suspensions of 10^3–10^7 spores per milliliter were prepared from 7- to 10-day-old cultures on PDA, and 0.5 ml was injected into the first internode above the water with a syringe. Uninoculated control plants were either wounded or injected with 0.5 ml of sterile distilled water in the same manner as inoculated plants. Three isolates of *P. carolinianum* cultured from aquatic weeds other than parrotfeather (1) were also tested for pathogenicity to parrotfeather by inserting mycelium into stems as described.

Parrotfeather cuttings were also grown in pairs in 0.1× Hoagland's solution (7) in 1-L glass jars placed in paper bags to exclude light. Jars were held in growth chambers with a 16-hr photoperiod. Nine-day-old cuttings in jars at day/night temperatures of 32/21 C were inoculated with zoospores of Ag 23-81-12 to achieve a concentration of 4×10^3/ml of solution (about 10^6 zoospores per plant). Zoospore suspensions were prepared by incubating 6-mm disks from 5-day-old V-8 juice agar cultures in 2% soil extract for 24 hr. One-week-old cuttings grown at day/night temperatures of 21/15 and 25/19 C and 3-wk-old cuttings grown at 21/15 and 32/21 C were inoculated with 15 ml of rinsed vermiculite culture material (about 10^5 zoospores per plant). Plants inoculated with rinsed, uninfested vermiculite culture material served as controls. Similar inoculations were performed with cuttings grown in jars of nutrient solutions maintained in water baths at constant water temperatures of

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15, 20, and 25 C in a greenhouse.

Inoculation of parrotfeather in the field. Sixteen basins (3.0 × 1.8 × 0.8 m deep) were constructed in an agricultural field at Davis, CA. The basins were lined with black polyethylene (254 μm thick) and filled to a depth of 15 cm with Yolo loam from an adjacent field. On 1 June 1982, each basin was planted with 32 parrotfeather cuttings (10 cm long) in eight rows of four plants, and the soil was immediately flooded with water from a domestic well to a depth of 2 cm. As the plants grew, the water level was gradually increased to a final depth of 28 ± 5 cm. Twelve basins were infested on 2 July (31 days after planting) and reinfested on 1 September 1982 with 2, 6, or 18 L of the vermiculite culture material of Ag 23-81-12. The two highest inoculum doses were broadcast evenly over the water surface of individual basins. The lowest dose was applied in a band within 0.3 m of the basin edge where a water inlet was to be placed. Control basins were not infested. Treatments were randomized using a modified Latin square design with four basins per treatment.

One week after inoculation, each basin was fitted with an individual water inflow and a separate overflow. Well water flowed into the basins at a constant rate of 10 ml/sec; at this rate, the volume of water in each basin was replaced every 2 wk. Each basin was fertilized with 280 g of ammonium sulfate on 21 July, giving an initial nitrogen concentration of 21 μg/g. On 6 August (66 days after planting), the flow rate was increased to 100 ml/sec to lower the pH of water in the basins. Water temperature at a depth of 28 cm was monitored with a recording thermograph set on the hydrosoil in one of the basins. The pH of freshly collected water samples was measured periodically.

Isolations of the pathogen were attempted from parrotfeather roots in each basin 1, 2, and 3 mo after the first inoculation. Identification of *P. carolinianum* isolates as Ag 23-81-12 was based on cultural morphology, amount of radial growth at 6, 27, 30, 33, and 42 C, and pathogenicity to parrotfeather when inoculated into stems.

On 31 August (60 days after inoculation), the terminal 12.5 cm of each shoot longer than 12.5 cm was cut from the eight plants in the two rows nearest the overflow. The number of shoots harvested and their fresh and dry weights were recorded for each basin. Because early storms in mid-September had greatly increased the risk of cross-contamination among treatments, the basins were harvested again on 27 September 1982. All above-soil portions of the 16 plants in the four center rows of each basin were harvested and their fresh weights determined. Data were analyzed with a two-way analysis of variance (3).

Inoculation of crop plants. A number of crop species commonly cultivated in California were inoculated with Ag 23-81-12 in the greenhouse. Those tested were the following: pea (*Pisum sativum* L. 'Alaskan'), black-eyed pea (*Vigna sinensis* Savi 'Wisconsin'), celery (*Apium graveolens* L. 'Utah 5270'), cantaloupe (*Cucumis melo* L. 'Top Mark'), barley (*Hordeum vulgare* L. 'CM67'), bush bean (*Phaseolus vulgaris* L. 'Bountiful'), faba bean (*Vicia faba* L. 'Windsor'), lettuce (*Lactuca sativa* L. 'Salinas'), sunflower (*Helianthus annuus* L. '7976'), sugar beet (*Beta vulgaris* L. 'Ruby Queen'), oats (*Avena sativa* L. 'Ceres'), pumpkin (*Cucurbita pepo* L. 'Sugar Sweet'), safflower (*Carthamus tinctorius* L. 'N10' and 'Biggs'), watermelon (*Citrullus vulgaris* L. 'Cal Sweet'), carrot (*Daucus carota* L.), and wheat (*Triticum aestivum* L.). Seeds of each variety were sown in flats 10 cm deep containing a U.C. potting mix of sand and peat (1:1, v/v) (11) infested with one part vermiculite culture material to 125 parts potting mix. Germinating seedlings were examined for symptoms of damping-off. Three weeks after planting, three to five seedlings of each variety were inoculated with mycelium inserted into slits in the stems by the method used to inoculate parrotfeather. Five seeds of each variety were also planted in 10-cm pots (two varieties per pot). At seedling emergence, each pot received a suspension containing 10^4 zoospores from a newly reisolated culture and was flooded for 24 hr.

The rice (*Oryza sativa* L.) varieties M-9, M-302, S-201, and L-201, which are representative of varieties grown in California, were also tested for susceptibility to Ag 23-81-12. Rice varieties were grown in 25-cm tubs (one variety per tub) containing 5 cm of Yolo fine sandy loam soil and 5 L of water and inoculated in several ways. Fifteen milliliters of rinsed vermiculite culture material or 20 plugs from V-8 juice agar cultures were added to each tub when the seeds started to

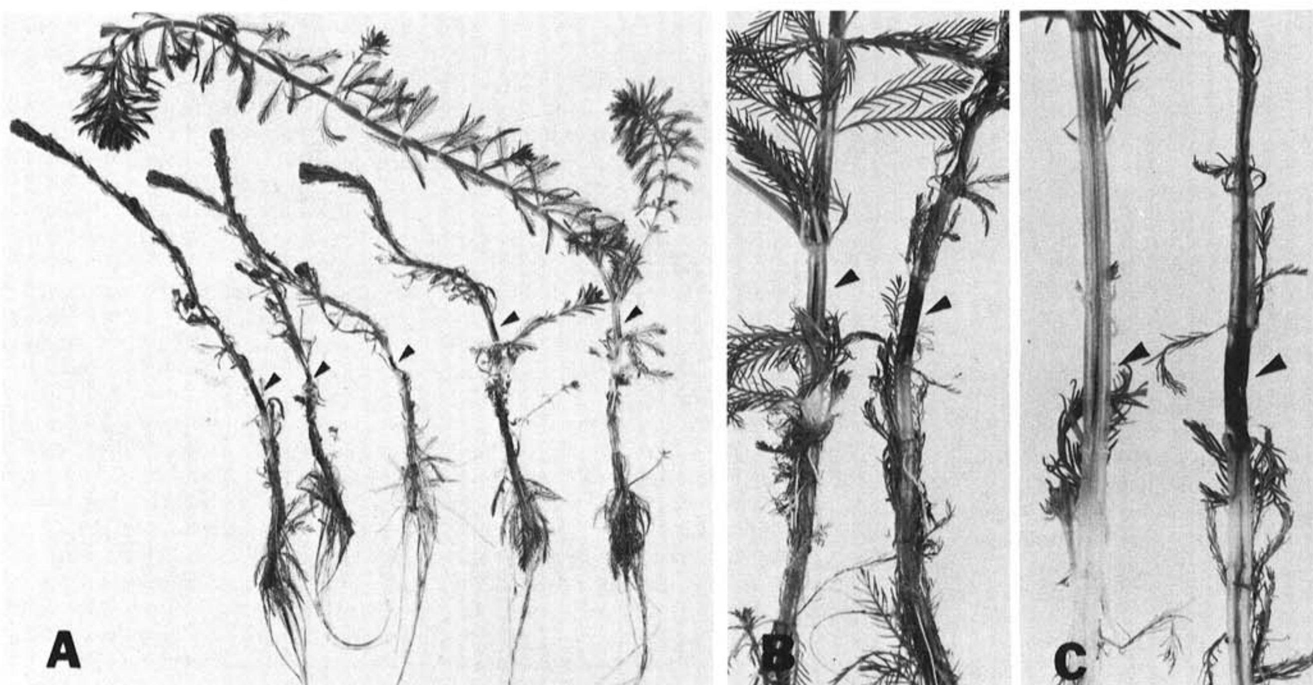

Fig. 1. Effect of stem inoculation with mycelium of *Pythium carolinianum* isolate Ag 23-81-12 on parrotfeather grown in a growth chamber. Eight-day-old cuttings grown at day/night temperatures of 27/20 C were inoculated with mycelium from 4-day-old colonies on potato-dextrose agar. Inoculated internodes are shown by arrows. **(A)** Cuttings 3 days after inoculation, with four inoculated plants on the left and one wounded, uninoculated plant on the right. **(B)** Close-up of (right) inoculated and (left) uninoculated stems. **(C)** Longitudinal sections through (right) inoculated and (left) uninoculated stems. Note darkening of stele of inoculated plant.

germinate. Zoospore suspensions from newly reisolated cultures were added to other tubs to give final concentrations of 10–100 zoospores per milliliter. Uninoculated plants served as controls.

RESULTS

Isolations and pathogenicity tests. Among fungi isolated from diseased parrotfeather collected from the field during 1980 and 1981, only isolate Ag 23-81-12 was pathogenic to parrotfeather in greenhouse tests. Shoots of parrotfeather collapsed 2–4 days after mycelium of this fungus was inserted into stems (Fig. 1A). The inoculated internode blackened, and a brown discoloration was visible in the stele up to six internodes above the inoculated internode (Fig. 1B,C). No external discoloration was evident above or below the inoculated internode (Fig. 1). The internal discoloration did not extend more than half of an internode below the inoculated internode, and the root system appeared unaffected by the inoculation. Ag 23-81-12 could be reisolated only from the inoculated internode. All other fungi that were inoculated into stems as spores or mycelium, including other isolates of *P. carolinianum* obtained from submersed aquatic weeds (1), had no adverse effect on the growth or vigor of parrotfeather grown in containers. Although dark lesions occurred at the point of inoculation on some of these plants, the lesions never encircled the stem. Twice during the experiments, isolates of Ag 23-81-12 that had been maintained on CMA lost pathogenicity to parrotfeather. Pathogenic isolates were recovered by isolating from plants in the field area where the disease was originally observed and from plants in the infested basins of the field plot.

Characteristics of Ag 23-81-12. Oospores were not detected on any of the media tested or in infected parrotfeather tissue. Spherical to elliptical sporangia with definite papillae formed within 1 day when mycelium was placed in water. Sporangia germinated either by zoospore release from a vesicle formed on a short evacuation tube or by producing one to several germ tubes. Successive sporangia were produced either within old sporangia or on sporangiophores that grew through or around the old sporangia. Sporangia averaged 26 μm in diameter, with a range of 20–32 μm. Hyphal diameter on PDA ranged from 2 to 6 μm. On the basis of these characters, the isolate was identified as *P. carolinianum* (12,24). According to a recent monograph on the genus *Pythium* (23), isolate Ag 23-81-12 would be designated as a Group P *Pythium* because it lacks hyphal swellings. The characteristics of our isolate, however, do conform to the existing description of the species *P. carolinianum* (12). The optimum temperature for linear growth of Ag 23-81-12 on cornmeal agar was 30 C and the limits for growth were 9 and 39 C. The optimum temperature for sporangium formation on disks of V-8 juice agar in soil extract was 27 C and sporangium formation occurred at temperatures from 12 to 39 C. After 24 hr of incubation, zoospore release from infested vermiculite was maximal at 24 C, but after 36 hr of incubation, equal numbers of zoospores were present at temperatures from 24 to 30 C. In agar cultures, Ag 23-81-12 produced sparse aerial mycelium and showed an indistinct rose pattern that distinguished it from other isolates of *P. carolinianum* obtained from aquatic weeds (Fig. 2).

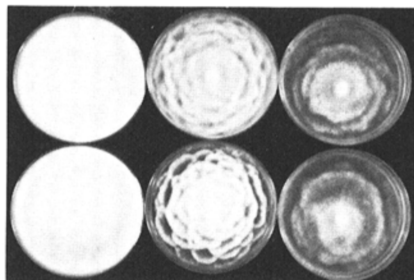

Fig. 2. Isolates of *Pythium carolinianum* on potato-dextrose agar after 5 days of incubation at 22–24 C. The left pair, (top) Ag 21-81-1 and (bottom) Ag 23-81-2, were isolated from parrotfeather but were not pathogenic to it; the center pair, (top) Jan 27-81-3 and (bottom) F11-81-10, were isolated from overwintering tubers of American pondweed and turions of curly leaf pondweed, respectively; and the right pair, of the pathogen Ag 23-81-12, were reisolated from inoculated parrotfeather in field plots.

Pathogenicity of Ag 23-81-12. Nine-day-old parrotfeather cuttings grown in solution culture at day/night temperatures of 32/21 C and inoculated with 10^6 zoospores wilted and collapsed within 6 days of inoculation. Roots of inoculated plants turned dark and rotted, but no extensive rot developed in the underwater stems. In other experiments, 7-day-old cuttings were grown at day/night temperatures of 21/15 and 25/19 C and inoculated with 15 ml of vermiculite infested with Ag 23-81-12 (Table 1). In both temperature regimes, some plants wilted within 48 hr of inoculation. Microscopic examination of roots from inoculated plants showed encysted zoospores and associated necrotic areas within 24 hr of inoculation. Sporangium production on inoculated roots was also evident within 24 hr. Root infection was more extensive among plants grown at 25/19 C than at 21/15 C. A two-way analysis of variance indicated inoculation had a significant effect on plant fresh weights (Table 1), but the effects of temperature and the interactions between temperature and inoculation were not significant at $P = 0.05$. Similar results were obtained when 3-wk-old cuttings were inoculated (Table 1). Wilting occurred only among plants grown at 32/21 C. Fresh weight was significantly reduced by inoculation, but there was no significant effect of temperature and no significant interaction between inoculation and temperature. No symptoms or growth decreases were observed among inoculated plants grown in constant water temperatures of 15, 20, and 25 C in a glasshouse.

None of the cultivated plant varieties inoculated with Ag 23-81-12 in the greenhouse developed symptoms of damping-off. Plant varieties inoculated by inserting mycelium into their stems were also unaffected by the inoculation.

Field inoculations. The water temperature measured in one of the basins closely followed the diurnal fluctuations in air temperatures (1). For the first month after inoculation, maximum and minimum daily water temperatures varied between 30 and 20 C, but when the water flow rate was increased on 5 August, daily maximum and minimum temperatures fell to 25 and 18 C, respectively. The average water pH ranged from 8.3 at the beginning of the experiment to 7.7 after the water flow rate through the basins was increased. The pH of water in the basins was not affected by the treatments.

In uninfested control basins, the roots of the parrotfeather plants were tan and firm. Individual roots averaged about 12.5 cm long. Roots in all infested basins were black, soft, and stunted, frequently as short as 2.5 cm. Similar symptoms were also observed on parrotfeather plants in one uninfested control basin shortly after inoculation. Ag 23-81-12 was isolated from parrotfeather roots in this basin and it was excluded from the analysis of the results. By the final harvest, Ag 23-81-12 had been reisolated from roots in half of the infested basins.

Shoot production by parrotfeather in the field was also affected by inoculation with Ag 23-81-12. Although there were similar numbers of new underwater shoots in all basins 3 wk after inoculation,

Table 1. Influence of inoculation[a] with *Pythium carolinianum* isolate Ag 23-81-12 on the fresh weight of parrotfeather grown in solution culture under controlled conditions

Temperature		Plant age (wk)		Average fresh weight (g)	
Day	Night	Inoculation	Harvest	Uninoculated	Inoculated
21	15	1	3	3.1	2.1*[b]
25	19	1	3	3.6	1.4*
21	15	3	5	2.7	1.7*
32	21	3	5	3.8	1.6*

[a] Fifteen milliliters of vermiculite infested with Ag 23-81-12 was added to each container.
[b] * = Value significantly less than the control according to LSD ($P = 0.05$).

greater percentages were dead in infested basins (Table 2). At the first harvest, 9 wk after infestation, inoculated plants had fewer emersed shoots than plants in the control basins (Table 3). This suppression was significant in basins receiving 6 L of broadcast inoculum and 2 L of inoculum at one point. The total fresh and dry weights of the shoots collected from infested basins were also less than those of the controls (Table 3). Although the inoculated plants produced fewer shoots than did the uninoculated plants, the average weight per shoot was similar in inoculated and control plants 9 wk after inoculation. At the final harvest, 13 wk after inoculation, the total fresh weights of plants from infested basins were significantly less than those of plants from uninfested control basins (Table 3).

DISCUSSION

Isolate Ag 23-81-12 of *P. carolinianum* is a pathogen of the emersed weed parrotfeather. This isolate killed shoots when inoculated directly into stems as mycelium and suppressed growth when plants were inoculated with either zoospores or infested vermiculite under both controlled and field conditions.

Isolates of *P. carolinianum* were obtained from parrotfeather at the field site where disease was first observed as well as from shoots and dormant overwintering propagules of the submersed aquatic weeds Eurasian watermilfoil (*Myriophyllum spicatum* L.), sago pondweed (*Potamogeton pectinatus* L.), curly leaf pondweed (*P. crispus* L.), and American pondweed (*P. nodosus* L.) (1). Ag 23-81-12 was the only isolate of *P. carolinianum* obtained from aquatic weeds that was pathogenic to parrotfeather. Ag 23-81-12 also differed from other isolates from aquatic weeds in cultural morphology (Fig. 2).

P. carolinianum was originally described by Matthews, who found it parasitizing the alga *Spirogyra* (12). It has been isolated infrequently from soil (6) and was reported to be pathogenic to field-grown chrysanthemums in North Carolina (9) and taro in Hawaii (16,17). Ag 23-81-12 was not pathogenic to any of the cultivated plants in our host range experiments. The host range experiments were not exhaustive, however, and further evaluation of the isolate's potential pathogenicity to cultivated plants will be needed if Ag 23-81-12 is developed further as a biocontrol agent.

Inoculation with Ag 23-81-12 reduced growth of parrotfeather growing in basins in the field by 20–33% (Table 3). Application of 2 L of inoculum at a point source was the most effective treatment, indicating that Ag 23-81-12 was able to reproduce and spread under conditions of the field experiment. The broadcast treatments of 6 and 18 L of inoculum were less effective in reducing growth (Table 3). The vermiculite culture material used as inoculum was not rinsed before use, and it is possible that unutilized V-8 juice in the vermiculite may have favored the growth of bacteria or other organisms antagonistic to or competitive with Ag 23-81-12. If so, the effect is likely to be greatest at the higher inoculum levels and it may have resulted in less disease in high-inoculum treatments.

Significant growth suppression occurred when parrotfeather was inoculated at fluctuating temperatures in growth chambers (Table 1) and in the field (Table 3) but not when parrotfeather cuttings were inoculated at constant water temperatures in the greenhouse. Changes in water temperature are known to stimulate zoospore release from sporangia of *Pythium* spp. (12,13,24). Fluctuating water temperatures may be more favorable for the continued growth and survival of the pathogen than constant temperatures.

Ag 23-81-12 is difficult to isolate. It was obtained only twice in numerous isolations from diseased parrotfeather collected from the Yuba City site and was reisolated infrequently from parrotfeather roots showing disease symptoms after artificial inoculation in the field at Davis. This isolate grows more slowly than other *Pythium* spp. commonly isolated from parrotfeather. As a result, it may be overrun by other *Pythium* spp. during isolation, leading to a low efficiency of isolation.

Although Ag 23-81-12 is clearly pathogenic to parrotfeather, it is not clear whether it was the primary cause of the dieback originally observed near Yuba City. Mycelial inoculation in stem wounds caused stem lesions and shoot death, but inoculation with zoospores or vermiculite cultures generally resulted only in root rot. In one instance, the entire syndrome of root rot, stem lesions, and shoot death was seen in 9-day-old parrotfeather cuttings inoculated with 10^6 zoospores. However, methods used rarely yielded this number of zoospores, which may have been necessary for severe disease development.

Ag 23-81-12 is a potential agent for biological control of parrotfeather. However, difficulties encountered during this investigation, including loss of pathogenicity while the organism was in culture and low levels of zoospore production, would have to be overcome. Nevertheless, it is our opinion that further research on this interesting host-pathogen interaction, including the possibility of its use in integrated control, may yield rewarding results.

Table 2. Number of new underwater shoots of parrotfeather in basins and the percentages of shoots dead 3 wk after inoculation with vermiculite infested with *Pythium carolinianum* isolate Ag 23-81-12

Treatment		New shoots	
Method of inoculation	Volume of inoculum (L)	Number per basin	Proportion of shoots dead
None	0	14.5 ± 7[a]	0.31
Broadcast	18	24.5 ± 10	0.41
Broadcast	6	18.5 ± 5	0.56*[b]
Point source	2	14.8 ± 8	0.61*

[a] Standard deviation of the mean.
[b] * = Proportion of shoots dead significantly greater than controls at $P<0.05$ as determined by difference using the normal approximation to the binomial distribution given by Snedecor and Cochran (20).

Table 3. Shoot production by parrotfeather in basins 9 and 13 wk after inoculation with vermiculite infested with *Pythium carolinianum* isolate Ag 23-81-12

Treatment		Weeks after inoculation			
		9[a]			13[b]
Method of inoculation	Volume of inoculum (L)	Number of shoots	Fresh weight (g)	Dry weight (g)	Fresh weight (g)
None	0	232 ± 26[c]	516 ± 79	121 ± 22	5,963 ± 743
Broadcast	18	176 ± 36	411 ± 118	92 ± 30	4,448 ± 757*[d]
Broadcast	6	142 ± 44*	368 ± 104	81 ± 22*	4,720 ± 937*
Point source	2	144 ± 26*	339 ± 67*	77 ± 20*	3,997 ± 985**

[a] Shoots tips 12.5 cm long were harvested from the eight plants nearest the outflow in each basin.
[b] The entire above-soil portions of the center 16 plants in each basin were harvested.
[c] Standard deviation of the mean.
[d] Mean significantly different from the uninoculated control as determined by Dunnett's method (4); * = $P=0.05$ and ** = $P=0.01$.

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