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Nimbya alternantherae a potential biocontrol agent for alligatorweed, *Alternanthera philoxeroides*

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Abstract. Alligatorweed, (*Alternanthera philoxeroides* (Mart.) Griseb.), an aquatic and wetland plant native to South America, is an aggressive weed in many parts of the world. Its ability to compete with other native plants and to impede waterways has made it a serious threat to aquatic ecosystems. Although biological control with insects has been fairly successful in aquatic habitats, there is a need for additional agents to manage the weed in upland sites. Accordingly, in a survey in Brazil in 1997 a fungus, *Nimbya (=Alternaria) alternantherae* (Holcomb and Antonopoulus) Simmons and Alcorn, was discovered and confirmed to be highly damaging to alligatorweed. Studies were conducted to determine the potential of this fungus for controlling this weed. Several isolates from Brazil, USA, and Puerto Rico were compared and no differences in virulence were observed, although a lower dew requirement was demonstrated for the Brazilian isolates. Conidia were more effective than mycelial suspension, and inoculum concentrations of 1×10^5 and 1×10^6 conidia per ml provided significant levels of control of the weed in greenhouse and field experiments, respectively. In a host-range study, *N. alternantherae* infected 6 plant species from a total of 42 species belonging to 23 families. *N. alternantherae* has the potential to be an effective mycoherbicide for alligatorweed.

Key words: *Nimbya alternantherae*, alligatorweed, *Alternanthera philoxeroides*, mycoherbicide, aquatic weed, Amaranthaceae, hyphomycetes

Introduction

Alternanthera philoxeroides (Mart.) Griseb. (alligatorweed; Amaranthaceae) is an amphibious perennial plant that is native to South America and an important weed of freshwater systems around the

world (Holm et al., 1977). It alters the ecology of the infested areas, restricting light penetration, and interfering with the growth of submerged flora and fauna (Holm et al., 1997). It is listed as one of 20 weeds of national significance for Australia (Thorp and Lynch, 1999). It is among the worst aquatic weeds in Indonesia, New Zealand, Thailand, and the United States, and is also found infesting crops and pastures in 30 countries (Holm et al., 1997).

Although chemical control can be effective for managing this weed, this method is not adequate for large infestations of natural ecosystems, particularly of water bodies. Biological control has been investigated, and four host-specific insect species from South America have been released in several parts of the world against this weed (Julien and Griffiths, 1998). Of these, the flea beetle *Agasicles hygrophila* Selman & Vogt (Coleoptera: Chrysomelidae) and the moth *Arcola malloi* Pastrana (Lepidoptera: Pyralidae) were introduced into the USA and are exerting good control of floating mats of the weed, except in the extreme tropical and temperate areas (Julien and Griffiths, 1998; Stewart et al., 2000). Unlike its effectiveness in controlling floating mats, these insects have not controlled upland stands effectively. Hence, additional biocontrol agents are needed.

Recently, a search for fungal pathogens that may be used as biocontrol agents of this weed was initiated by Barreto and Torres (1999) who reported the occurrence of *Nimbya (=Alternaria) alternantherae* (Holcomb and Antonopoulus) Simmons and Alcorn and *Cercospora alternantherae* Ellis & Langlois (Hyphomycetes), on *A. philoxeroides* in Brazil. Preliminary inoculation studies suggested that the former might have potential as a mycoherbicide. This fungus is also known to occur in the USA (Holcomb and Antonopoulos, 1976) and Australia (Simmonds, 1995), where it was recently recollected by B. Auld (Orange Agricultural Institute, Australia, personal communication). It causes purple foliar and stem lesions that later become brown and necrotic resulting in defoliation under natural conditions (Figure 1).

The objective of the study reported in this article was to evaluate *N. alternantherae* as a possible mycoherbicide for alligatorweed. Comparison of disease severity produced by different isolates, effect of inoculum concentration and inoculum formulation on disease development, host-range studies, and field trial were performed.

Figure 1. Symptoms from a natural infection of *Alternanthera philoxeroides* by *Nimbya alternantherae*.

Materials and methods

Fungal cultures and study site

Isolates were obtained from diseased alligatorweed plants collected in Brazil (states of Rio de Janeiro and Minas Gerais), United States, and Puerto Rico. Leaf tissues with disease symptoms (4 mm^2) were excised and surface sterilized with 2% sodium hypochlorite (v:v) for 3, 6, 9, 12, and 15 min, washed with sterile water, transferred to V-8 juice agar plates, and the plates incubated at 25 °C in the dark. After 3–4 days, stock cultures were prepared by transferring mycelial tips to potato carrot agar (PCA). All isolates were preserved in slants with soil, water, and mineral oil (Dhingra and Sinclair, 1995). An isolate from Florida was used in all the experiments unless otherwise stated.

Greenhouse experiments in the USA involving the American, Brazilian, and Puerto Rican isolates were performed and conducted in the Plant Pathology Quarantine Greenhouse at the University of Florida IFAS, Gainesville. The field experiments were done at the

Biven's Arm Lake in Gainesville, and along Waldo road using the local isolate. All experiments were performed in 1997 and 1998.

All the experiments were repeated at least once.

Inoculum production

The inoculum was produced with a biphasic solid–liquid fermentation method based on the technique developed by Walker (1980) and modified by Chandramohan and Charudattan (2003). Mycelial mats were produced in Erlenmeyer flasks containing a liquid medium composed of 400 ml of V-8 juice (Campbell Soup Company, Camden, NJ) per liter of tap water and grown for 7 days on a shaker (100 rpm at 25 °C). A mycelial disk from a 10-day-old culture of *N. alternantherae* was used as the seed inoculum. At harvest, the contents of the flask were blended for 30 s with 1% (v/v) antibiotic solution (3.5 ppm of streptomycin and 1.5 ppm of chloramphenicol). About 50 ml of the resulting suspension was poured into each of several trays ($30 \times 20 \times 4$ cm) lined with aluminum foil and previously filled with 250 ml of oat meal agar (Difco laboratories, Detroit, MI) containing antibiotics (as above). The trays were then transferred to racks, covered with plastic sheeting, and incubated under a 12-h light regime at room temperature. After 48 h, the trays were flooded with 100 ml of sterile tap water and conidia were collected by scraping the culture surface with a rubber spatula. The trays were then reincubated as before and the conidia were harvested daily during the next 5 days. While still fresh, the resulting suspensions were filtered through a layer of cheesecloth, vacuum filtered, and air dried for 48 h. The resulting conidial mass was mixed with kaolin (1:1 – w/w) and stored in screw cap vials at 5 °C.

Plant production

Healthy alligatorweed plants were collected from a field population on the border of a pond located at the Center for Aquatic and Invasive Plants, University of Florida-IFAS, Gainesville. Plants were washed, sectioned, and one 5- to 7-cm stem piece was planted in each of several 500-cm^3 plastic pots containing a commercial potting mixture (Metro-Mix 300; W.A. Grace & Co, Cambridge, MA). The pots were kept on a greenhouse bench and watered and fertilized as needed. Each pot containing one plant was an experimental unit. Each alligatorweed plant was pruned and standardized to have all plants with

three branches per pot before inoculation. The inoculations were made using 1-month-old plants unless otherwise stated.

Seedlings prepared for formulation studies were planted in 10-l plastic pots filled with potting mixture as above. Five seedlings were planted per pot and each pot was spaced1 m apart. All plant species used for the host-range trials were grown from seeds obtained from commercial sources, sown in pots filled with the same substrate, and maintained at the same conditions described above.

Inoculation and disease assessment procedures for greenhouse tests

Brazilian isolates were evaluated in a quarantine greenhouse following standard safety requirements. The conidial suspension (inoculum), was prepared by hydrating the dried conidia with 0.005% Tween® 85 in water (v/v; Fluka Chemical Corp, Ronkonkoma, NY) and adjusting the conidial concentration with the aid of a hemacytometer. Before starting each experiment, a sample of the conidial suspension was plated on water–agar plates to determine the percentage of conidial germination. All experiments were performed with conidia having $\geq 80\%$ germination.

Plants were inoculated to complete wetness with a hand-held aerosol sprayer and then incubated in a dew chamber (Percival Manufacturing Co., Boone, IA) set at 100% relative humidity (RH) for 18 h at 25 °C in the dark. Subsequently, the pots were transferred to a greenhouse. Control plants were treated similarly except they were sprayed with sterile distilled water only.

Disease severity for each plant or all plants in the experimental plot was estimated using a rating system based on a scale from 0 to 6, where 0 = no visible symptoms; 1 = 0–20; 2 = 20–40%; 3 = 40–60%; 4 = 60–80%; $5 \geq 80\%$ diseased area; and 6 = dead plant. Disease severity was estimated at 4 days following inoculation.

Comparison of isolates

The experiment was designed to evaluate possible differences in the virulence of *Nimbya* isolates. Four isolates were used, two from Brazil (Minas Gerais and Rio de Janeiro States) and one each from USA (Florida) and Puerto Rico. The experiment had a completely randomized design with 5 replicates. The conidial suspension was adjusted $1-2 \times 10^5$ conidia per ml. The number of leaves per plant was recorded at the beginning of each experiment. The plants were

inoculated and subjected to a dew period as described under Section Inoculation and disease assessment procedures for greenhouse effects. The disease severity and percentage of dead leaves were evaluated daily. The third day of evaluation was chosen for statistical analysis.

Effect of inoculum level on disease severity

Four conidial concentrations were tested to determine the inoculum threshold needed to produce high disease severity levels on inoculated plants. The experiment was set in a completely randomized design with four replicates. The plants were sprayed with a conidial suspension containing 1×10^3; 1×10^4; 1×10^5 or 5×10^5 conidia per ml. Plants sprayed with distilled water served as controls. The plants were inoculated and subjected to a dew period as described under Section Inoculation and disease assessment procedures for greenhouse effects. Disease severity was evaluated after 4 days on the disease severity scale described.

Effect of dew period on disease severity

Three-month-old plants were inoculated with a conidial suspension adjusted to 1×10^5 conidia per ml. Two isolates from Brazil (CP 21 and CP 22) and the Florida isolate were evaluated. After inoculation, plants were placed in a dew chamber maintained at 25 °C in the dark. Following 0, 6, 12, or 14-h dew periods, groups of six plants for each isolate were then kept in the quarantine greenhouse. The disease severity was evaluated after 4 days.

Comparison of mycelial fragments and conidia as inoculum

Conidia and mycelial fragments were evaluated as inoculum against alligatorweed. Mycelial mat was obtained from a 5-day-old growth in liquid culture (400 ml of V8 per liter of water), sieved through two layers of cheesecloth, washed with sterile tap water, and partially dried by spreading the mat over a layer of a sterile tissue paper. A 3-g (fresh weight) aliquot was then mixed with 30 ml of water and blended for 10 s. The resulting suspension was sprayed on 2-month-old alligatorweed seedlings with a hand-held atomizer until the shoots were completely wet. A conidial suspension containing 1×10^5 conidia per ml was sprayed on another group of plants. After spraying, all plants were incubated in the dew chamber (100% RH, 25 °C, complete darkness) for 18 h. Seedlings

sprayed with sterile tap water served as controls. The disease severity was evaluated after 4 days. Each treatment had six replicates.

Effect of different formulations on disease severity

This trial was carried out using healthy plants in microplots placed outside the greenhouse. The experiment had a completely randomized design with four replicates. Three-month-old plants were inoculated by spraying 20 ml suspension per pot containing 1×10^5 conidia per ml. The Florida isolate was used in this test. Care was taken to avoid inoculum drift that might lead to interplot interference. The adjuvants added to the conidial suspension were: (1) unrefined corn oil (UCO) at 50% (v:v); (2) conidia in water; (3) conidia in 0.2% Silwet L77 in water (v:v) (a copolymer of siliconpolyether, OSI Corporation, Loveland Industries, Inc., Greeley CO); (4) conidia in 0.5% Metamucil in water (w:v) (a hydrophilic mucilloid, a polysaccharide from the psyllium plant, Procter & Gamble, Cincinnati, OH); (5) conidia in an emulsion based on 2% Sunspray oil (agricultural mineral oil, Ipiranga Química, Rio de Janeiro, RJ Brazil) and 5% paraffinic oil (v:v); and (6) control plants sprayed with sterile tap water only. The disease severity was evaluated as previously described. Evaluations were performed 6 times at 3-day intervals. Inoculation was made late in the afternoon to avoid problems with reduced conidial viability because of UV exposure and desiccation.

Host range

Pathogenicity of the local isolate (Florida) of *N. alternantherae* to 42 plant species belonging to 23 different families was tested according to the testing strategy proposed by Wapshere (1974). Three replicates per plant were sprayed with a suspension of 5×10^5 conidia per ml until the shoots were completely wet. Alligatorweed plants were sprayed as a positive control. Two replicates of each species were treated with distilled water only as a negative control. After inoculation, all plants were kept in the dew chamber (100% RH, 25 °C, total darkness) for 18 h. Plants were considered either immune (no visible reaction), resistant, (non-spreading, <1-mm diam necrotic spots), or susceptible (spreading, >2-mm diam necrotic spots) by visual observation 14 days after inoculation.

Field trials

For field trials, a naturally occurring homogeneous mat of alligatorweed was used. The experimental unit or replicate, was a 0.25 m^2

area, marked with 0.50−m high iron poles. Only populations growing rooted in shallow water near shoreline (i.e., not floating mats) were chosen.

Inoculum was prepared as previously described and adjusted to 1 to 2×10^6 conidia per ml and amended with unrefined corn oil (UCO) at 20% (v:v). Field plots (0.5×0.5 m) were located along the shoreline, 1 m apart from each other. The trials were done once during May through July, 1998. The plots consisted of a natural alligatorweed infestation forming homogeneous mats. Treatments were applied in the evening with the aid of a hand-held aerosol sprayer (40 ml of inoculum suspension per plot). The treatments were: (1) conidial suspension + UCO and (2) water + UCO (control). Control plots were sprayed with sterile distilled water only. The experiments were completely randomized with nine replicates per treatment, except control plots, which had three replicates. Disease severity was evaluated, as previously described, every 3 days during 35 days. The experiment was repeated once.

Statistical analysis

All data were analyzed using the statistical software SAEG version 8.0 (Fundação Arthur Bernardes, UFV, Viçosa, Brazil). All multiple comparisons were first subjected to analysis of variance (ANOVA). Significant differences among treatment means were determined using Tukey's Studentized Test (Gomez and Gomes, 1984).

Results

Comparison of isolates

All four isolates were pathogenic to alligatorweed and killed 100% of the leaves 3 days after inoculation (Figure 2). No differences in virulence were observed among the isolates based on either disease severity or percentage of leaf death after this 3-day period.

Effect of inoculum concentration on disease severity

Inoculum density of $>1 \times 10^4$ conidia per ml produced higher plant mortality compared to the lower density used (Figure 3). A minimum of 1×10^4 conidia per ml was necessary to completely kill the seedlings after 4 days. No regrowth was observed on plants with disease severity (DS) of 100%; the seedlings inoculated with 1×10^3 conidia

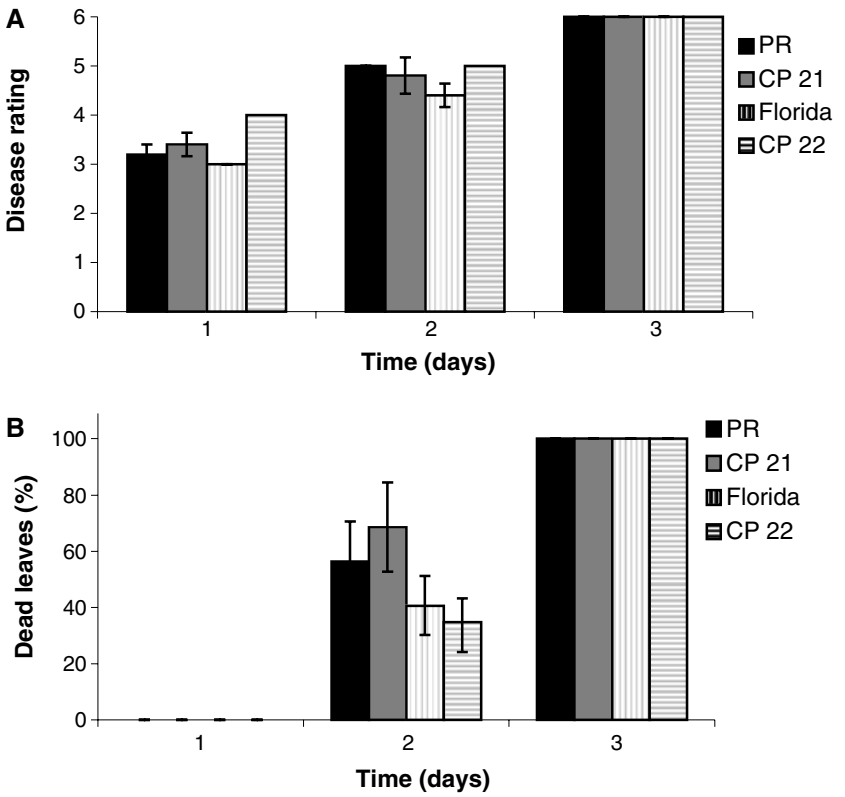

Figure 2. (a) Virulence of different *Nimbya alternantherae* isolates from Brazil (CP21 and CP22), USA (Florida), and Puerto Rico (PR). Disease severity was rated on a 0–6 scale, where 6 indicates plant death; (b) Percentage of dead leaves. Vertical bars represent standard error of mean.

per ml developed only 16.3% DS and were able to produce healthy sprouts within a few weeks. The first symptoms appeared 24 h after inoculation. Inoculum density of $>1 \times 10^4$ conidia per ml produced numerous small purple spots over the leaf and stem surface, extensive blighting and necrosis of tissues, and death of the entire leaf. Frequently, the lesions also girdled the stems, killing them upwards.

Effect of dew period on disease severity

Maximum disease severity was obtained when plants were exposed to dew periods of 12 h and longer (Figure 4). No difference on virulence was observed between the Brazilian isolates (CP 21 and CP 22), but

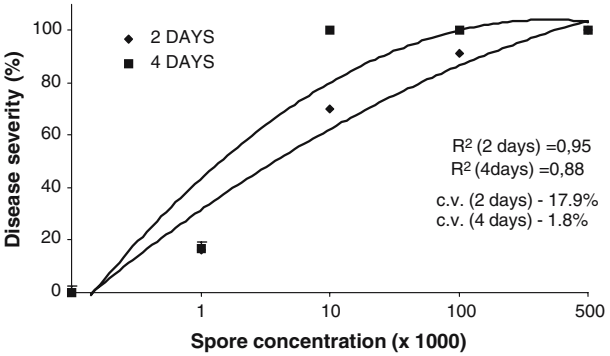

Figure 3. Effect of conidial concentration of *N. alternantherae* on disease severity on alligatorweed. Disease severity as a mean from 4 plants per treatment. Assessments were made 4 days after inoculation. Plants were exposed to 100% RH for 18 h at 25 ± 2 °C after inoculation.

the Florida isolate (FL) caused 20% lower disease severity than that produced by the Brazilian isolates. Although plants inoculated with the Florida isolate showed a high disease severity after a 12-h dew period, no mortality was registered; the plants were able to recover after a few weeks.

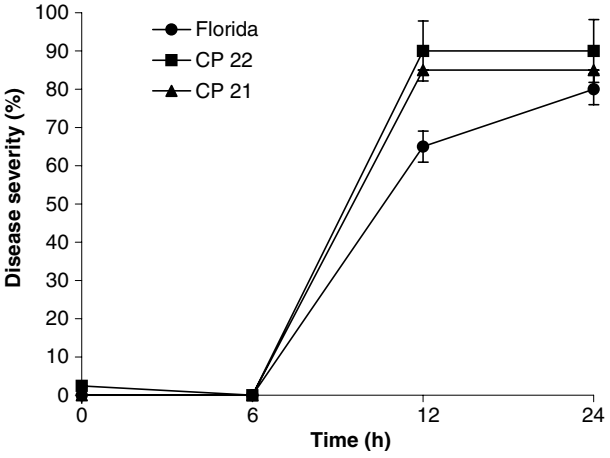

Figure 4. Effect of dew period on disease severity caused by *Nimbya alternantherae* on *Alternanthera philoxeroides*. Plants were evaluated 4 days after inoculation with 1×10^5 conidia per ml. Data points represent means of 6 replicates. Vertical bars represent standard error of mean.

Comparison of mycelial fragments and conidia as inoculum

Conidia provided a higher disease severity level ($p = 0.01$) compared to that obtained with mycelium inoculation. Seventy percent of the plants sprayed with conidia were killed after 4 days, whereas no plants died in the group inoculated with the mycelial fragments. No disease was observed in the control plants.

Effect of different formulations on disease severity

The adjuvants added to the conidial formulation influenced the disease severity. The maximum disease rate observed (equivalent to 60–80% disease severity) was obtained with the UCO formulation, although not statistically different from Metamucil and Emulsion (Table 1). This formulation produced a disease severity level that was higher than that obtained from conidia applied in water or in Silwet L77. The third disease severity evaluation, done on the 9th day after inoculation, was the one selected for comparisons and statistical analysis. After this time, a general reduction in disease severity was registered in all treatments because of new growth that remained healthy and the plants recovered. No disease occurred on the control plants treated with sterile tap water.

Table 1. Effect of formulations on disease severity caused by *N. alternantherae* on *A. philoxeroides*

Formulations	DR[a]
Unrefined corn oil (UCO – 50% v:v)	4.25 A[b]
Metamucil (0.5% w:v)	3.75 AB
Emulsion (Paraffinic oil 5%, Sunspray oil 2%)	3.50 AB
Silwet L77 (0.2% v:v)	2.50 B
Conidia in water	2.50 B
Sterile tap water	0.00 C
	c.v. – 27.7%

[a]Disease rate: evaluated on a disease rating scale of 0–6, where 0 = no visible symptoms; 1 = 0–20%; 2 = 20–40%; 3 = 40–60%; 4 = 60–80%; 5 = >80% diseased area; 6 = dead plant. Each value was obtained from 4 replicates; each pot (replicate) had 5 plants.

[b]Values followed by the same letter are not significantly different (Tukey test, $p = 0.01$).

Host range

Six of 42 plant species tested (in addition to alligatorweed) in the host-range study developed disease symptoms (Table 2). None of the control plants showed any disease. Some necrotic foliar flecks were observed on endive but they were considered as a hypersensitive resistant reaction response. Three members of the Amaranthaceae, including alligatorweed, two members of the Chenopodiaceae and one of the Portulacaceae developed disease symptoms, out of the 22 families evaluated. By visual estimation, sugar beet was most severely damaged.

Field trials

No natural infection caused by *N. alternantherae* was observed in the plots before the experiment started. The fungus produced a great impact on plants in the inoculated plots, 3 days after inoculation. By then, 80% disease severity was observed in those plots (disease rate of 5), and after 6 days, all leaves on the treated plants were dead (Figure 5).

A slight reduction of the disease severity was observed over this period, caused by the production of new healthy leaves. Nevertheless, after 1 month, the plants in the inoculated plots still had a disease rate close to 5. Unexpectedly, the disease also appeared on control plots. Although severity levels in control plots were initially low, they increased steadily, reaching rate 2 after 1 month. The disease incidence on the control plots developed similarly on plots sprayed with UCO and on those sprayed with water alone.

Discussion

The work reported in this paper was limited to a few isolates because of quarantine restrictions. The choice of the isolate is a critical factor for the success in any mycoherbicide program. Surveys can be oriented in order to find isolates showing lower dew requirement for infection, higher virulence against the host, prolonged shelf life, as well as other desirable characters. In this study no differences were observed between the Brazilian isolates and the isolates from Puerto Rico and Florida. The Florida isolate appeared to have a higher dew requirement when compared with the Brazilian isolates. The Florida isolate caused disease of lesser severity in relation to the Brazilian

Table 2. Reaction of nontarget plants in a host-range test of *N. alternantherae*

Family	Plant species	Common name	Disease reaction [a]
Amaranthaceae	*Alternanthera ficoidea* (L.) Sm. in Rees.		I[b]
	A. philoxeroides (Mart.) Griseb.	Alligatorweed	S
	Amaranthus spinosus L.	Spiny amaranth	I
	Celosia cristata L.	Celosia	S
	*C. plumosa*Hort.ex Burvenich	Celosia	S
	Gomphrena globosa L.	Globe amaranth	I
	Pfaffia paniculata (Mart.) Kuntze.	Suma	I
Apiaceae	*Daucus carota* L.	Carrot	I
	Petroselinum crispum (Mill.) Nyman	Parsley	I
Araceae	*Alocasia* sp.	Alocasia	I
Asteraceae	*Cichorium endivia* L.	Endive	R
	C. intybus L.	Chicory	I
	Lactuca sativa L.	Lettuce	I
	Senecio brasiliensis (Spreng.) Less	Maria mole	I
Brassicaceae	*Brassica oleracea* L. var. *botrytis* L.	Cauliflower	I
	B. oleracea L. var. *capitata* L.	Cabbage	I
	B. oleracae L. var. *italica* Plenck	Broccoli	I
	B. oleracea L. var. *viridis* L.	Collards	I
	B. rapa L.	Turnip	I
	Raphanus sativus L.	Radish	I
	Sinapis alba L.	White mustard	I
Bromeliaceae	*Ananas sativus* Schult.	Pineapple	I
Cactaceae	*Opuntia sp.*	Cactus	I
Chenopodiaceae	*Beta vulgaris* L.	Sugar beet	S
	Spinacia oleracea L.	Spinach	S
Cucurbitaceae	*Cucumis sativus* L.	Cucumber	I
	Cucurbita pepo L.	Squash	I
Cyperaceae	*Cyperus rotundus* L.	Purple nutsedge	I
Fabaceae	*Phaseolus vulgaris* L.	Bean	I
	Pisum sativum L.	Pea	I
Liliaceae	*Allium cepa* L.	Onion	I
Malvaceae	*Abelmoschus esculentus Moench*	Okra	I
Melastomataceae	*Tibouchina herbacea* Cogn.	Tibouchina	I
Musaceae	*Musa paradisiaca* L.	Plantain	I
Plumbaginaceae	*Plumbago sp.*	Plumbago	I
Polygonaceae	*Polygonum sp.*	Polygonum	I

Table 2. Continued

Family	Plant species	Common name	Disease reaction [a]
Portulacaceae	*Portulaca halimoides* L.	Silkcotton purslane	S
Rubiaceae	*Coffea arabica* L.	Coffee	I
Solanaceae	*Capsicum annuum* L.	Pepper	I
	Lycopersicum esculentum Mill.	Tomato	I
	Solanum melongena L.	Eggplant	I
Tetragoniaceae	*Tetroagonia tetragonioides* (Pall.) Kuntze	New Zealand spinach	I
Verbenaceae	*Lantana camara* L.	Lantana	I

[a]Disease incidence was evaluated 14 days after inoculation. I = immune reaction; R = resistant reaction; S = susceptible reaction.
[b]Results based on three replicates from each plant species.

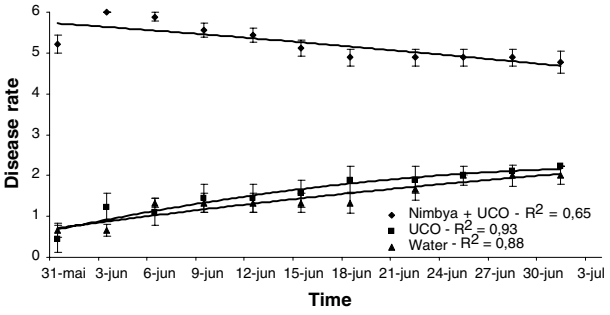

Figure 5. Disease progress on a naturally occurring population of alligatorweed sprayed with: (1) *Nimbya alternantherae* + unrefined corn oil (Nimbya + UCO); (2) UCO + water (UCO), and (3) sterile distilled water alone (water). Disease rate: evaluated on a scale of 0–6, where 0 = no visible symptoms; 1 = 0–20%; 2 = 20–40%; 3 = 40–60%; 4 = 60–80%; 5 = >80% diseased area; 6 = dead plant. Each data point was obtained from 9 replicates except for the control plants, where the data were from 3 replicates. Vertical bars represent standard error of mean.

isolates when the dew period was of only 12 h. South America is the center of origin of alligatorweed and the likely center of origin of *N. alternantherae*. This region therefore may contain the largest genetic diversity for this fungus, offering the prospect for finding more desirable isolates for alligatorweed control than those used in this study. Dew requirement was the main problem for *Colletotrichum orbiculare* (Berk. & Mont.) Arx, a pathogen probably brought along

accidentally with *Xanthium spinosum* L. from Argentina into Australia. It was conjectured that further exploration and collections of *C. orbiculare* in Argentina might yield a superior isolate to the Australian one (Auld and Say, 1999).

Low and erratic production of conidia of *N. alternatherae* on PDA was the major constraint at the beginning of this work. Solid fermentation for conidial production using rice as a substrate was also attempted without success (Pomella, unpublished data), mycelial preparations, although infective, were not as virulent as conidia. Adequate conidial production was finally obtained by using the methodology proposed by Walker (1980) as modified by Chandramohan and Charudattan (2003). This method is, nevertheless, impractical for commercial production due to cost and labor involved. Conidial production by a ''mesh screen method'' was used for *Nimbya scirpicola* (Fuckel) E.G. Simmons, a fungus tested for controlling *Eleocharis kumguwai* Ohwi, a troublesome weed in paddy fields in Japan (Matsuki et al., 1997, 1998). The prepared conidia showed sufficient stability and efficacy. However, this method proved commercially unsuitable, because nearly 318 m^2 of medium would be required to produce sufficient conidia to treat just1 ha of infested area at an effective dose (Matsuki et al., 1997, 1998). Further improvement in mass production of conidia is therefore required before *N. alternantherae* can be considered further for practical use. Liquid fermentation techniques should be tested as this is the favored method for the production of commercial bioherbicides.

Under controlled conditions, an inoculum concentration of just 10^4 conidia per ml was sufficient to kill newly growing shoots within 4 days. These conidial concentrations are low when compared to those needed for other related fungi studied as potential mycoherbicides which are normally applied at a rate of 10^6-10^7 conidia per ml under similar conditions (Mortensen, 1988; Pfirter and Défago, 1998). Nevertheless the low concentration of inoculum of *N. alternantherae* sufficient to cause high disease severity in greenhouse trials were not as effective in the field, probably because of environmental constraints and the older age of test plants.

Several formulations were evaluated to improve the efficacy of *N. alternantherae*. Unrefined corn oil (UCO) was the only adjuvant that provided a significant increase on disease severity as compared with conidia suspended in water. At comparable use rates, UCO was helpful in reducing dew requirement and application volume of *Alternaria helianthi* (Hansf.) Tubaki & Nishih. (Abbas and Egley, 1996).

However, the 50% v:v concentration of UCO is prohibitively expensive for commercial use. Therefore, in our field trials the concentration of UCO in the formulation was reduced to 20%. Even after the amount of UCO in the formulation was significantly reduced, *N. alternantherae* caused a substantial level of disease severity on alligatorweed in the field. After just one application, the disease severity reached 100% after 6 days and remained at $\geq 80\%$ for 1 month. The moderate decline on the disease levels after 1 month following applications was caused by the production of new healthy shoots by the plant mat. This effect could probably be reduced if a larger area had been treated, or if the isolate used had a good capacity to sporulate and incite secondary disease cycles. It is possible that drift of conidial suspensions might have infected the control plots, despite the care taken to avoid it. Background inoculum could also be provided by the secondary cycle of the pathogen from treated plots, but it may have occurred in a later stage, since characteristic disease symptoms on the control plots were observed a few days after inoculation.

Integration of *N. alternantherae* with insect natural enemies may offer an attractive approach to achieve adequate levels of control of this weed. There will be situations where chemical control may also be required to be used in combination with biological control agents, because the choice in the method of control (biological, chemical, and physical) will vary over time and between sites, so any integrated control approach needs to be flexible (Wright and Julien, 1999). No insect damage was observed at the experimental field site in Florida; the possibility of a synergistic effect between *N. alternantherae* and insects at different temperature ranges should be experimentally investigated. Likewise, the potential for integration of *N. alternantherae* with low dose chemical herbicides should also be explored.

The host-range data confirm that *N. alternantherae* is relatively host specific at the familial level. The disease symptoms observed on the two members of Chenopodiaceae and one of Portulacaceae, out of 42 species within 22 families tested, may represent an experimental anomaly resulting from the high inoculum level that was used. Such anomaly has been demonstrated for other fungi, as in the case of *Alternaria cassiae* Jurair & A. Khan, a fungus belonging to a genus related to *Nimbya*. Among 30 plant species in nine families that were screened against *A. cassiae*, phytotoxity was observed initially on some plants, but only low disease incidence was obtained when the trial was repeated with the application of a low inoculum concentration (Walker, 1982). Apart from members of the Amaranthaceae,

N. alternantherae was able to sporulate and be recovered only from dead leaves of spinach (*Spinacia oleracea*). Therefore, if a commercial mycoherbicide based on *N. alternantherae* is developed, and further studies confirm its pathogenicity to crop plants in the Chenopodiaceae and Amaranthaceae, particularly celosia (an important species for the cut flower industry in Florida), spinach and beet, special care will be needed while preparing a label for the product so that crops of such crops are spared from direct spray and spray drifts. It is also possible that more specific pathogens may be found in the future that become more attractive candidates for the development of a mycoherbicide to be used against alligatorweed.

In general, *N. alternantherae* has the potential to be an effective mycoherbicide against alligatorweed. The report (Simmonds, 1995) of the occurrence of *N. alternantherae* in Australia offers some hope for the development of this fungus as a mycoherbicide for use in that country.

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