An Analysis of Genetic Diversity in Hyphal Tip Isolates of Promising Mycoherbicide *Alternaria alternata* for Control of *Amaranthus retroflexus*

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The potential mycoherbicide *Alternaria alternata* was isolated from one lesion on a leaf of *Amaranthus retroflexus* which had been inoculated with conidia of the fungus. The isolate was cultured on V-8 agar medium. Hyphal tip isolates (HTI) were derived from young germ tubes of single spores and cultured on a semi-defined culture medium with a C:N ratio of 15:1. Amplified fragment length polymorphism analysis detected genetic variation in four HTIs and revealed 29% polymorphism. Selected HTIs that were different in at least one DNA fragment pattern varied in degree of sporulation, length of conidial germ tube, virulence and desiccation tolerance. The results indicated that the original culture of *A. alternata* is heterogeneous and, thus, cannot be considered as a suitable candidate for development as a commercial biocontrol agent.

Key words: Single spores, C:N ratio:, genetic variation, polymorphism, *Alternaria alternate*, *Amaranthus retroflexus.*

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پاتوژنی که برای کنترل میکروبی علف‌های‌هرز در نظر گرفته می‌شود، باید دارای ثبات ژنتیکی باشد. از این رو، گوناگونی ژنتیکی جمعیت‌های چنین پاتوژنی، بویژه اگر بطور طبیعی هتروکاریوتیک باشد، باید تعیین شود. در این پژوهش، نخست در شرایط گلخانه‌ای، بوته‌های تاج خروس وحشی *Amaranthus retroflexus* با کنیدی‌های قارچ *Alternaria alternata* جدایه ۴۲۳ اسپورپاشی شد. پس از یک هفته، از یک لکه در سطح برگ میزبان که آثار بیماری را بروز داده بود، نمونه برداری شده و در محیط V-8 کشت داده شد. پس از تک اسپور کردن، از انتهای لوله تندشی اسپورهای جوانه‌زده نمونه برداری شده و هر کدام به یک پتری دارای محیط کشت نیمه تعریف شده‌ای که نسبت کربن به نیتروژن آن ۱۵:۱ بود، انتقال داده شد. آنگاه، از ۷۶ جدایه نوک ریسه‌ای تولید شده، ۲۴ جدایه بطور تصادفی انتخاب گردید. با بهره بردن از روش AFLP، ۲۹٪ پلی‌مرفیسم DNA در میان آنها تشخیص داده شد. جدایه‌های نوک ریسه‌ای که دست کم در یک اثر DNA (باند) با یکدیگر متفاوت بودند، از نظر اسپورزایی، درازی لوله تندشی اسپور، بیماری‌زایی و تاب آنها به خشکی با یکدیگر مقایسه شدند. نتایج این پژوهش نشان داد که کشت اولیه این پاتوژن از نظر ژنتیکی ناهمگون (heterogeneous) بوده و نمی‌توان آن را به عنوان یک مایکوهربیساید توسعه داده و به سطح بازرگانی رساند. اما، چون در *A. alternata* تنها یک هسته وارد لوله تندشی می‌شود، از این رو کلنی بدست آمده هتروکاریوتیک نخواهد بود که به همین دلیل، جدایه‌های نوک ریسه‌ای تولید شده، از نظر ژنتیکی خالص در نظر گرفته می‌شوند. در میان این جدایه‌ها، چون جدایه شماره ۱۳، در اسپورزایی، بیماری‌زایی و تاب به خشکی وضعیت مناسبی داشت (شکل ۳، ۴و ۵)، می‌توان از آن به عنوان یک عامل کنترل میکروبی برای توسعه و رسانیدن به سطح یک میکوهربساید بهره برد.

INTRODUCTION

Genetic manipulation may be a useful strategy for developing more effective biological weed control agent (Greaves *et al*., 1989). Improvement of virulence to target plants, ability to spread, competitive ability, safety and tolerance to environmental stress may all be used to improve agent efficacy (Kistler, 1991). At the same time, a prerequisite for any potential biological control agent is that it should be genetically stable. On both these counts it is essential to investigate any genetic diversity or instability present in the natural population of any pathogenic fungus identified as having potential as a biocontrol agent.

Molecular markers, which are used to determine population dynamics of fungi, are revolutionizing the analysis of population biology of phytopathogenic fungi (McDermott & McDonald, 1993; Assigbetse *et al*., 1994; Milgroom & Fry, 1997; Tran-Dinh *et al*., 1999). Polymerase chain reaction (PCR)-based techniques have been developed to evaluate DNA polymorphisms in a wide variety of organisms (Welsh & McClelland, 1990; Williams *et al*., 1990). One of these techniques is amplified fragment length polymorphism (AFLP, Vos *et al*., 1995) a powerful DNA fingerprinting technique that has been used to identify fungi and bacteria and to study their genetic variation (Bonants *et al*., 1999). AFLP is based on the selective PCR amplification of restriction fragments from a total digest of genomic

DNA. This technique delivers large numbers of bands (typically 50-100 restriction fragments), is reproducible, has a good distinctive capacity, is easily standardized and can be automated (Vos *et al*., 1995).

This investigation was conducted to study genetic diversity in hyphal tip isolates (HTI) of *A. alternata* (isolate 423), which exhibit differences of vegetative growth, morphology, sporulation, desiccation tolerance and pathogenicity (Montazeri, 2000).

MATERIALS AND METHODS

Cultural Conditions and Conidia Production

A. alternata (isolate 423) was isolated from *Amaranthus powelii* (S. Wats) at East Leach, Oxfordshire, UK (Lawrie *et al*, 2000). Stock cultures of the fungus were stored at 4 ^{o}C on V-8 agar (200 mL V-8 Juice [Campbell Grocery Products Ltd., Norfolk, UK], 12 g agar; Technical No 3 [Oxoid Ltd, Basingstoke, UK], 800 mL deionized water, pH 6).

For conidia production, a solid medium, prepared by adding 20 g L^{-1} agar, Technical No 3, to a semi-defined medium (Schisler *et al*., 1991), with a C:N ratio of 15:1 (calculated as described by Jackson and Schisler, 1992) was used. The pH of the autoclaved medium was adjusted to 6 with 1 M HCl or 1 M NaOH. The fungus was cultured in Petri dishes by inoculating centrally with 3 mm plugs taken from stock cultures. Plates were sealed with parafilm and incubated for 14 days at 20 ^{o}C in a cycle of 12 h dark, 12 h near ultraviolet light received from two 24 inch 8W UV tube light (Philips, Holand).

Conidia were harvested by lightly scraping the surface of the culture into sterile deionized water using a razor blade. The suspension was filtered through two layers of sterile muslin and the filtrate adjusted to the required concentration, using sterile deionized water, after counting conidia in a haemocytometer.

Hyphal Tip Isolates

The leaves of a red root pigweed (*Amaranthus retroflexus*) plant at the 4-leaf stage were treated by applying a 2-µL droplet of conidial suspension, at $3x10^{6}$ conidia

mL^{-1}, obtained from a two-week-old culture, using a micropipette. After one week, a portion (5x5 mm) of leaf showing a single lesion was excised and cultured for 14 days at 20 ^{o}C on a semi-defined agar medium with a C:N ratio of 15:1. A conidial suspension (10 µL, 10^3 conidia mL^{-1}) was prepared from the resulting culture and spread over semi-defined agar medium in a Petri dish to produce single spore germlings. After 72 h incubation at 20 ^{o}C, plate was examined under a microscope and tips of germtubes clearly identified as arising from a single spore were excised and cultured on semi-defined agar medium. From the 76 HTIs so obtained, 24 were arbitrary chosen for further investigation.

DNA Preparation

Genomic DNA was isolated essentially as described by Pie *et al.* (1997). For each HTI, approximately 10 mg conidia were added to 2 ml screw-cap tubes containing two 3-mm-diam and 20-30 2-mm-diam glass beads. Then, 200 µL SDS extraction buffer (150 mM EDTA, pH 8.0; 5 mM Tris-HCl, pH 8.0; 1% SDS) was added to each tube. The tubes were shaken vigorously for 10 min at 2000 rpm on a VXR shaker fixed with a VX2E tube platform (BDH Laboratory Supplies, Poole, UK) and incubated at 90 ^{o}C on a tube platform heater (BDH) for 30 min. Phenol:chloroform:isoamy alcohol mixture (25:24:1; 800 µL) was added, and the tubes gently shaken for 20 min at 37 ^{o}C before diluting with 600 µL distilled sterile water. The tubes were centrifuged at 4000 ×g for 30 min and 650 µL of supernatant transferred to new tubes. Two volumes of ethanol were added to the supernatant and the tubes were kept at room temperature for 10 min before centrifuging at 11200 ×g for 10 min. The pellets were dried and resuspended in 20 µL sterile distilled water.

AFLP Analysis

The AFLP procedure used was that described by Pei and Ruiz (2000). Adapters and primers (Table1) were synthesized by Genosys, Cambridge, UK. Approximately 200 ng genomic DNA was digested with 2 U *Eco*R I (Pharmacia) and 2 U *Pst* I (Amersham Pharmacia Biotech, Amersham, UK) in 25 µL of 1x

One-Phor-All (OPA) buffer (10 mM Tris-acetate, pH 7.5, 10 mM Mg-acetate, 50 mM K-acetate; Pharmacia) for 1 h at 37 °C. Then, 5 µL of a ligation reaction mixture containing 10 ng *Eco*R I adapter, 10 ng *Pst* adapter, 1 mM ATP (Genosys) and 0.55 U T4 DNA-ligase (Amersham Pharmacia Biotech) in 0.5 µL 1x OPA buffer was added and incubation continued for a further 3 h. Adapters were prepared by mixing equimolar amounts of both strands in 10x OPA buffer, heating to 95 °C and allowing cooling to room temperature slowly.

A two-step PCR procedure was adopted for selective amplification. In the first step (pre-amplification), both E-A and P-A primers (Table 1), each having 1-bp 3'-extension, were used. Each 25 µL of pre-amplification reaction mixture contained 5 ng digested and ligated genomic DNA, 200 ng each of the E-A and P-A primers, 200 µM of dNTP (dATP, dCTP, dGTP and dTTP), 1xPCR buffer (Promega, Madison, USA), 2 mM Mg Cl_2 and 0.5 U Taq polymerase (Promega). The surface was overlaid with a drop of mineral oil. The pre-amplification PCR was carried out in a GeneAmp PCR system 9700 (PE Applied Biosystems, Cheshire, UK) programmed with 20 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. The PCR products were diluted 10-fold with distilled water and stored at –20 °C. In the second amplification, *Eco*R I and *Pst* I primers with 3-bp 3'-extensions were used in combination (E-AAA and P-ACA, E-AAT and P-ACA, E-AAG and P-ACA). Each 10 µL reaction mixture contained 1µL diluted PCR product from the preamplification, 10 ng unlabelled *Eco*R I primer, 15 ng *Pst* I end-labeled with [γ-^{33}P] ATP, 200 µM of dNTP, 1x PCR buffer (Promega), 2 mM $MgCl_2$, and 0.5 U Taq polymerase (Promega). The selective amplification was performed in the GeneAmp PCR system 9700 programmed with 10 cycles of the following profile: 30 s at 94 °C, 30 s at 62 °C and 60 s at 72 °C. The PCR continued for an additional 25 cycles for 30 s at 94 °C, 30 s at 56 °C and 60 s at 72 °C followed by a final extension cycle of 72 °C for 30 min. The PCR products were denatured and separated on a 5% polyacrylamide DNA sequencing gel (National Diagnostic Company, Hessle Hul, UK) and autoradiographs were obtained using Hyperfilm MP (Amersham Pharmacia Biotech).

Polymorphic AFLP markers were scored manually as presence (1) or absence

(0) of a band for a putative locus. A similarity matrix was constructed using the Jaccard coefficient (Sneath & Sokal, 1973) for the markers from different primer combinations together. Based on Nei & Li's coefficients (Nei & Li, 1979), a dendrogram was constructed and principal coordinate analysis was performed using UPGMA (Sneath & Sokal, 1973). All analyses were conducted using the Genstat 5 statistical package (Genstat 5 Committee 1993).

Table 1. Adapter and primer sequences (5'-3') used in AFLP analysis.

Adapters:	
*Eco*R I (*Escherichia coli*)	-CTCGTAGACTGCGTACC-
	-AATTGGTACGCAGTC-
Pst I (*Providencia stuartii*)	-CTCGTAGACTGCGTACATGCA-
	-TGTACGCAGTCTAC-
Primers:	
E-A	-GACTGCGTACCAATTCA-
P-A	- GACTGCGTACATGCAGA-
E-AAA	- GACTGCGTACCAATTCAAA-
E-AAT	-GACTGCGTACCAATTCAAT-
E-AAG	-GACTGCGTACCAATTCAAG-

Biological Variability in Hyphal Tip Isolates

Conidiation, desiccation tolerance and virulence of HTIs 1, 13, 14, 16, 19, which were different in at least one DNA fragment pattern, were studied.

Conidiation: The HTIs were cultured on semi-defined agar media with a C:N ratio of 15:1 and incubated at 20 ^{o}C in a cycle of 12 h dark, 12 h ultraviolet light. Conidia were harvested from each of 4 replicate 14 day-old cultures. Ten discs (1-cm-diam) were cut from 8-10 days growing area of the colony using a cork borer and placed in tubes containing 5 mL 0.1% (v/v) Tween 40 in deionized water. The tubes were shaken on a vortex mixer at 200 rpm for 2 min. The resulting suspensions were filtered through two layers of muslin and a haemocytometer used to determine the conidial concentration.

Virulence: The conidia from 3 week-old cultures were harvested in 0.01% (v/v) Tween 40 and filtered in the usual way. The resulting suspensions from 4 replicate plates were mixed and adjusted to $5x10^6$ conidia mL^{-1}, if necessary after centrifuging at 2700 ×g for 5 min to concentrate the suspension. Host plants (*A. retroflexus*) were grown in pots, each containing 3 plants. At the 2-leaf stage (3 weeks old) a 2-µL droplet of conidial suspension was put on each true and each cotyledonous leaf of each plant. The check plants were treated with 2-µL droplets of deionized water containing 0.01% (v/v) Tween 40. The experiment was designed as a randomized complete block with 4 replicates (pots). After drying the propagules onto the leaves at 21 °C and 45% RH for 1 h, all the pots were transferred to propagator chambers at 92 ± 2 % RH, 25 °C and illuminated with two 24 inch 18 W white pluslux 3500 fluorescent tubes (Osram Ltd, St Albans, UK). Disease development was evaluated after six days by counting the leaves or cotyledons of those plants which showed lesions formation.

Desiccation Tolerance: Conidia of each HTI were harvested, in deionized water, from 2 week-old cultures on plates of V-8 agar media. The conidial suspensions from 4 replicate plates were mixed, filtered and adjusted to 0.5 x 10^6 conidia mL^{-1}. Desiccation tolerance of conidia was evaluated on discs (0.6-cm diameter) of semi-permeable membrane (Medicell International Ltd, London, UK) at 25 °C with constant 15% RH as described by Montazeri and Greaves (2002).

After 120 days storage, 5 replicate discs of each HTI were incubated on tap water agar at 20 °C for 6 h when a 5 µL droplet of aniline blue / lactophenol (0.1 g aniline blue [Riedel-de-Haen, Seelze, Germany] + 67 ml lactophenl [BDH Laboratory Supplies, Poole, UK] + 20 mL distilled water] was added to the disc to stop germination and stain the conidia. The germ tubes of about 60 conidia were measured using a microscope on each disc.

Statistical Analysis: The results were subjected to analysis of variance using Genstat (Payne *et al*., 1987). Treatment means were compared with the least

significant difference (LSD).

RESULTS

Analysis of Genomic DNA

In total, 110 markers were scored from three primer combinations. Of these, 32 (29%) were polymorphic, demonstrating genetic differences among the HTIs (Figure 1). This data was used to construct the dendrogram (Figure 2) that clearly shows that the AFLP analysis detected genetic variation in the 24 HTIs. Thus, 5 genotypic variations HTIs were apparent. HTIs 1 and 19 are quite different from the other HTIs, showing 74% and 92% similarity, respectively. This was expected due to the number of unique bands detected in these two HTIs. HTIs 13 and 14, with one and two unique bands respectively, showed 99% and 97% similarity. It was decided to use HTI 16 to represent the remaining HTIs, which were identical.

Biological Differences

Conidiation: Conidiation of the HTIs, on semi-defined culture medium with a C:N ratio of 15:1 showed significant differences. Among these isolates, HTIs 13 and 19 showed the highest conidium production (Figure 3).

Virulence: All 5 HTIs types were pathogenic on the host plant (*A. retroflexus*). All produced similar symptoms, including chlorosis at the site of inoculation, which extended with time, developing into a necrotic lesion. Eventually, defoliation and decreased growth of the host plant was obvious. However, HTIs differed in the severity of symptoms and numbers of lesions they caused on treated plants. Thus, HTI 14 produced greater disease expression than any other, and HTI 1 was the weakest pathogen (Figure 4).

Desiccation Tolerance: Germination of the fresh conidia of all selected HTIs was not significantly different, being more than 94%. But, over 120 to 210 days desiccation at 25 ^{o}C and 15% RH, the ability to germinate declined (Figure 5) with significant differences. Thus, after 210 days, HTIs 13 and 19 gave the higher germination, i.e. greater desiccation tolerance, than HTIs 1 and 14. After 210 days, HTI 1 showed the lowest germination (6%), whereas HTIs 13 and 19 gave the

highest germination (77% & 73%, respectively) (Figure 5).

After 120 days storage, HTIs showed significant differences in the size of germ tubes produced by conidia. After 6 h incubation on semi-permeable discs on TWA, the germ tubes of HTIs 16 and 19 were significantly longer than those of others (Figure 6).

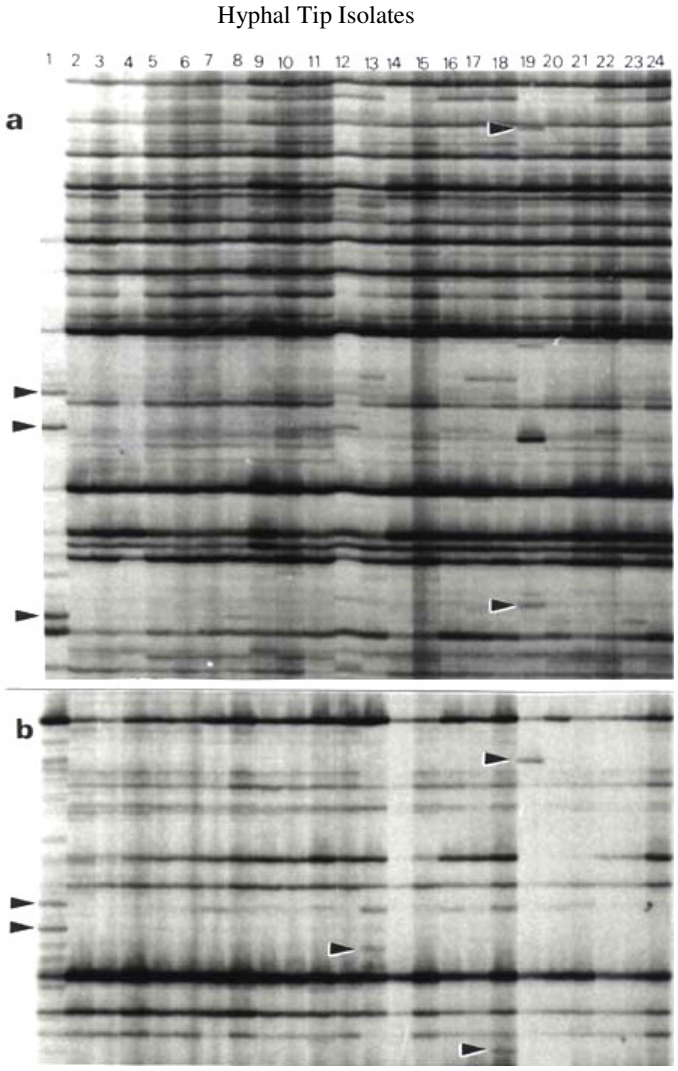

Figure 1. Amplification profile using AFLP primer *Pst*I-ACA + *Eco*RI-AAA (a) and *Pst*I-ACA + *Eco*RI-AAG (b). The patterns were chosen to display examples of markers which are indicated by arrows.

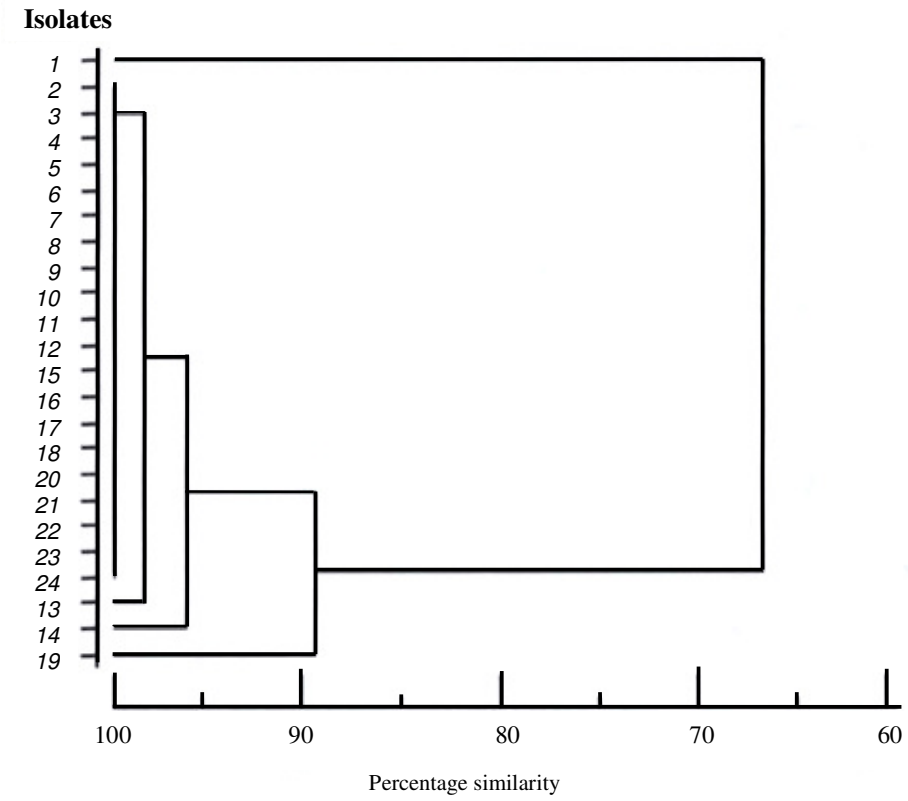

Figure 2. UPGMA dendrogram of hyphal tip isolates of *Alternaria alternata* based on 32 polymorphic AFLP markers obtained from three primer combinations.

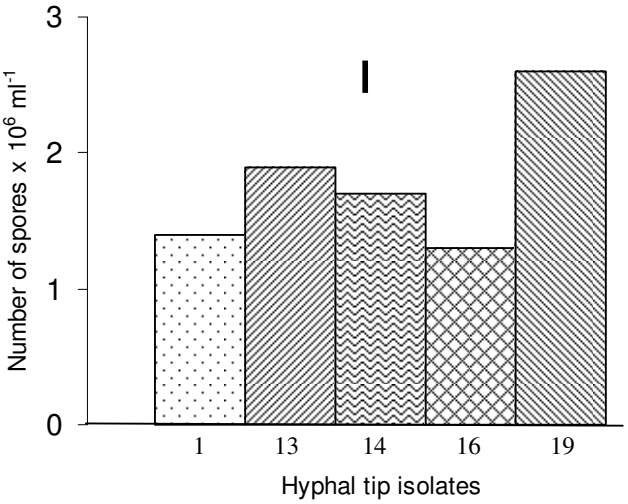

Figure 3. Sporulation of hyphal tip isolates of *Alternaria alternate* measured after 14 days growth on a solid medium with a C: N ratio of 15:1. Bar indicates LSD at P= 0.05.

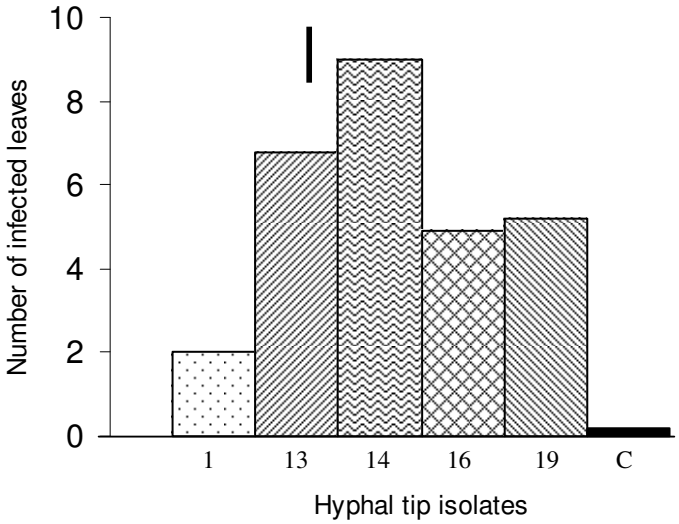

Figure 4. Virulence of hyphal tip isolates of *Alternaria alternata* on *Amaranthus retroflexus*, 6 days after application of conidia. Bar indicates LSD at P = 0.05.

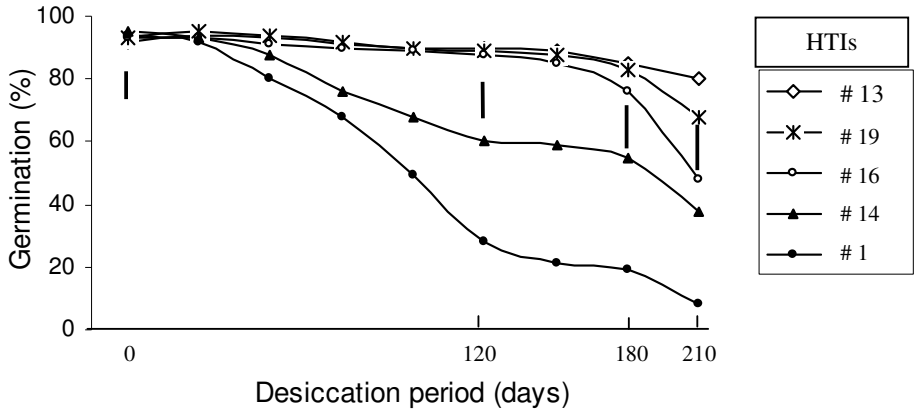

Figure 5. Germination of stored conidia of hyphal tip isolates (HTIs) of *Alternaria alternata* on tap water agar. Vertical bars indicate LSD at P=0.05.

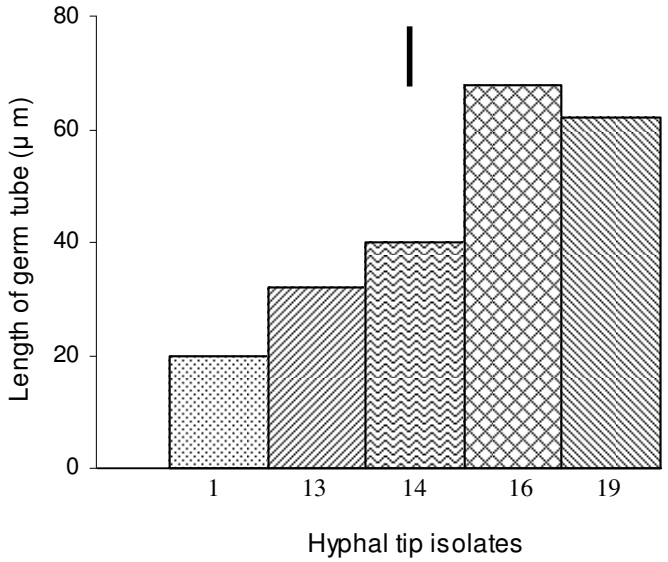

Figure 6. Desiccation tolerance of conidia produced by hyphal tip isolates of *Alternaria alternata* and stored for 120 days at 25 °C and 15% RH, measured as the length of germ tubes 6 h after germination at 29 °C on tap water agar. Vertical bar indicates LSD at P=0.05.

DISCUSSION

The multinucleate arrangement and heterokariosis that are common in *Alternaria* spp. (Su & Sun, 1985; Rotem, 1994) result in the production of different genotypes (Stall & Alexander, 1957). Morris *et al*. (2000) using RAPD analysis observed a high level of genetic diversity among 69 isolates of *A. alternata* collected from tomato fruits in California, USA. They suggested that one possible explanation for this diversity is that there exists an unknown sexual stage. In their study, no genetic diversity was observed among the isolates that were obtained from one lesion. However, in the current study, analysis of DNA by AFLP demonstrated that 29% of the marker derived from 24 HTIs of a culture of *A. alternata* isolated from one lesion were polymorphic. The HTIs varied also in degree of conidiation, length of germ tube, virulence and desiccation tolerance of conidia.

According to Su and Sun (1985), in *A. alternaria* only one nucleus migrates into the germ tube, it divides or does not divide and accordingly produces binucleate or uninucleate cell which, obviously, are not heterokaryotic. In this study, in which each HTI obtained from the tip of a young hypha, homogeneity might have been expected. The result may, also, suggest that the lesion was caused by several different strains, i.e. a population of original culture of *A. alternata*.

For a mycoherbicide, homogenesis and genetic stability are essential to give constant results in conidiation, desiccation tolerance, virulence and host range i.e. to give a reliable commercial product. It is also essential to avoid the risk that undesirable traits, which may pose environmental threats, do not develop in the released population of biocontrol agent. Thus, the original culture of *A. alternata* (isolate 423) which is heterogeneous and/or a combination of several strains cannot be considered as an ideally suitable candidate for development as a commercial biocontrol agen. If further research demonstrates that any of the HTIs produced in this study is genetically homogeneous and has, desirable characteristics of virulence, host selectivity and desiccation tolerance, it could be further developed for practical use. On the basis of spore yield, desiccation tolerance and virulence on *A. retroflexus*, HTI 13 may be such a candidate.

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